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RESEARCH ARTICLE

Two independent sulfation processes regulate mouth-form plasticity in the nematode *Pristionchus pacificus*

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ABSTRACT

Sulfation of biomolecules, like phosphorylation, is one of the most fundamental and ubiquitous biochemical modifications with important functions during detoxification. This process is reversible, involving two enzyme classes: a sulfotransferase, which adds a sulfo group to a substrate; and a sulfatase that removes the sulfo group. However, unlike phosphorylation, the role of sulfation in organismal development is poorly understood. In this study, we find that two independent sulfation events regulate the development of mouth morphology in the nematode *Pristionchus pacificus*. This nematode has the ability to form two alternative mouth morphologies depending on environmental cues, an example of phenotypic plasticity. We found that, in addition to a previously described sulfatase, a sulfotransferase is involved in regulating the mouth-form dimorphism in *P. pacificus*. However, it is unlikely that both of these sulfation-associated enzymes act upon the same substrates, as they are expressed in different cell types. Furthermore, animals mutant in genes encoding both enzymes show condition-dependent epistatic interactions. Thus, our study highlights the role of sulfation-associated enzymes in phenotypic plasticity of mouth structures in *Pristionchus*.

KEY WORDS: *Pristionchus pacificus*, Developmental plasticity, Sulfotransferases, Developmental switch gene, *Eud-1*/sulfatase

INTRODUCTION

Phenotypic plasticity is the ability of an organism to develop different phenotypes from the same genotype in response to environmental cues, and has been suggested to facilitate the evolution of phenotypic novelty and diversity (Pigliucci, 2001; West-Eberhard, 2003; Moczek et al., 2011; Susoy and Sommer, 2016). Often also referred to as 'developmental plasticity', the phenomenon is widespread in nature, and is best known in plants and insects. For example, beetle horn development, butterfly wing polyphenisms, phase transition in locusts and the formation of castes in social insects all represent plastic traits, indicating the importance of plasticity for the physiology, ecology and evolution of these organisms (Moczek, 1998; Beldade and Brakefield, 2002; Ernst et al., 2015; Corona et al., 2016; Fischman et al., 2017). Not surprisingly, plasticity has become a prominent idea extensively discussed in the literature (Bateson et al., 2017).

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Experimental studies of phenotypic plasticity have been restricted, owing to the scarcity of model organisms that provide genetic and molecular tools for mechanistic insight. However, several recent studies in insects provide molecular mechanisms of plasticity (Wang and Kang, 2014). In addition, investigations in the nematode model organism *Pristionchus pacificus* focus on phenotypic plasticity in the context of the formation of alternative feeding structures (Sommer and McGaughran, 2013; Sommer et al., 2017). *P. pacificus* has two alternative and discontinuous mouth-form morphologies, the eurystomatous (Eu) and stenostomatous (St) forms (Bento et al., 2010). The Eu mouth form is wider and has a large claw-like dorsal tooth and an opposing right subventral tooth, whereas the St mouth form is narrower, having only a flint-shaped dorsal tooth (Fig. 1A,B). Mouth morphology once formed is irreversible. The dimorphism is associated with predatory feeding behavior, as Eu worms have the extraordinary ability to prey on other nematodes including *Caenorhabditis elegans* (Fig. 1C), whereas St worms are strictly bacteriovorous (Serobyan et al., 2014; Wilecki et al., 2015; Lightfoot et al., 2016).

P. pacificus is amenable to the genetic analysis of mouth-form plasticity for several reasons (Sommer et al., 2017). First, *P. pacificus* is a self-fertilizing hermaphrodite, which can be propagated as isogenic strains, thereby simplifying the analysis of plasticity. Similar to the distantly related *C. elegans*, *P. pacificus* has a fast generation time of 4 days under laboratory conditions (see Materials and Methods) and can be grown on *Escherichia coli* OP50, all of which resulted in the development of forward genetic protocols for mutagenesis (Sommer and Carta, 1996). Second, a chromosome-scale genome assembly, as well as methods for transgenesis and CRISPR/Cas9 engineering are available for *P. pacificus* (Dieterich et al., 2008; Rödelsperger et al., 2017; Schlager et al., 2009; Witte et al., 2014). Third, the wild-type strain RS2333 of *P. pacificus* exhibits mouth-form plasticity that – in addition to conditional factors, such as starvation and pheromones (Serobyan et al., 2013; Bose et al., 2012) – also depends on stochasticity (Susoy and Sommer, 2016). Regardless of the mouth form of the hermaphroditic mother, offspring will have 70-90% Eu and 10-30% St mouth forms under standard laboratory conditions with only OP50 as food (Serobyan et al., 2014). Thus, even in the absence of environmental perturbations, both plastic traits are developed in parallel, making *P. pacificus* an ideal system for genetic screens and the identification of monomorphic mutants.

