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# Functional Conservation and Divergence of *daf-22* Paralogs in *Pristionchus pacificus* Dauer Development

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## Abstract

**Small-molecule signaling in nematode dauer formation has emerged as a major model to study chemical communication in development and evolution. Developmental arrest as nonfeeding and stress-resistant dauer larvae represents the major survival and dispersal strategy. Detailed studies in *Caenorhabditis elegans* and *Pristionchus pacificus* revealed that small-molecule communication changes rapidly in evolution resulting in extreme structural diversity of small-molecule compounds. In *C. elegans*, a blend of ascarosides constitutes the dauer pheromone, whereas the *P. pacificus* dauer pheromone includes additional paratosides and integrates building blocks from diverse primary metabolic pathways. Despite this complexity of small-molecule structures and functions, little is known about the biosynthesis of small molecules in nematodes outside *C. elegans*. Here, we show that the genes encoding enzymes of the peroxisomal β-oxidation pathway involved in small-molecule biosynthesis evolve rapidly, including gene duplications and domain switching. The thiolase *daf-22*, the most downstream factor in *C. elegans* peroxisomal β-oxidation, has duplicated in *P. pacificus*, resulting in *Ppa-daf-22.1*, which still contains the sterol-carrier-protein (SCP) domain that was lost in *C. elegans daf-22*, and *Ppa-daf-22.2*. Using the CRISPR/Cas9 system, we induced mutations in both *P. pacificus daf-22* genes and identified an unexpected complexity of functional conservation and divergence. Under well-fed conditions, ascaroside biosynthesis proceeds exclusively via *Ppa-daf-22.1*. In contrast, starvation conditions induce *Ppa-daf-22.2* activity, resulting in the production of a specific subset of ascarosides. Gene expression studies indicate a reciprocal up-regulation of both *Ppa-daf-22* genes, which is, however, independent of starvation. Thus, our study reveals an unexpected functional complexity of dauer development and evolution.**

***Key words:*** **dauer development, phenotypic plasticity, *C. elegans*, *Pristionchus pacificus*, *daf-22*, ascarosides, β-oxidation.**

Article

## Introduction

Chemical communication by small molecules is crucial for animal development and behavior and represents an important mechanism for the interaction of organisms with the environment. In recent years, selected nematode species became widely used models to study the chemical nature of small molecule communication and its mechanisms of action and evolution (for review, see Schroeder 2015). In *Caenorhabditis elegans*, a modular library of small-molecule signals, the ascarosides, regulate several important behaviors, such as mating, aggregation, and dispersal (Srinivasan et al., 2008; Pungaliya et al. 2009; Bose et al. 2012; Ludewig et al. 2013; Ludewig and Schroeder 2013). Ascarosides derive from the combination of the dideoxysugar ascarylose with a variety of fatty acid-like side chains (fig. 1). Small-molecule pheromones usually function as a blend and often act in a concentration-dependent manner. These molecules are also major regulators of developmental processes, for example, dauer formation. Dauer larvae are arrested, stress-resistant, and long-lived alternative juveniles that are characterized by many specific morphological, physiological, and behavioral adaptations (fig. 1A) (for review, see Sommer and Ogawa 2011). The dauer stage represents the major survival and dispersal strategy in nematodes, and many nematode species are regularly found in the wild in the dauer stage (for review, see Sommer and Mayer 2015). Among several stress factors that can induce dauer formation under unfavorable conditions, population density is special in that it requires interactions between conspecifics. In this context, the function of ascaroside secretion in *C. elegans* is thought to indicate population density: Young larvae will develop into dauers when they sense small-molecule signals above a certain threshold (Golden and Riddle 1984).

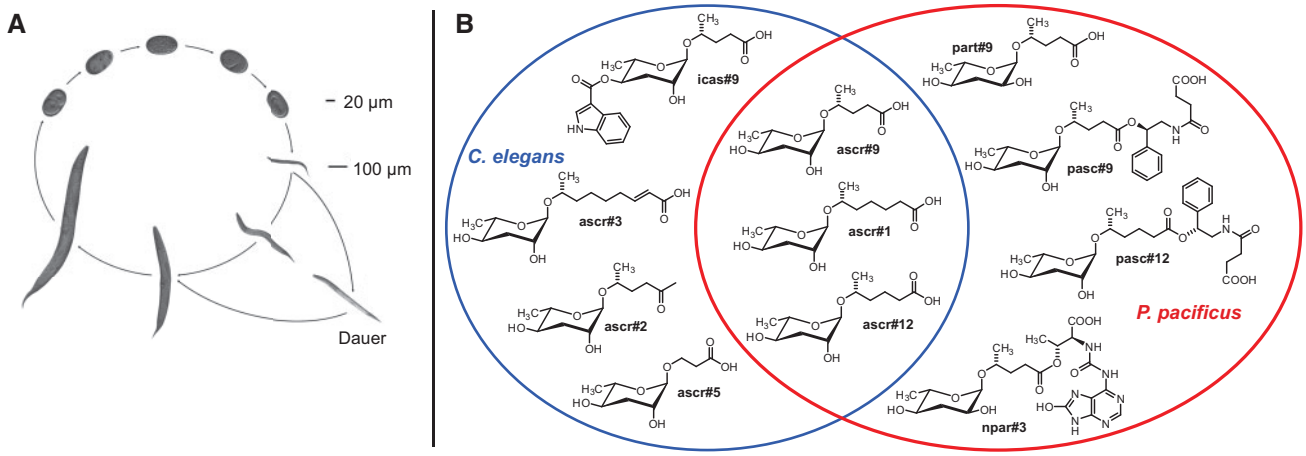


**FIG. 1.** The *P. pacificus* life cycle and variation in ascarosides between *C. elegans* and *P. pacificus*. (*A*) The life cycle of the nematode *P. pacificus*. Animals stay in the direct life cycle indefinitely if sufficient food is provided. In contrast, animals enter the dauer stage under harsh environmental conditions, such as elevated temperature, starvation, or high population density. (*B*) Selected ascarosides and ascaroside-derivatives identified from *C. elegans* and *P. pacificus*, respectively, illustrating their structural diversity and common biosynthetic origin from modular assembly of primary metabolic building blocks, including a peroxisomal β-oxidation-derived side chain. Although different compounds induce dauer in the two species, some simple short-chained ascarosides, for example, ascr#1 and ascr#9, are produced by both species.

