

Pristionchus pacificus daf-16 is essential for dauer formation but dispensable for mouth form dimorphism

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SUMMARY
The nematode Pristionchus pacificus shows two forms of phenotypic plasticity: dauer formation and dimorphism of mouth form morphologies. It can therefore serve as a model for studying the evolutionary mechanisms that underlie phenotypic plasticity. Formation of dauer larvae is observed in many other species and constitutes one of the most crucial survival strategies in nematodes, whereas the mouth form dimorphism is an evolutionary novelty observed only in P. pacificus and related nematodes. We have previously shown that the same environmental cues and steroid signaling control both dauer formation and mouth form dimorphism. Here, we examine by mutational analysis and whole-genome sequencing the function of P. pacificus (Ppa) daf-16, which encodes a forkhead transcription factor; in C. elegans, daf-16 is the target of insulin signaling and plays important roles in dauer formation. We found that mutations in Ppa-daf-16 cause strong dauer formation-defective phenotypes, suggesting that Ppa-daf-16 represents one of the evolutionarily conserved regulators of dauer formation. Upon strong dauer induction with lophenol, Ppa-daf-16 individuals formed arrested larvae that partially resemble wild-type dauer larvae, indicating that Ppa-daf-16 is also required for dauer morphogenesis. By contrast, regulation of mouth form dimorphism was unaffected by Ppa-daf-16 mutations and mutant animals responded normally to environmental cues. Our results suggest that mechanisms for dauer formation and mouth form regulation overlap partially, but not completely, and one of two key transcriptional regulators of the dauer regulatory network was either independently co-opted for, or subsequently lost by, the mouth form regulatory network.

KEY WORDS: C. elegans, P. pacificus, Dauer formation, DAF-16/FOXO, Phenotypic plasticity

INTRODUCTION
Many organisms show an ability to adopt multiple phenotypes depending on environmental conditions. Such plasticity of phenotypes not only enables these organisms to adapt to changing environments but may also facilitate phenotypic evolution (West-Eberhard, 2003). Despite their importance, the molecular mechanisms that underlie the evolution of phenotypic plasticity have only recently begun to be understood. One example of phenotypic plasticity is nematode dauer formation (Riddle and Albert, 1997). Nematodes go through four larval stages before they reproducibly mature. When environmental conditions are unsuitable for reproduction, many nematode species arrest their development at the third larval stage and form specialized larvae called dauer larvae (Lee, 2002). Dauer larvae are resistant to various environmental stresses and can survive for months without feeding, representing one of the major survival strategies in these organisms (Lee, 2002; Ogawa and Sommer, 2009).

In C. elegans dauer formation, environmental cues indicating harsh conditions are processed through several signaling pathways and eventually lower the levels of a class of steroid hormones called the dafachronic acids (DAs). The decrease in DA levels shifts the nuclear hormone receptor DAF-12 from the ligand-bound to ligand-free form, which specifies the dauer fate (Antebi et al., 2000; Motola et al., 2006). DAF-12 and the forkhead transcription factor DAF-16 [the C. elegans forkhead box O (FOXO) homolog], represent two crucial transcription factors that are required for the formation of dauer larvae. Loss-of-function mutations in C. elegans (Cel) daf-12 completely abrogate the ability to form dauer larvae, resulting in strong dauer formation-defective (Daf-d) phenotypes (Antebi et al., 1998). Cel-daf-12 mutations suppress dauer formation constitutive (Daf-c) mutations in multiple signaling pathways (Gerisch et al., 2001; Jia et al., 2002; Thomas et al., 1993; Vowels and Thomas, 1992), suggesting that DAF-12 is one of the ultimate regulators of dauer formation. We have previously shown that DAs and DAF-12 regulate the formation of dauer larvae in P. pacificus, which is distantly related to C. elegans (Fig. 1A) (Ogawa et al., 2009).

Mutations in Cel-daf-16 severely impair the ability to form dauer larvae and cause a Daf-d phenotype (Lin et al., 1997; Ogga et al., 1997), but they show a phenotypic spectrum different from that of Cel-daf-12. In combination with other Daf-c mutations or under dauer-inducing environmental conditions, individuals carrying a Cel-daf-16 mutation fail to achieve a fully developed dauer morphology, suggesting that Cel-daf-16 is essential for the normal morphogenesis of dauer larvae (Gottlieb and Ruvkun, 1994; Vowels and Thomas, 1992). However, several Daf-c mutations and strong dauer-inducing environmental conditions can partially suppress the Daf-d phenotype of Cel-daf-16 mutations. When sterol precursors of DAs that antagonize DAF-12 activity are completely depleted from the culture media, the daf-16 Daf-d phenotype is partially suppressed (Matyash et al., 2004). Under these conditions, Cel-daf-16 animals form partial dauer larvae that are characterized by both dauer-like and non-dauer-like features, such as a non-constricted pharynx and SDS susceptibility, suggesting that Cel-daf-16 is dispensable for the developmental arrest and for part of dauer morphogenesis upon DAF-12 activation.