Over the years, several genetic, epigenetic and environmental factors were identified to play a role in mouth-form determination of *P. pacificus* (Fig. 1D). Environmental cues such as starvation and crowding can shift the mouth-form ratio towards more Eu animals (Serobyan et al., 2013). In earlier studies, it was shown that treatment with the steroid hormone dafachronic acid results in fewer animals with Eu form (Bento et al., 2010), whereas treatment with the pheromone dasc#1 induces Eu forms (Bose et al., 2012). More

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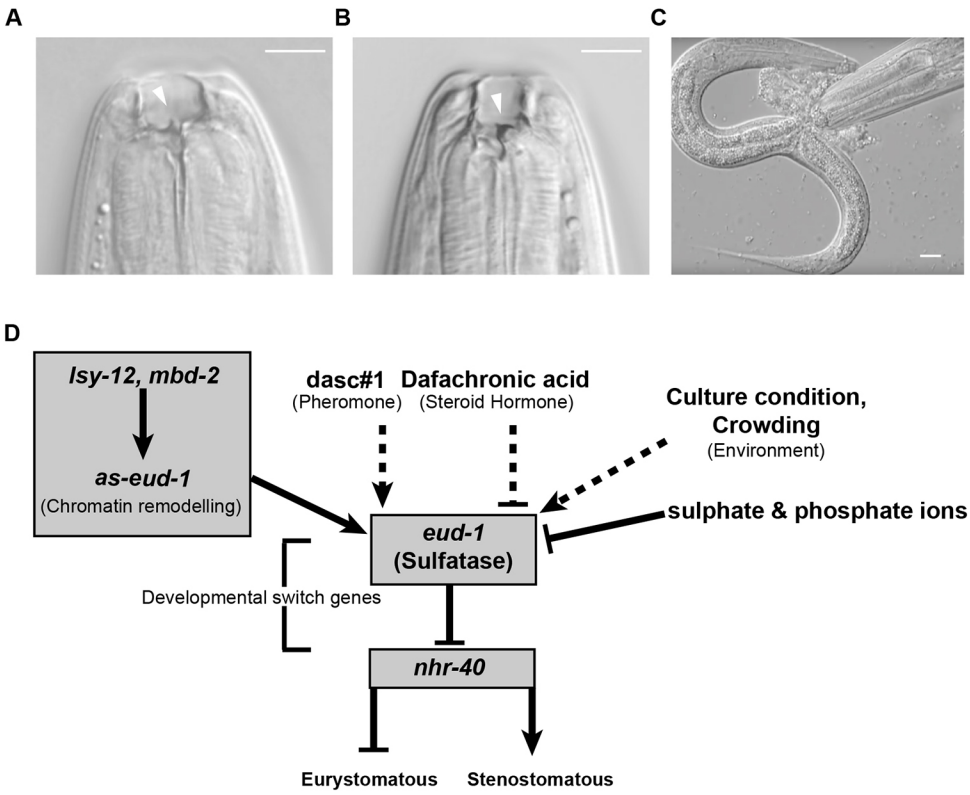

Fig. 1. *P. pacificus* exhibits mouth-form dimorphism. (A-C) Representative images of eurystomatous mouth form of *P. pacificus* (A), stenostomatous mouth morph of *P. pacificus* (B), *P. pacificus* killing and feeding on *C. elegans* (C). The arrowhead in A indicates the subventral denticle of an Eu animal, whereas the arrowhead in B indicates the dorsal tooth in an St animal. The smaller worm in C is an L2-stage *C. elegans*. Internal body material is coming out of the *C. elegans* owing to the *P. pacificus* (larger worm) biting it. (D) Representation of relationships among various known factors that affect mouth form. Scale bars: 10 µm.

recently, liquid culture of worms in S-medium was identified as a growth condition that also lowers the abundance of Eu animals (Werner et al., 2017).