Chemical communication systems using small molecules are highly evolvable and can likely be modified throughout evolution to generate novel types of interactions. Indeed, comparative studies among different nematode species indicated substantial diversification of the composition of small molecules (fig. 1*B*) and the involvement of small-molecule pheromones in novel types of interactions. One organism that has been studied in greater detail in this regard is *Pristionchus pacificus*. *Pristionchus pacificus* was developed as a second nematode model system with a specific focus on comparative and evolutionary biology (Sommer 2015). In the wild, *P. pacificus* is associated with different scarab beetles around the world, and on the living beetle *P. pacificus* is exclusively found in the dauer stage, providing a system where laboratory studies can be combined with fieldwork (Herrmann et al. 2007, 2010; Weller et al. 2010; Morgan et al. 2012; Ragsdale et al. 2015). The chemical architecture of *P. pacificus* small-molecule pheromones is surprisingly diverse, containing besides ascarosides also paratosides, which are derived from the dideoxysugar paratose (fig. 1*B*) (Bose et al. 2012). The chemical structures of the ascarosides and paratosides identified from *P. pacificus* and *C. elegans* incorporate building blocks from all major primary metabolic pathways. For example, *P. pacificus* forms a pentamodular metabolite, pasa#9, and an 8-oxoadenine-containing compound npar#3, suggesting the co-option of tryptophan and nucleoside metabolism pathways for the biosynthesis of endogenous metabolites (Yim et al. 2015). Analysis of the ascaroside profiles of *P. pacificus*, *C. elegans*, and other nematodes further showed that the chemical structures of these small-molecule signals are highly species-specific (Bose et al. 2012; Choe et al. 2012). Together, these observations indicate that nematodes must contain sophisticated biosynthesis pathways that can generate the observed diversity of secondary metabolites.

Despite the complexity of ascaroside structures and functions, little is known about the synthesis of these small molecules in nematodes other than *C. elegans*. Several recent studies identified and characterized the peroxisomal β-oxidation pathway to be involved in ascaroside biosynthesis in *C. elegans*. Peroxisomal β-oxidation in *C. elegans* proceeds iteratively via a four-step process that involves enzymes belonging to four different families (fig. 2*A*) (von Reuss et al. 2012). All of them are members of multigenic families that are present across eukaryotes in varying numbers (Poirier et al. 2006), raising the possibility that multiple paralogous genes from the same family contribute to ascaroside biosynthesis. Indeed, already three *acox* gene paralogs were recently shown to be involved in the synthesis of dauer-inducing ascarosides in *C. elegans* (Zhang et al. 2015). As *P. pacificus* and *C. elegans* are separated by more than 200 million years of evolution, these nematodes have largely divergent genomes and the *P. pacificus* genome was shown to encode more than 26,000 genes, around 6,000 more than *C. elegans* (Dieterich et al. 2008). Interestingly, our previous work indicated that enzyme-encoding genes, in particular those that are members of multigene families, undergo rapid birth and death processes resulting in the near absence of 1:1 orthology relationships between *P. pacificus* and *C. elegans* (Markov et al. 2015). Therefore, given the diversity of small molecules identified in *P. pacificus* and *C. elegans*, we set out to study the biochemical synthesis of small molecules by combining bioinformatic, genetic, and chemical tools.

Here, we start to investigate the biosynthesis of ascarosides and paratosides in *P. pacificus*. Genome-wide phylogenetic analysis of potential biosynthetic enzymes reveals that multiple genes encoding enzymes of the β-oxidation pathway have undergone rapid gene duplications involving substantial domain switching. We focus on the thiolase *daf-22*, which in *C. elegans* represents a single gene. In *P. pacificus*, two genes with different domain architectures are present. We induced mutations in *Ppa-daf-22.1* and *Ppa-daf-22.2* using CRISPR-Cas9 genome engineering and studied small-molecule