In addition to dauer formation, P. pacificus and related nematodes have a second, unique phenotypic plasticity, which is a polyphenism in the mouth form morphology (Fig. 1A). P. pacificus individuals can have either a narrow and long mouth opening,
called stenostomatous (St), or a wide and shallow mouth opening, which is referred to as eurystomatous (Eu) (Bento et al., 2010; von Lieven and Sudhaus, 2000). This dimorphism is regulated by environmental cues, including starvation and population density, which are also known to regulate dauer formation. Δ7-DA and mutations in DAF-12 strongly affect the regulation of the mouth form dimorphism, indicating that these signaling mechanisms for the regulation of dauer formation were co-opted for the regulation of this novel plastic trait (Bento et al., 2010).

**MATERIALS AND METHODS**

**Nematode strains and cultures**

Worms were grown on NGM plates with a lawn of *E. coli* strain OP50. The nematode strains used in this study were PS312 (the wild-type *P. pacificus* strain) and *Ppa-daf-16* (*tu414, tu415, tu416*).

**Complementation tests**

We constructed a double-mutant strain carrying *Ppa-daf-16* (*tu416*) and a dumpy mutation *Ppa-pdl-1*. Homophrodites of this double-mutant strain were crossed with males of other Daf-d strains. Non-dumpy F1 progeny resulting from these crosses were individually placed into wells of 96-well plates containing S-medium and *E. coli* food. After replenishing the *E. coli* food several times, we obtained densely grown worm cultures in these wells. After crosses of *tu416* with *tu414* or *tu415*, no dauer larvae were found in the cultures, indicating that these mutants do not complement each other and that they are allelic.

**Preparation of concentrated dauer phenomone extracts and dauer formation assay**

We prepared concentrated dauer phenomone extracts as previously described with slight modifications (Ogawa et al., 2009). The phenomone bound to charcoal was sequentially eluted with methanol and 1%, 5%, 10% and 20% toluene in methanol. Eluates were combined and evaporated under vacuum. The dauer formation assay was performed as previously described (Ogawa et al., 2009).

**Preparation of lophenol plates**

We slightly modified the reported protocol (Matyas et al., 2004). Agarose washed with 1:1 chloroform:methanol was dissolved in S-medium without cholesterol. We used *E. coli* strain NA22 grown in Dulbecco’s Modified Eagle Medium (Invitrogen) as food source. Lophenol (5 µg/ml; gift of F. Mende and T. Kurzchalia, Max-Planck Institute for Molecular Cell Biology and Genetics, Dresden, Germany) was added to the bacterial suspension before being placed on agarose plates.

**Mutant allele identification by Illumina genome analyzer and *Ppa-daf-16* molecular structure**

We sequenced the genomes of the two alleles *tu415* and *tu416* and generated 42 bp paired-end reads on the Illumina Solexa platform. The strains carrying *tu415* and *tu416* were sequenced to an average depth of 32× and 20×, respectively. Short reads were aligned with Maq 0.7.1 (Li et al., 2008) using default settings. Single-nucleotide polymorphism (SNP) calling was performed with the Maq cns2snp command using default settings. Initially, we retained only homozygous SNP calls in genomic positions with a minimal coverage of three short reads. We further masked out SNPs that were already present in a resequencing run of our nematode wild-type strain or that fell into genomic positions with a low Phred score (<90) in the reference genome assembly.

After identifying a nonsense mutation in *tu416* by Illumina sequencing, we sequenced Ppa-daf-16 cDNAs from *tu416* and the two other strains and found nonsense mutations in exons that are missing in the genome assembly. The mutations identified are G-to-A (W212 stop), C-to-T (R300 stop) and C-to-T (R222 stop) substitutions in *tu414, tu415* and *tu416*, respectively.

**Assays for mouth form dimorphism**

The proportions of Eu and St worms were visually examined and starvation and application of dauer phenomone were performed (Bento et al., 2010) as described. Assays involving DA followed the previously described protocol (Bento et al., 2010), except that DA was in a water solution instead of ethanol; this change was made because *Ppa-daf-16* strains have been shown to be ethanol intolerant and to have extremely reduced broods when treated with ethanol solution. The datasets were analyzed with one-way ANOVAs and ANOVA assumptions were graphically tested (Quinn and Keough, 2002).

**RESULTS AND DISCUSSION**

During our previous study, we performed genetic screens for Daf-d mutants using low-cholesterol liquid medium to obtain *P. pacificus* (*Ppa*) daf-12 mutations (Ogawa et al., 2009). Cholesterol serves as a precursor for the synthesis of DAs, and cholesterol deficiency strongly enhances dauer formation. Therefore, we reasoned that screening for Daf-d mutants in cholesterol-deficient medium should bias the mutations to genes that act in parallel to, or downstream of, DAs such as *Ppa-daf-12*. Whereas three previously described mutations constitute the *Ppa-daf-12* locus on chromosome I, we found three other mutations, *tu414, tu415* and *tu416*, that map to chromosome V. Complementation tests indicated that these mutations are in the same complementation group.