Using the forward genetic tools available in *P. pacificus* with its hermaphroditic mode of reproduction resulted in the discovery and characterization of several genes involved in mouth-form regulatory pathways. *eud-1*, a gene coding for an aryl-sulfatase, was described as a developmental switch because *eud-1* mutants are all-St animals, whereas worms overexpressing *eud-1* all have the Eu mouth form (Ragsdale et al., 2013). The discovery of a developmental switch gene in the regulation of plasticity confirmed a long-standing theory that was originally developed in the context of caste differentiation in wasps and other hymenopterans (West-Eberhard, 2003). *eud-1* is expressed in neurons and is thought to be involved in either environmental sensing or decision making in the mouth-form determination process. NHR-40, a nuclear hormone receptor was identified in a suppressor screen of *eud-1* (Kieninger et al., 2016). *nhr-40* null mutants have only Eu mouth forms, whereas animals overexpressing *nhr-40* have only St forms, indicating that the developmental switch of *P. pacificus* mouth-form plasticity is not a single gene but a genetic network. *nhr-40* is also expressed in neurons and, similar to *eud-1*, acts in cells far away from those that form the teeth in the mouth (Kieninger et al., 2016).

Finally, multiple epigenetic factors – including the histone acetyltransferase *lsy-12*, the methyl binding protein *mbd*-2 and an antisense RNA at the *eud-1* locus, as-*eud-1* – were also shown to affect mouth-form plasticity (Serobyan et al., 2016). Interestingly, all these factors positively regulate *eud-1* levels suggesting that the switch gene *eud-1* is a primary target of mouth-form regulation. In agreement with this observation, EUD-1 protein is sensitive to small molecules. For example, treatment of animals with small molecules like sulfate and phosphate ions has been shown to induce the St form, and is thought to act by inhibition of EUD-1 (Ragsdale et al., 2013). However, only a very small number of bioactive compounds were tested for their potential role in mouth-form regulation.

The key role of the sulfatase EUD-1 in controlling mouth-form plasticity hints at the importance of sulfation processes. Sulfation, also described as sulfonation or sulfoconjugation, is one of the most fundamental biochemical modifications in various biomolecules including proteins, steroids, glycolipids and glycoproteins (Strott, 2002). It is present in organisms ranging from bacteria to humans, and has been shown to be essential for a multitude of biological processes, such as hormone metabolism, xenobiotic metabolism, and intra- and extracellular localization of sulfated molecules (Strott, 2002; Kauffman, 2004). The sulfation pathway is considered to be reversible, and it consists of two major enzyme families, sulfotransferases and sulfatases (Coughtrie et al., 1998). Sulfotransferases are responsible for the transfer of a sulfonate or sulfo group ($-SO_3^-$) to a substrate. They are further divided into cytosolic and membrane-bound categories. The cytosolic sulfotransferases are responsible for sulfation of small molecules including xenobiotics, whereas their membrane-bound counterparts are associated with Golgi membranes and catalyze sulfation as a post-translational modification of proteins (Negishi et al., 2001). In general, sulfation of xenobiotics by cytosolic sulfotransferases is essential for detoxification and elimination of these compounds. In contrast, sulfatases catalyze the hydrolysis of sulfate esters formed by the action of a sulfotransferase (Hanson et al., 2004). This enzymatic module of sulfation (sulfotransferase-sulfatase) is well studied in various cancers, especially breast and ovarian cancers, for which tumor growth is often associated with steroid metabolism (Rižner, 2016). However, unlike the phosphorylation module (kinase-phosphatase), sulfation has not been studied extensively in the context of model systems and, as a result, relatively little is known about the involvement of sulfation in organismal processes in the context of development or ecology. One of the possible

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functions could be the perception of environmental cues based on the known role of sulfation in steroid and hormonal metabolism, and the fact that many environmental biochemical signals are lipophilic in nature. Another potential function could be a role in intracellular signaling, because sulfation can change the nature and activity of various bioactive compounds in a cell.

Here, we performed a pharmacological screen by treating *P. pacificus* with several bioactive compounds and examined their effect on mouth-form ratio. We found that bisphenol A, tyramine and dopamine induce St mouth forms. As all three of these phenolic compounds are potential substrates of cytosolic sulfotransferases, we examined a potential role of sulfotransferases in regulating mouth-form plasticity. We generated knockout mutants in all five cytosolic sulfotransferases in *P. pacificus* (arbitrarily named *sult-1* to *sult-5*), and found that *sult-1* mutants resulted in all or preferentially Eu animals. Surprisingly, *sult-1* expression studies and epistasis analysis between *sult-1* and *eud-1* suggest that both genes act in different cells. Our analysis provides the first evidence for the function of a sulfotransferase in the regulation of phenotypic plasticity and indicates that at least two independent sulfation processes are involved in mouth-form determination in *P. pacificus*.