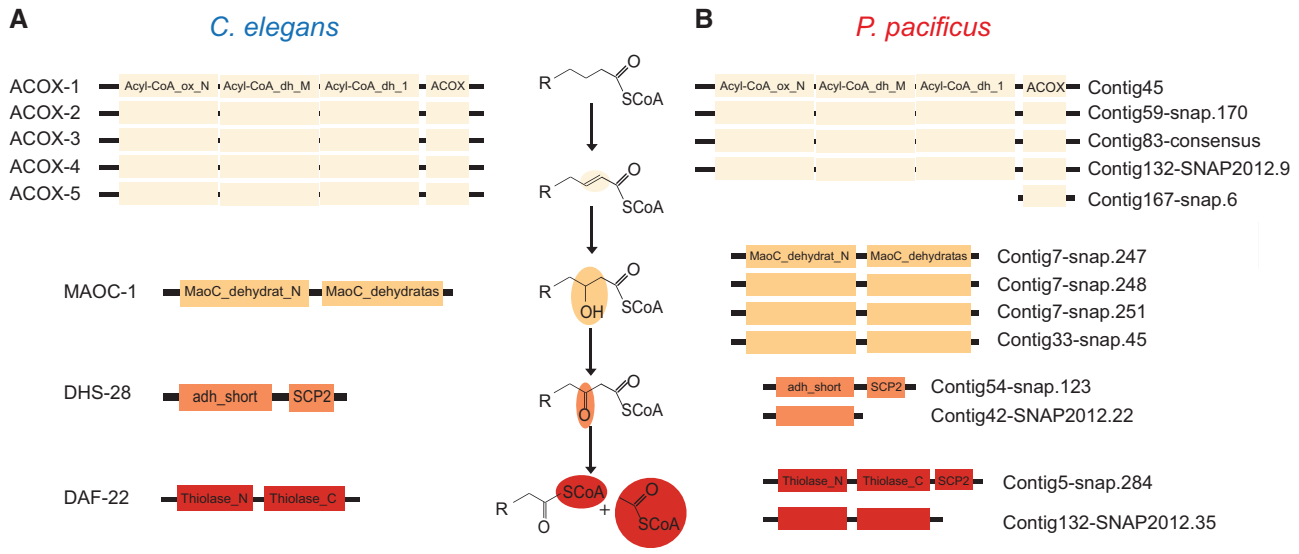


**Fig. 2.** Comparison of the peroxisomal β-oxidation pathway in *C. elegans* and *P. pacificus*. (*A*) Genes encoding enzymes that were characterized as components of the β-oxidation pathway in *C. elegans*. (*B*) Closest paralogs of genes in *P. pacificus* encoding enzymes of the β-oxidation pathway. For maximum-likelihood phylogenetic trees of individual gene families, see supplementary figs. S2–S5, Supplementary Material online.

production in these mutants under fed and starved conditions. Our work identified an unexpected complexity of functional conservation and divergence of *daf-22* and β-oxidation of ascarosides.

## Results

### Ascaroside Biosynthetic Genes are Nonorthologous Between *C. elegans* and *P. pacificus*

To study the biosynthesis of small molecules in *P. pacificus*, we first evaluated the conservation of the putative peroxisomal β-oxidation pathway among nematodes based on orthology relationships. Analysis of the phylogenetic relationships of all closer paralogs of *C. elegans* enzymes involved in β-oxidation revealed that none of them is conserved as a 1:1 ortholog between *P. pacificus* and *C. elegans* (fig. 2 and supplementary figs. S1–S5, Supplementary Material online). Figure 2 summarizes the minimal number of closest paralogs between both species, based on maximum likelihood trees of all nematode genes with the same PFAM domain structure (supplementary figs. S1–S5, Supplementary Material online). The highest divergence is found in the *acox* gene family, with multiple paralogs coming from parallel lineage-specific amplifications in *C. elegans* and *P. pacificus* (fig. 2 and supplementary fig. S2, Supplementary Material online). The three other β-oxidation genes, *Cel-maoc-1*, *Cel-dhs-28*, and *Cel-daf-22*, are present as single copies in the genome of *C. elegans*, whereas they underwent gene duplication and domain shuffling events in *P. pacificus*. Specifically, *maoc-1* underwent several rounds of duplications leading to four paralogs in the *maoc* family (fig. 2 and supplementary fig. S3, Supplementary Material online), whereas a single duplication followed by losses of the C-terminal sterol-carrier-protein-2 domain (SCP-2) in one of the paralogs occurred in the *dhs-28* (fig. 2 and supplementary fig. S4, Supplementary Material online) and *daf-22* families (fig. 2 and supplementary fig. S5, Supplementary Material online). Together, these phylogenetic studies indicate the absence of 1:1 orthology relationships between *C. elegans* and *P. pacificus* for all genes with putative functions in β-oxidation.

### *daf-22* Duplicated Specifically in the *Pristionchus* Genus

Given the absence of 1:1 orthology relationships between the β-oxidation genes, we focused further studies in *P. pacificus* on the *daf-22* paralogs, which encode the most downstream acting enzymes in the *C. elegans* pathway. *Cel-daf-22* contains two thiolase domains, but lacks a SCP-2 domain that is known from thiolase genes in vertebrates, insects, and fungi (Pereto et al. 2005). Interestingly, *daf-22* is present as a single copy gene bearing a C-terminal SCP-2 domain in a diversity of nematodes including parasites of Clade III (*Ascaris suum, Brugia malayi, Loa loa*), Clade IV (*Strongyloides ratti, Panagrellus redivivus and Meloidogyne hapla*), and Clade V (*Necator americanus and Haemonchus contortus*) (supplementary fig. S5, Supplementary Material online). These findings suggest that the ancestral domain organization has been lost in *C. elegans* and some other nematode lineages.

In the genus *Pristionchus*, gene duplication led to two genes, *daf-22.1* and *daf-22.2*, the latter of which lost the SCP-2 domain secondarily (fig. 2*B*). Both paralogs differ in their chromosomal location and in their exon–intron structure (www.pristionchus.org; fig. 3). *Ppa-daf-22.1* is expressed in dauer larvae, late larval stages and adults, and to a lesser extent in early larvae (Baskaran et al. 2015). In contrast, *Ppa-daf-22.2* is only weakly expressed in dauers, and no expression was detectable in RNAseq experiments in other stages. Using quantitative PCR (qPCR) experiments, expression of *Ppa-daf-22.2* was observed at low levels, but *Ppa-daf-22.1* was expressed roughly 35× higher than *Ppa-daf-22.2* under well-fed conditions (supplementary fig. S6, Supplementary Material online). To study the function of the *Ppa-daf-22* genes, we separately inactivated the two

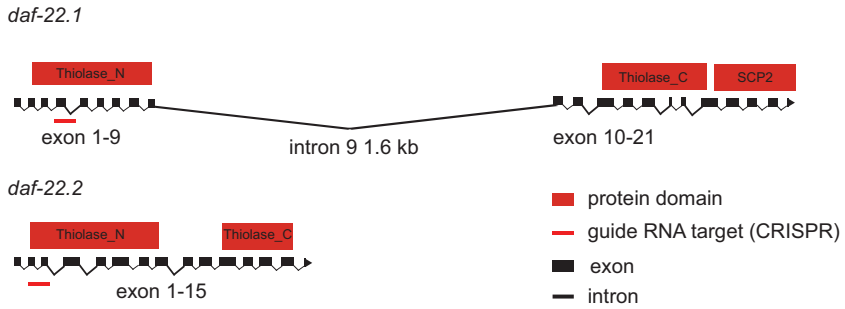


**Fig. 3.** The gene structure and protein domains of the *daf-22.1* and *daf-22.2* genes respectively. Thin red dashes indicate the exons targeted by CRISPR.