To clone the second Daf-d locus, we subjected genomic DNA of two of the mutant strains to whole-genome sequencing by Illumina genome analyzer (see Materials and methods for details) (Sarin et al., 2008). In one of the strains (*tu416*) we found a nonsense mutation in an exon of *Ppa-daf-16* (Fig. 2A and see Fig. S1 in the supplementary material). cDNA sequencing of *Ppa-daf-16* in *tu416* confirmed this mutation. Furthermore, cDNA sequencing of *Ppa-daf-16* in the alleles *tu414* and *tu415* revealed that these strains also contain a nonsense mutation in *Ppa-daf-16* (Fig. 2A and see Fig. S1 in the supplementary material). *Ppa-daf-16* encodes a forkhead (FH) transcription factor that shows greater than 90% identity to C.
Fig. 2. Structure and conservation of P. pacificus DAF-16. (A) Primary structure of DAF-16. The forkhead (FH) domain is indicated. Three akt phosphorylation motifs are indicated by arrows. Amino acids mutated in the three alleles are indicated by arrowheads. The amino acid stretch that shows similarity to human FOXO3 is indicated by a double-headed arrow. (B) Amino acid sequence alignment of the FH domain of P. pacificus and C. elegans DAF-16. Identical amino acids are indicated by dots.

elegans DAF-16a within the FH domain (Fig. 2B). There are three akt/protein kinase B phosphorylation consensus sites (RXRXXS/T), which correspond to the four consensus sites in akt/protein kinase B phosphorylation consensus sites (RXRXXS/T), indicating by a double-headed arrow.

P. pacificus (shows similarity to human FOXO3 is indicated by a double-headed arrow. The three alleles are indicated by arrowheads. The amino acid stretch that phosphorylation motifs are indicated by arrows. Amino acids mutated in structure of DAF-16. The forkhead (FH) domain is indicated. Three akt

Fig. 3. daf-16 is involved in dauer formation in P. pacificus. (A) Response of the three Ppa-daf-16 alleles tu414, tu415 and tu416 to dauer pheromone. Error bars denote 95% binomial confidence intervals. (B) Morphologies of dauer larvae induced on a lophenol plate. Arrowheads indicate the terminal bulb, which is completely remodeled in the wild type but incompletely remodeled in Ppa-daf-16 (tu415).

Next, we examined whether Ppa-daf-16 is also involved in the regulation of the mouth form dimorphism, as is the case for DA and Ppa-daf-12, and found that Ppa-daf-16 mutant animals behave like wild type. First, all three alleles of Ppa-daf-16 showed a ratio of Eu to St worms that was similar to that of wild-type animals under well-fed conditions (Fig. 4A). Second, under starved conditions, the proportions of Eu worms were similar for all Ppa-daf-16 alleles and the wild type (Fig. 4A). We also tested the effect of dauer pheromone. Wild-type worms respond to dauer pheromone by increasing the proportion of Eu worms when treated with dauer pheromone that was comparable to the response of wild type (Fig. 3B). Finally, we tested the effect of 7-DA (Fig. 4C). Administration of 7-DA strongly influenced mouth form dimorphism in a Ppa-daf-16 mutant background and decreased the proportion of Eu worms to a comparable degree as in wild-type worms. Taken together, these results suggest that, in contrast to DAF-12, DAF-16 has no or only a minor role in the regulation of mouth form dimorphism in P. pacificus.

Our results suggest that DAF-16 is an evolutionarily conserved regulator of dauer formation in free-living nematodes. Mutations in Cel-daf-16 result in impairment of dauer regulation, as well as defects in the morphogenesis of dauer larvae (Vowels and Thomas, 1992). Mutations in the daf-16 ortholog in P. pacificus also affect dauer formation and result in dauer phenotypes similar to those observed in C. elegans (Fig. 3A,B). As P. pacificus and C. elegans are only distantly related (Dieterich et al., 2008), our results demonstrate deep evolutionary conservation of the function of daf-16 in dauer formation.
In contrast to dauer formation, mutations in Ppa-daf-16 did not affect the regulation of mouth form dimorphism. We have shown previously that the polyphenism of mouth form is regulated by starvation and dauer pheromone, two environmental cues that also regulate dauer formation (Bento et al., 2010). In addition, DAF-12, a nuclear receptor that regulates the dauer diapause and developmental age in C. elegans. (2010). A hormonal signaling pathway influencing C. elegans metabolism, reproductive development, and adult longevity. Develop. Cell 129, 221-231.


Fig. 4. daf-16 is dispensable for mouth form dimorphism in P. pacificus. The effect of (A) starvation, (B) dauer pheromone and (C) Δ7-dafachronic acid on the regulation of mouth form dimorphism in wild-type and Ppa-daf-16 strains. Data are mean ± s.d.