RESULTS

Bisphenol A, tyramine and dopamine induce St mouth forms

To extend previous studies on the effect of bioactive compounds on the regulation of mouth-form plasticity (Ragsdale et al., 2013), we decided to study the effect of a series of small molecules. We treated the highly Eu wild-type strain RS2333 from California with 23 different bioactive compounds (Fig. 2A). Because we would not be able to identify Eu form-inducing chemicals in this screen, we performed similar assays on another *P. pacificus* strain, RSC019, from La Réunion Island, which has equal frequencies of Eu and St animals (Fig. 2B). For all assays, three J4 hermaphrodites were placed on agar plates containing *E. coli* OP50 and one of the compounds. The progeny of these three hermaphrodites were scored for mouth-form phenotypes once they reached adulthood. Animals of both RS2333 and RSC019 strains showed strongest reduction in Eu form frequency in bisphenol A, tyramine and dopamine, out of all the compounds tested, at the applied concentration (10 µM) (Fig. 2A,B, Fig. S1). In addition, synephrine, octopamine, insulin and S-adenosyl methionine showed a relatively weak reduction of Eu form frequency in RS2333, but not in RSC019. Some molecules (cyclic AMP, chondroitin sulfate and acetyl-CoA) have a Eu form-inducing effect in RSC019 only (Fig. 2B). For this study, we focused on molecules that induce the strongest reduction of Eu frequency in both the RS2333 and RSC019 strains, i.e. bisphenol A, tyramine and dopamine. Interestingly, all three phenolic compounds are potential substrates of cytosolic sulfotransferases (Brix et al., 1999; Hattori et al., 2006; Yasuda et al., 2007). For example, in *C. elegans*, the sulfotransferase *ssu-1* has been described to most efficiently sulfate bisphenol A out of all the endogenous and xenobiotic compounds tested (Hattori et al., 2006). This intrigued us because the sulfatase *eud-1* was already described as a developmental switch gene for mouth-form regulation (Ragsdale et al., 2013), and thus a sulfation module consisting of a sulfatase and a sulfotransferase might modify common substrates during mouth-form regulation. Also, *C. elegans ssu-1* (*Cel-ssu-1*) is expressed in neurons including amphid neurons, which are related to those cells expressing *P. pacificus eud-1* (Ragsdale et al., 2013; Carroll et al., 2006). Therefore, we targeted cytosolic sulfotransferases in *P. pacificus* as potential regulators of mouth-form plasticity.

A mutant library of *P. pacificus* cytosolic sulfotransferases

Next, we examined the presence and phylogenetic relationship of sulfotransferase genes in the nematodes *P. pacificus*, *C. elegans* and *Caenorhabditis briggsae* using the fruit fly *Drosophila melanogaster* as an outgroup for phylogenetic reconstruction (Fig. 3). *P. pacificus* has 17 sulfotransferases, a massive expansion in comparison to *C. elegans* and *C. briggsae*, which have seven genes each. In particular, *P. pacificus* has a large number of paralogs to the single *C. elegans* cytosolic sulfotransferase *ssu-1*. Five *P. pacificus* genes are most closely related to *Cel-ssu-1* and four additional genes cluster with a *Drosophila*-specific expansion. Given these phylogenetic clusters, we decided to systematically study the five closest paralogs of *Cel-ssu-1* in *P. pacificus*. For this, we arbitrarily named these five genes *sult-1* to *sult-5*. We employed the CRISPR/Cas9 technique to obtain knockout mutants for all five genes (Fig. 4). In total, we isolated ten mutants as described in Fig. 4, Fig. S2 and Table 1. Only nonsense alleles or those resulting in frame-shift mutations were selected for phenotypic characterization.