*P. pacificus* paralogs by CRISPR-Cas9-induced deletion in the *N*-terminal part of the thiolase domain. The *sgen* sequences were targeted to exon 4 of *Ppa-daf-22.1* and to exons 2 and 3 of *Ppa-daf-22.2*, ensuring that the catalytic activity of the encoded enzyme would be fully abolished (fig. 3). We obtained two mutant lines, a 7-bp deletion in *Ppa-daf-22.1* (*tu489*) and a 7-bp insertion in *Ppa-daf-22.2* (*tu504*), both resulting in frameshift mutations. A double mutant was generated to study the effect of knockout of both genes.

## *Ppa-daf-22.1* (*tu489*) and *Ppa-daf-22.1; Ppa-daf-22.2* Double Mutant are Deficient in Ascaroside Biosynthesis

To investigate the roles of the two *Ppa-daf-22* genes in ascaroside and paratoside biosynthesis in *P. pacificus*, we compared the metabolomes of both single mutants and *Ppa-daf-22.1; Ppa-daf-22.2* double mutants with that of wild-type animals. Given that ascaroside production in *C. elegans* is known to depend on environmental conditions (Kaplan et al. 2011; von Reuss et al. 2012), we analyzed sets of samples derived from two different culture protocols, in which worm liquid cultures were either fed with bacteria continuously ("*ad lib*" condition) or starved for an extended period of time prior to harvest ("starved" condition). LC/MS analysis of *ad lib* mixed-stage as well as synchronized cultures showed that short-chain ascaroside and paratoside biosynthesis is fully abolished in the double mutant *Ppa-daf-22.1; Ppa-daf-22.2* (fig. 4A, supplementary fig. S7, Supplementary Material online). Analysis of the single mutants revealed similar ascaroside profiles for *Ppa-daf-22.2 (tu504)* and wild-type (fig. 4A and supplementary fig. S7, Supplementary Material online), whereas *Ppa-daf-22.1 (tu489)* was found to produce only trace amounts of a few specific ascarosides. Similar to *C. elegans* mutants with impaired peroxisomal β-oxidation, for example, *Cel-daf-22* (Izrayelit et al. 2012; von Reuss et al. 2012), we observed accumulation of large amounts of long-chain ascarosides as shunt metabolites in the *Ppa-daf-22.1; Ppa-daf-22.2* double mutant and *Ppa-daf-22.1 (tu489)*. In contrast, the amounts of long-chain ascarosides in *Ppa-daf-22.2 (tu504)* were only slightly elevated compared with wild type (fig. 4C and D). These results indicate that in *ad lib* worms, ascaroside and paratoside biosynthesis proceeds almost exclusively via *Ppa*-DAF-22.1, whereas *Ppa*-DAF-22.2 does not significantly contribute.

In contrast, analysis of metabolome samples from starved cultures revealed that *Ppa*-DAF-22.2 can produce substantial amounts of certain ascarosides. Whereas the ascaroside profiles of starved wild-type and *Ppa-daf-22.2 (tu504)* cultures remained largely unchanged from those observed for *ad lib* cultures, starved *Ppa-daf-22.1 (tu489)* worms produced near-wild-type levels of the ascaroside pasc#12, corresponding to at least 20-fold up-regulation of this compound relative to the amounts observed in *ad lib Ppa-daf-22.1 (tu489)* worms (fig. 4B). In addition, the production of several other ascarosides, including ascr#1 and ascr#12, was up-regulated in starved *Ppa-daf-22.1 (tu489)* cultures, though less markedly. The double mutant *Ppa-daf-22.1; Ppa-daf-22.2* did not produce any ascarosides under starvation conditions, indicating that upregulation of ascaroside production in *Ppa-daf-22.1 (tu489)* in these conditions is due to *Ppa-daf-22.2* rather than some other, yet unidentified gene (fig. 4C and D). Notably, the chemical structures of pasc#12 and ascr#12 feature a 6-carbon carboxylic acid side chain, whereas all other major ascarosides in *P. pacificus* and *C. elegans* feature fatty acid side chains with an odd number of carbons. However, *Ppa*-DAF-22.2 does not seem to be exclusively involved in the production of ascarosides with a six-carbon side chain, since we also observed up-regulation of ascr#1 (seven-carbon side chain) and ascr#9 (five-carbon side chain) production in starved *Ppa-daf-22.1 (tu489)* cultures (figs. 1 and 4B).