sult-1 has a strong mouth-form phenotype

To study the potential role of *sult-1* to *sult-5* in mouth-form regulation, we have grown all mutant lines under two different culture conditions that generate preferentially Eu or St mouth forms in wild-type animals, respectively (Werner et al., 2017). Additionally, we tested hermaphrodites and males separately, as wild-type RS2333 males are highly St, unlike hermaphrodites (Serobyan et al., 2013). When grown on standard nematode growth medium (NGM) agar plates, hermaphrodites of all mutant lines showed highly Eu mouth-form ratios that were not different from RS2333 animals (Fig. 5A). In contrast, we found that in males, alleles of *sult-1* are either completely Eu (*tu1061*) or highly Eu (*tu1232*), whereas wild-type males are highly St (Fig. 5B). Similarly, *sult-2(tu1063)* mutant males also had a significant increase in Eu frequency compared with wild type (Fig. 5B). Therefore, we employed liquid culture conditions for scoring mouth-form phenotypes because such conditions were previously shown to reduce Eu frequency in hermaphrodites (Werner et al., 2017). Indeed, *sult-1(tu1061)* and *sult-1(tu1232)* mutant hermaphrodites exhibited a strong Eu phenotype in liquid culture, with 100% of the observed animals being Eu (Fig. 5C). In contrast, mutant alleles of the other Sult genes resulted in high St phenotypes as in wild-type animals. Similarly, mouth-form ratios of *sult-1(tu1061)* and *sult-1(tu1232)* mutant males grown under liquid culture conditions were highly biased towards the Eu form, whereas wild-type males and mutants of the other four genes were Eu defective (Fig. 5D). Together, these experiments suggest a role of *sult-1* in mouth-form regulation in *P. pacificus*. It is important to note that both *sult-1* alleles have frame-shift mutations that result in premature stop codons (Fig. S3). However, the *sult-1* guide RNA was designed to target the central part of the coding region to avoid translation from several known alternative open reading frames when targeting more 5′ regions of the gene (Fig. S2). Therefore, these frame-shift mutations most likely result in reduction-of-function, rather than loss-of-function, alleles.

Given these results, we overexpressed *sult-1* in a wild-type background using two genomic constructs with a 2.2 kb and an 8 kb promoter region, respectively. Indeed, we found that hermaphrodites overexpressing *sult-1* have significantly lower Eu frequency compared with wild-type animals in NGM agar plates (Fig. 5E). This phenotype is contrary to the *sult-1* knockout phenotype, further indicating that *sult-1* is an important regulator of mouth-form plasticity in *P. pacificus* and promotes the St mouth form.

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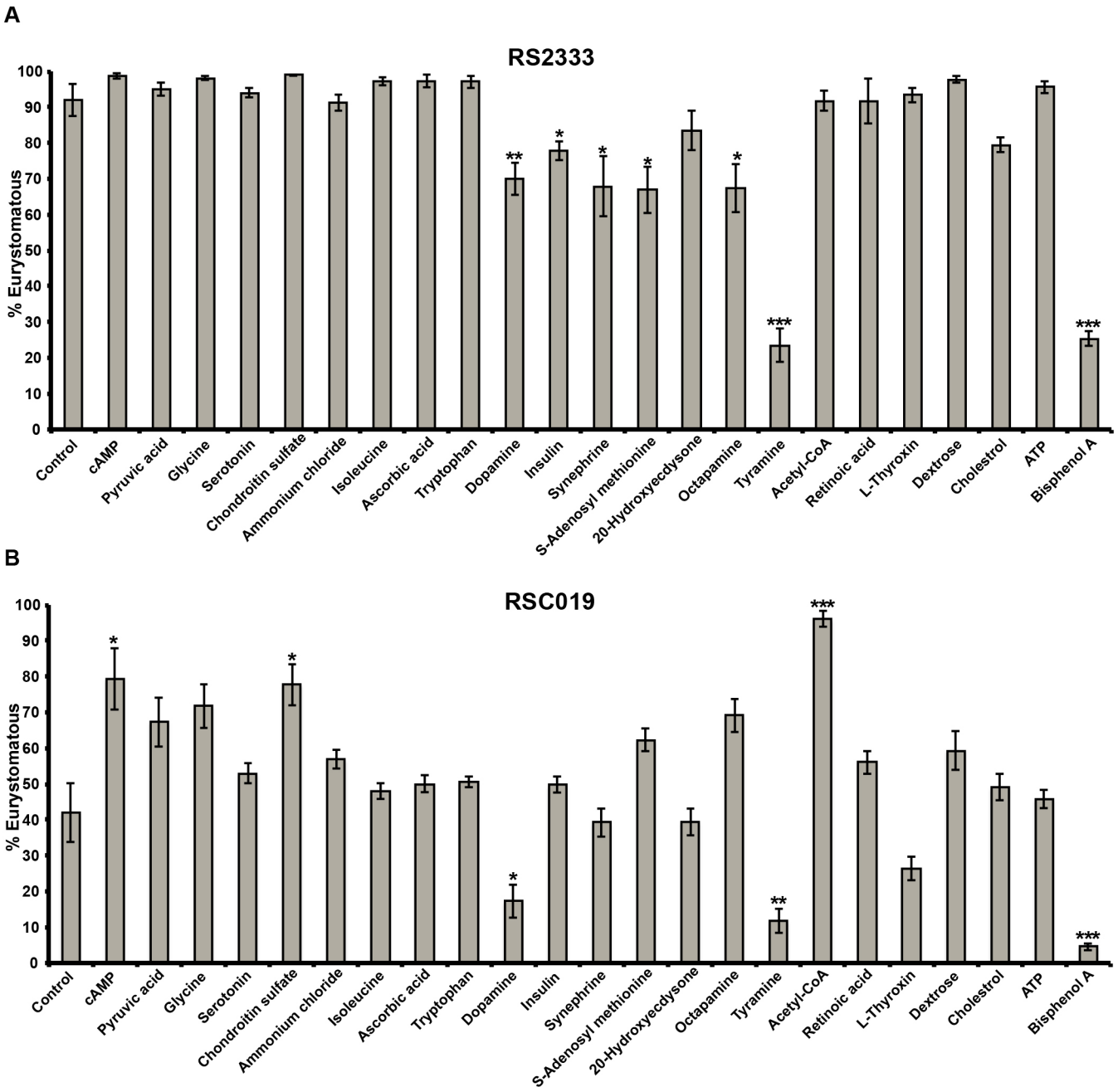