## Gene Deletion Causes Reciprocal Up-Regulation in a Starvation-Independent Manner

Next, we wanted to test if the observed changes in the ascaroside profile of *Ppa-daf-22.1 (tu489)* under starved conditions resulted from changes in gene expression in the *Ppa-daf-22.2* gene. However, qPCR analysis revealed no significant difference in *Ppa-daf-22.2* mRNA levels between fed and starved conditions (fig. 5A). This suggests that the induction of ascaroside biosynthesis in *Ppa-daf-22.1 (tu489)* mutant animals under starved conditions is regulated at the posttranscriptional level, or that starvation affects the expression of other genes upstream of *daf-22* in the β-oxidation pathway. We also compared expression of *Ppa-daf-22.1* between wild-type and *Ppa-daf-22.2 (tu504)* under *ad lib* and starved conditions, and similarly compared *Ppa-daf-22.2* expression between wild type and *Ppa-daf-22.1 (tu489)*. Interestingly, we found reciprocal upregulation resulting in a 2-fold

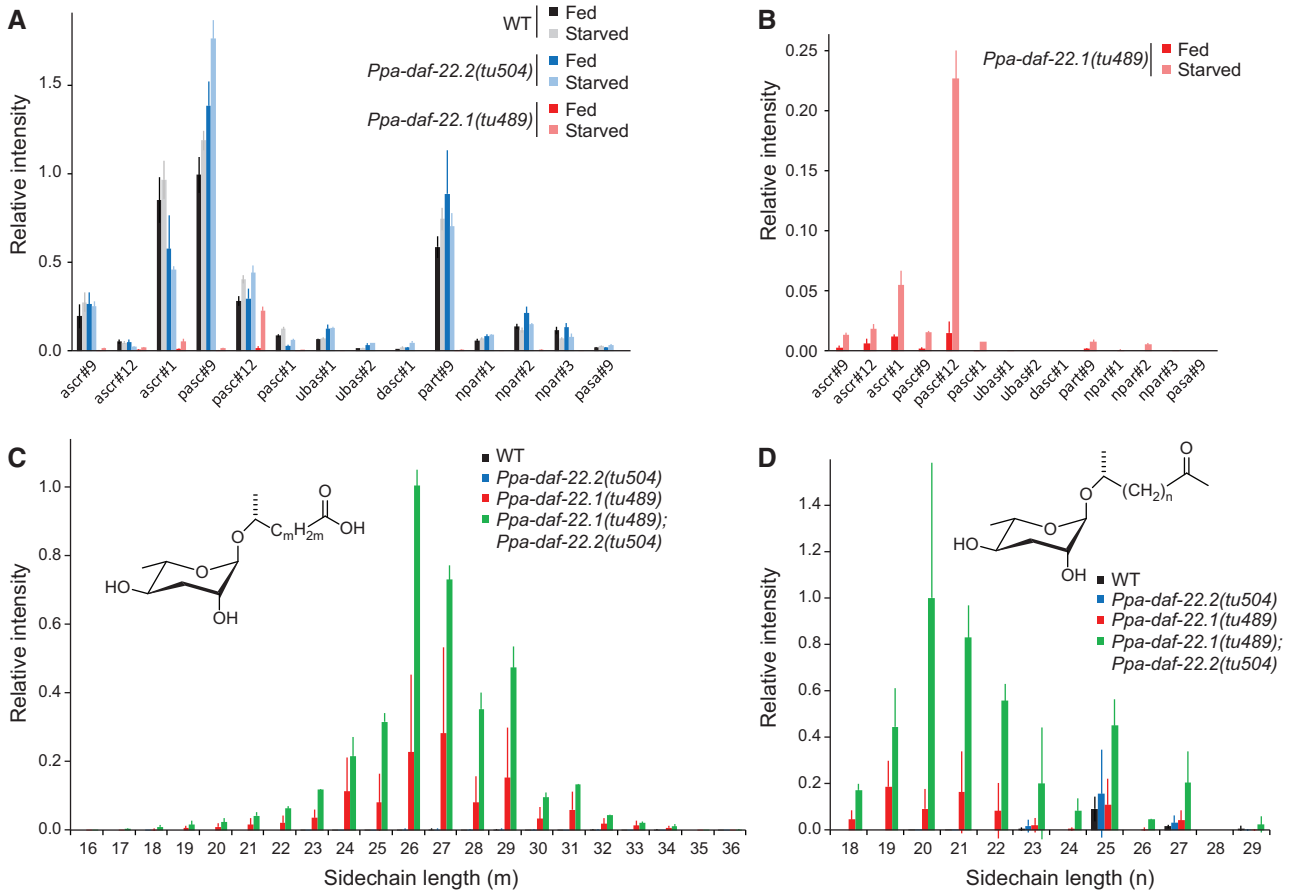


FIG. 4. Ascaroside profiles of *Ppa-daf-22* mutants as determined by LC–MS analysis. (*A*) Ascaroside profiles determined from *ad lib* and starved mixed-stage liquid cultures of *P. pacificus* wild-type and *daf-22* mutants (worm media extracts, exo-metabolomes). (*B*) Enlargement of data for *Ppa-daf-22.1(tu489)* shown in (*A*) to better visualize the effect of starvation on ascaroside production in this strain. Error bars in (*A*) and (*B*) are SD, $n = 3$. Relative intensities in (*A*, *B*) are normalized to the peak area of pasc#9 in the wild-type strain in the fed condition. (*C*, *D*) Long-chain carboxylic acid ascarosides (*C*) and methylketone ascarosides (*D*) accumulate in worm pellets (endo-metabolome) of the *Ppa-daf-22.1(tu489)* mutant and *Ppa-daf-22.1(tu489); Ppa-daf-22.2(tu504)* double mutant. No short-chain ascarosides were found in *Ppa-daf-22.1; Ppa-daf-22.2* double mutant. Error bars in (*C*, *D*) are SD, $n = 2$. Relative intensities in (*C*, *D*) are normalized to the peak area of the compound with the largest peak area in each panel.

(*Ppa-daf-22.1*) and 3- to 4-fold (*Ppa-daf-22.2*) increase of the remaining gene (fig. 5*A*).