Fig. 2. Pharmacological screening reveals that bisphenol A, tyramine and dopamine have strongest St form-inducing effects.
(A,B) Pharmacological screening with different bioactive compounds (10 µM) on RS2333 (wild-type) (A) and RSC019 (B) animals. For each compound and each strain (RS2333 and RSC019), more than 200 animals were scored for their mouth morphology phenotype. At least three biological replicates were performed, with each having more than 50 animals. Error bars represent standard deviation from all the biological replicates. **P*<0.01, ***P*<0.005, ****P*<0.001, two-tailed Student's *t*-test, with respect to control values. Only the compounds showing strongest St form-inducing effects were further analyzed.

Epistasis analysis of *sult-1* and *eud-1* shows strong conditional effects

The identification of the sulfotransferase *sult-1* as a regulator of mouth-form plasticity, in addition to the sulfatase *eud-1* and their opposing phenotypes, raises the question of their potential genetic and biochemical interaction. In theory, SULT-1 and EUD-1 could form a sulfation module that acts on identical target molecules, i.e. hormone ligands that can be activated or de-activated by changing their sulfate moieties. Alternatively, SULT-1 and EUD-1 might have different target molecules acting in parallel pathways, or in the same pathway, resulting in epistatic relationships. To unravel the functional relationship between *sult-1* and *eud-1*, we first performed epistasis tests by creating a *sult-1(tu1061);eud-1(tu455)* double mutant. The *sult-1(tu1061)* mutation weakly suppressed the Eu-defective phenotype of *eud-1(tu455)* in the double mutant when grown on NGM agar plates (Fig. 5F). Strikingly, however, epistasis between *sult-1* and *eud-1* is conditional as different Eu form-inducing factors can strongly influence the mouth-form ratio. First, animals were treated with dasc#1, a pheromone earlier found to increase the ratio of Eu animals (Bose et al., 2012). Wild-type and *sult-1(tu1061)* single mutant animals are 100% Eu after dasc#1 treatment, whereas *eud-1(tu455)* mutants remain 0% Eu (Fig. 5F).

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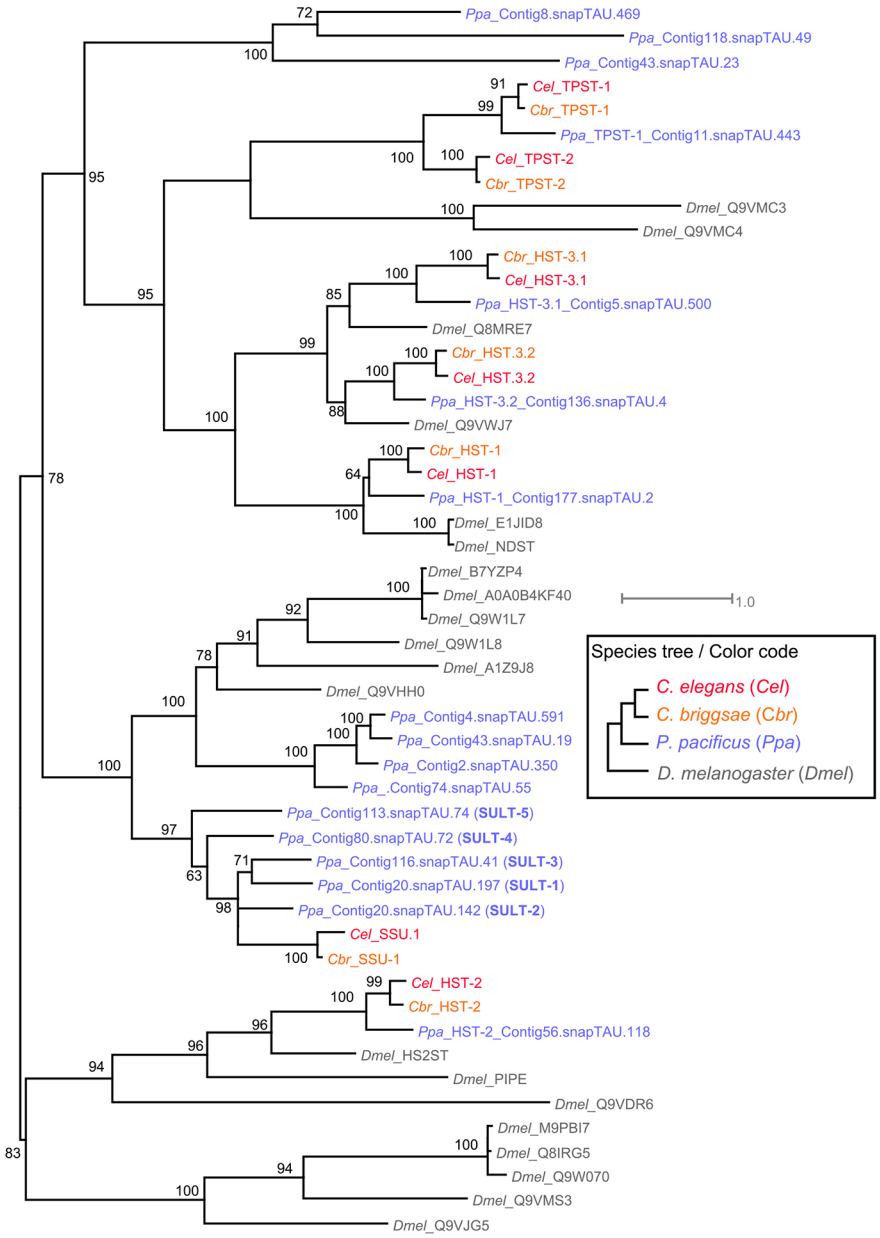

Fig. 3. *P. pacificus* has an expansion of cytosolic sulfotransferases. The tree shows the phylogenetic relationships between sulfotransferase genes in *C. elegans*, *C. briggsae*, *P. pacificus* and *D. melanogaster*. Although the number of sulfotransferases in *Caenorhabditis* nematodes remained rather constant, the *P. pacificus* lineage showed multiple independent expansions.

In contrast, *sult-1(tu1061);eud-1(tu455)* double mutants have significantly higher percentages of Eu animals when compared with untreated animals (Fig. 5F).

Ongoing experiments in our laboratory had shown that when nematodes are grown on bacteria isolated from the wild instead of on *E. coli* OP50, the ratio of the two mouth forms in the population does change. For example, the bacterial isolate *Pseudomonas* sp. LRB26 increases the ratio of Eu animals, such that in the case of wild-type animals, no St forms have ever been seen on *Pseudomonas* sp. LRB26 (Fig. 5F). Therefore, we tested the *sult-1(tu1061);eud-1(tu455)* double mutant on these bacteria and found a significant increase in the percentage of Eu worms, whereas *eud-1(tu455)* single mutants are unaffected at 0% Eu (Fig. 4F). Finally, similar patterns were observed when *sult-1(tu1061); eud-1(tu455)* worms were grown for 10 days under starvation conditions on agar plates, another condition known to increase the ratio of Eu worms (Serobyan et al., 2013). In all the mentioned Eu form-inducing conditions, *eud-1(tu455)* single mutant worms remained St, whereas *sult-1(tu1061);eud-1(tu455)* double mutants formed condition-specific ratios of Eu animals. Although the epistasis tests are likely influenced by the fact that the available *sult-1* mutant represents a reduction-of-function allele, these results suggest first, that *sult-1* is partially epistatic over *eud-1*, and second, that *sult-1(tu1061);eud-1(tu455)* double mutants are sensitized to environmental factors. This finding would be compatible with several hypotheses of the molecular interactions of SULT-1 and EUD-1, including one in which both proteins act in different cellular contexts. Therefore, we subsequently tested where *sult-1* is expressed in *P. pacificus*.