### Extracts from *Ppa-daf-22.1 (tu489)* and *Ppa-daf-22.1; Ppa-daf-22.2* Double Mutants Do Not Induce Dauers

Finally, we tested extracts from *Ppa-daf-22* mutant cultures in dauer formation assays (fig. 5*B* and *C*). In *C. elegans*, knockout of *Cel-daf-22* results in loss of all dauer-inducing activity of liquid culture metabolome extracts (Golden and Riddle 1985), a result that we confirmed independently as a control experiment for studies described below (fig. 5*B*). Testing liquid culture metabolome extracts of *Ppa-daf-22.1 (tu489)* and *Ppa-daf-22.2 (tu504)* single and double mutants on *P. pacificus* wild-type animals, we found that metabolome extracts of *Ppa-daf-22.1 (tu489)* and *Ppa-daf-22.1; Ppa-daf-22.2 ad lib* worms had no dauer-inducing activity, whereas extracts from *Ppa-daf-22.2 (tu504) ad lib* worms retained most dauer-inducing activity compared with that of wild-type extracts (fig. 5*C*). Considering that the production of short-chain ascarosides and paratosides is largely abolished in both *Ppa-daf-22.1 (tu489)* and *Ppa-daf-22.1; Ppa-daf-22.2* double mutant *ad lib* cultures, these results indicate that short-chain ascarosides and paratosides are required for the dauer induction in *P. pacificus* (fig. 5*C*). Therefore, it appears that *Ppa*-DAF-22.1 is primarily responsible for the generation of dauer-inducing pheromones in *P. pacificus* under our *ad lib* conditions.

## Discussion

Evolutionary developmental biology (evo–devo) in animals has provided detailed insight into the evolution of developmental processes and developmental mechanisms and revealed a striking conservation of transcription factor and signaling pathway evolution. In contrast, the evolution of the biosynthetic processes underlying small molecule structure and function has attracted little attention, although it is intensively studied in microbes and plants (Downs 2006; Pichersky and Lewinsohn 2011). In this study, we have analyzed the genes encoding enzymes of the peroxisomal β-oxidation pathway involved in ascaroside and paratoside

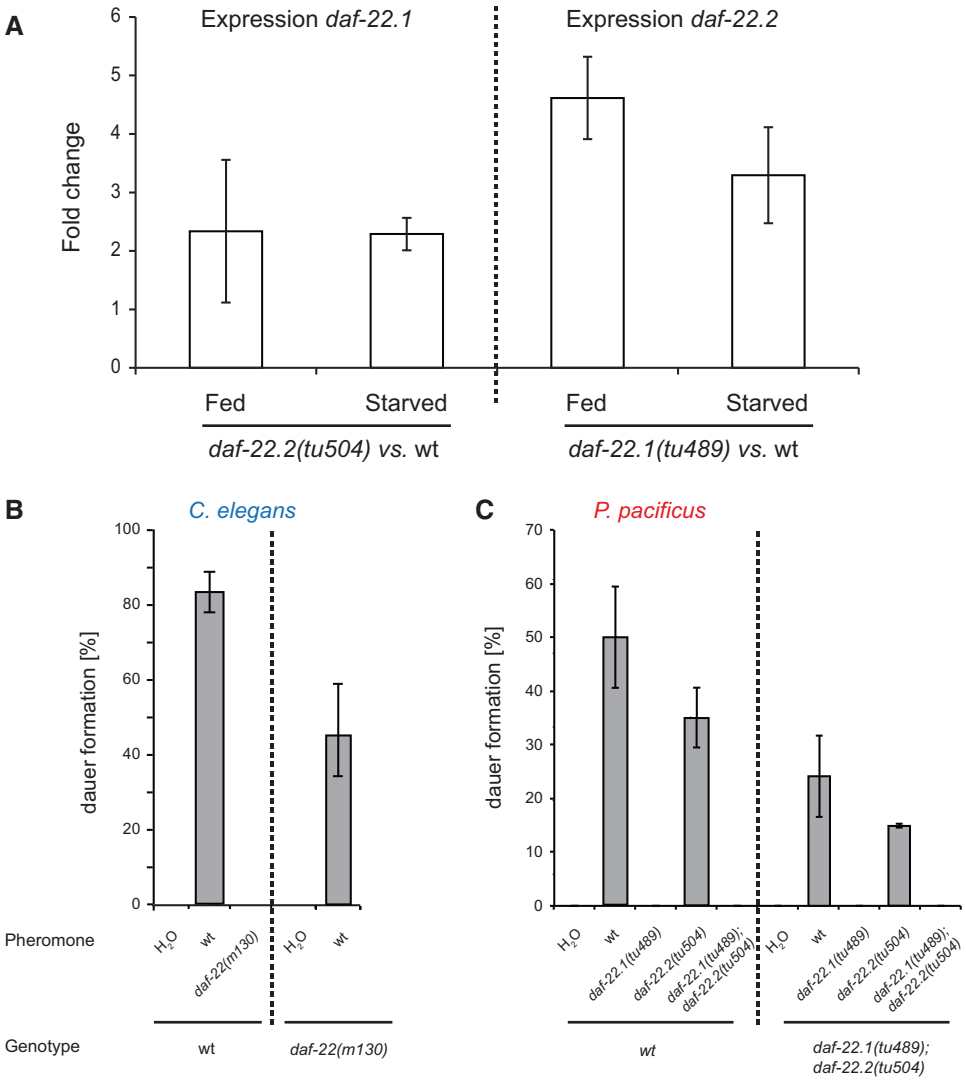


**Fig. 5.** *Ppa-daf-22.1* is the major factor in the synthesis of ascarosides in *P. pacificus*. (*A*) Up-regulation of *daf-22.1* and *daf-22.2*. Fold change relative to wild-type worms of *daf-22.1* and *daf-22.2* mutant worms determined by qRT-PCR under fed (*ad lib*) and starved conditions (mean and standard error for three biological replicates). (*B*) *Caenorhabditis elegans* dauer-induction assay using pheromone extracts from wild-type and *daf-22(m130)* on wild-type worms. Bars represent the mean with standard error of three independent biological replicates. (*C*) *Pristionchus pacificus* dauer-induction assay using pheromone extracts from wild-type, *daf-22.1*, *daf-22.2*, and the *daf-22.1;daf-22.2* double mutant on wild-type and double mutant worms. Bars represent the mean with standard error of three independent biological replicates.

biosynthesis in nematodes. Our analysis reveals rapid evolution, including gene duplications and domain switching of multiple genes, functional promiscuity, and environment-dependent regulation of individual enzymes. These alterations result in a complex mix of conservation and divergence of β-oxidation pathway structure and function.

First, bioinformatic comparison reveals that 1:1 orthologs of genes encoding the enzymes of peroxisomal β-oxidation are nearly absent above the genus level, with some genes showing even high birth and death rates within the genera *Caenorhabditis* and *Pristionchus*. This pattern is consistent with previous observations on the evolution of nematode detoxification-encoding enzymes (Markov et al. 2015). High evolutionary turnover rates seem to be the norm in nematode gene families and are in contrast to developmental control genes that are conserved as 1:1 orthologs in nematodes as well as other animals (Carroll 2005; Pires da Silva and Sommer 2003).

Second, domain shuffling is observed for multiple genes in the β-oxidation pathway, causing difficulties in the assignment of enzymatic function to individual proteins. To overcome these problems, we have used reverse genetic approaches by CRISPR/Cas9 engineered knockout mutants of individual paralogs of the thiolase *daf*-22 and have compared the small molecule profiles of wild-type and mutant strains, and their metabolome extracts in dauer-induction assays. Similar to *Cel-daf-22* worms (Izrayelit et al. 2012), along with disappearance of short-chain ascarosides, we observed build-up of very long-chain ascarosides and their corresponding methylketones as shunt metabolites in *Ppa-daf-22.1 (tu489)* (fig. 4C and D). These shunt metabolites are formed when very long-chain ascaroside precursors cannot be

processed normally due to the lack of thiolase enzymatic activity. This indicates that the *C. elegans* and *P. pacificus daf-22* genes catalyze similar biochemical processes.

Third, the analysis of *Ppa-daf-22* single and double mutants and the comparison of fed and starved cultures revealed another level of the regulation of peroxisomal β-oxidation. Under well-fed (*ad lib*) conditions mixed-stage or synchronized cultures of *Ppa-daf-22.1 (tu489)*, such as *Ppa-daf-22.1; Ppa-daf-22.2* double mutants, do not produce significant amounts of short-chain ascarosides and paratosides. In contrast, under starved conditions, *Ppa-daf-22.1 (tu489)*, mutants produce near-wild-type levels of a specific subset of ascarosides, indicating that a branch of peroxisomal β-oxidation involving DAF-22.2 becomes active under starvation conditions. This activity may require post-transcriptional regulation or upstream regulatory input, as *Ppa-daf-22.2* expression is not significantly upregulated under starvation.

In conclusion, our results show that orthology is a useful predictor of biosynthetic function of enzymes, but that metabolic pathways may diverge as a result of evolutionary changes that interact with other regulatory mechanisms. This partial uncoupling between the evolution of enzymes and the evolution of metabolic pathways implies that only interdisciplinary approaches of bioinformatics, genetic engineering, metabolomics, chemistry, and molecular biology can reveal the full scope of evolutionary alterations and their organismic consequences. Metabolomics is maturating as a research field that can provide such information and has already provided important results in a diversity of organisms including land plants, nematodes, and diatoms (Kuhlisch and Pohnert 2015).

## Materials and Methods

### Nematode Strains

All experimental analyses were done with the *P. pacificus* strain RS2333, a laboratory derivative of the original strain PS312 isolated in Pasadena, CA (USA) in 1988 (Sommer et al. 1996).

### Sequence Data Sampling and Phylogenetic Analyses

Phylogenetic analyses were performed as described previously (Markov et al. 2015). In short, protein sequences for *Caenorhabditis elegans, Caenorhabditis briggsae, Caenorhabditis remanei, Haemonchus contortus, Brugia malayi, Loa loa, Ascaris suum, and Trichinella spiralis* were collected from BLAST searches in GenBank. Sequences for *Bursaphelenchus xylophilus, Meloidogyne hapla, Panagrellus redivivus and Strongyloides ratti* were collected from BLAST searches in Wormbase version WS240 (Yook et al. 2012). Sequences of *P. pacificus* are from the HYBRID1 proteomics gene model dataset that is available on the website http://pristionchus.org (last accessed 10 May 2016) and were refined when necessarily by the new assembly dataset (SNAP2012). Sequences from the sister species *Pristionchus exspectatus* also come from the published genome assembly (Rödelsperger et al. 2014). Augustus predictions are available on http://pristionchus.org (last accessed 10 May 2016), whereas the SNAP predictions are available on http://parasite.wormbase.org/ (last accessed 10 May 2016). One additional experimental sequence from the Clade IV nematode *Heterodera glycines* was also incorporated. All manually improved sequence predictions are provided as dataset S1. Some corrected cDNA predictions for *P. pacificus* were added in the Hybrid1 community track database that is displayed in the genome browser from http://pristionchus.org. Collected sequences were aligned with Muscle (Edgar 2004) and alignments were checked by eye and edited with Seaview (Gouy et al. 2010). Phylogenetic trees were made using PHYML (Guindon and Gascuel 2003), a fast and accurate maximum likelihood heuristic method, using the best estimated substitution models, that turned out to be the LG model (Le et al. 2008) under slightly different detailed parameters detailed in the concerned figure captions. Reliability of nodes was assessed by the likelihood-ratio test (Anisimova and Gascuel 2006).

### CRISPR/Cas9 Deletion Mutants

CRISPR/Cas9-induced gene inactivation was performed as described previously (Witte et al. 2015). For *daf-22.1*, 288 F1 animals were screened for deletions by Sanger sequencing after injection. For *daf-22.2*, a coinjection procedure with the *dpy-1* marker gene enabled a reduction of screened animals to the progeny of 25 F1 animals. We obtained two mutant lines *Ppa-daf-22.1(tu489)* and *Ppa-daf-22.2(tu504)*, with a 7-bp deletion in *tu489* and a 7-bp insertion in *tu504*. All mutant lines including the double mutant were backcrossed twice.

### RNA Extraction

Nematodes were separated by centrifugation from the culture supernatant, which was extracted with methanol and analyzed for ascarosides using HPLC–MS. The worm pellet was frozen at −80 °C and then immediately transferred into Tri Reagent® (Sigma–Aldrich, Munich, Germany). After three rounds of freezing in liquid nitrogen and thawing at 37 °C to break open cells, RNA was extracted with the Direct-zol™ RNA MiniPrep (Zymo, Freiburg, Germany) following the manufacturer's protocol. RNA quality was determined with a NanoDrop ND 1000 spectrometer (PeqLab, Erlangen, Germany) and RNA integrity was checked with the Agilent RNA Nano Chip Assay (Agilent, Santa Clara, USA). Only RNA samples of high quality (OD 260/280 $>$ 2 and OD 260/230 $>$ 1.8) and high integrity were used for quantitative RT-PCR.

### Quantitative RT-PCR

In brief, cDNA was synthesized from complex RNA with oligoDT primers to enrich for mRNA with the Superscript II kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. RT-PCR was performed as described earlier (Schuster and Sommer 2012) using the LC-480 SybrGreen Mix and a LC480 light cycler (Roche, Mannheim, Germany). As reference genes *Ppa-β-tubulin* and *Ppa-actin* were used.

### Dauer Pheromone Extraction and Dauer Assays

Dauer pheromone purification was performed as described earlier (Ogawa et al. 2009). In brief, the pure cultures of all wild-type and mutant worms were grown in 500 ml liquid cultures. The supernatant was sterile-filtered and incubated with 30 g of activated charcoal under stirring. The charcoal was washed five times with water and the dauer pheromone was eluted from the charcoal with ethanol Ph. Eur. (Sigma–Aldrich). Ethanol was evaporated using a Rotavapor® R210 with cold trap (Buchi, Essen, Germany) and the remaining yellow pellet was re-suspended in 3 ml of water. Dauer formation assays were performed according to Bose et al. (2014). One hundred microliters of the pheromone extract were applied to the 6-cm NGM agar plates without peptone or cholesterol but with 50 μg/ml kanamycin (final concentration). Afterwards, kanamycin-inactivated OP50 and either *P. pacificus* freshly hatched J2 larvae or *C. elegans* L1 larvae were transferred to the assay plates. Note that in *P. pacificus*, the J1 stage hatches inside the eggshell. Therefore, the J2 is the first larval stage that can be collected and manipulated. Plates were incubated at 25 °C, and after 2 days dauer versus non-dauer larvae were counted. For all experiments, three independent replicates (three assay plates) for each treatment were performed.

### Metabolomic Profiling

To grow mixed-stage cultures, worms from a populated 10 cm NGM agar plate seeded with *Escherichia coli* OP50 were washed into 25 ml of S-complete medium and fed OP50 on Days 1 and 3 (starved) or on Days 1, 3, 5, and 8 (*ad lib*) for an 11-day culture period, whereas shaking at 22°C, 220 rpm. The cultures were then centrifuged and worm pellets and supernatant frozen separately, lyophilized and extracted with 95:5 ethanol:water for 12 h. The extract was dried *in vacuo*, resuspended in methanol and analyzed by LC–MS as described below. For synchronized cultures, we collected gravid adults and eggs from six 10-cm NGM agar plates fed with *E. coli* HB101, isolated eggs by alkali hypochlorite treatment, and let them hatch in M9 buffer overnight at room temperature. Hatched J2 larvae were transferred to S-complete medium with HB101 (70,000 worms per 25 ml) and grown at 22°C. After 3 days, we collected, froze, and lyophilized the medium. After overnight methanol extraction, the extract was dried in vacuo, resuspended in methanol and analyzed by LC/MS. LC/MS analysis was performed on a Dionex 3000 UPLC coupled with a Thermo Q Exactive high-resolution mass spectrometer. In the case of analyses targeted at short-chain ascarosides, metabolites were separated using water–acetonitrile gradient on Agilent Zorbax Eclipse XDB-C18 column, 150 mm × 2.1 mm, particle size 1.8 μm, and maintained at 40°C. Solvent A: 0.1% formic acid in water; Solvent B: 0.1% formic acid in acetonitrile. A/B gradient started at 5% B at 1.5 min after injection and increased linearly to 100% B at 12.5 min. Long-chain ascarosides were analyzed on the same column using an acetonitrile–propanol gradient. Solvent B: As above. Solvent C: 100% propanol-2. B/C gradient started at 5% C at 1.5 min after injection and increased linearly to 75% C at 12.5 min. Most ascarosides were detected as $[M-H]^-$ ions in the negative ionization mode. Methylketones were observed as $[M+Na]^+$ adducts in the positive ionization mode. Metabolites were identified based on their high-resolution masses (< 1 ppm), fragmentation spectra, and comparison of retention times with those of synthetic standards.

## Supplementary Material

Supplementary figures S1–S7 and dataset S1 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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