sult-1 and *eud-1* exhibit distinct expression profiles

To examine the spatiotemporal expression pattern of *sult-1*, we created two reporter lines, *tuEx282* and *tuEx283*, carrying an Ex[*sult-1*::Venus] construct in a wild-type background, each containing a 9 kb upstream fragment of *sult-1* fused to Venus fluorescent protein. We then compared the expression of *sult-1* with

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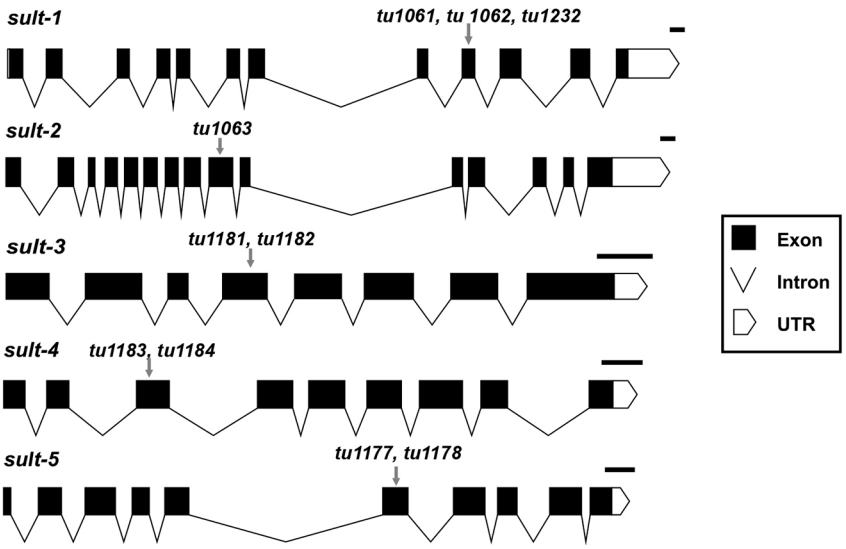

Fig. 4. CRISPR/Cas9-mediated knockout of cytosolic sulfotransferases: gene structures of sulfotransferases that were selected for knockout using CRISPR/Cas9 technique. The arrow indicates the mutated site. Scale bars: 100 bp. Black boxes represent exons, lines are introns and empty boxes are UTRs.

that of *eud-1*, which is expressed in several head neurons (Werner et al., 2017; Serobyan et al., 2016). Both reporter lines of *sult-1* resulted in similar expression patterns. Surprisingly, we found that *sult-1* and *eud-1* are expressed in different cells throughout the development of the worm. We confirmed the earlier reported expression of *eud-1* in sensory neurons, more specifically in amphid neurons (Fig. 6A). Surprisingly, however, we found that *sult-1* is expressed in pharyngeal muscle cells. Specifically, *sult-1* is expressed in cells homologous to *C. elegans* pm1, pm2 and pm3 (Fig. 6B). *sult-1* expression in pharyngeal muscle cells is seen throughout development, from the early juvenile stage (J2), and remains visible in adults. Thus, *sult-1* and *eud-1* are not co-expressed, which makes it unlikely that they compete for common target compounds. Instead, these findings indicate that *sult-1* expression in pharyngeal muscle cells might be involved in the execution of mouth formation and of the mouth structure itself. This would suggest that the mouth form-related function of *sult-1* is genetically downstream of or in parallel to *eud-1*.

sult-1 is downregulated in *nhr-40* mutant animals

The experiments described above revealed that (1) bisphenol A decreases the frequency of Eu hermaphrodites on agar plates, (2) *sult-1* mutants are highly Eu in both sexes and under different culture conditions, and (3) *eud-1* and *sult-1* act on different cells. These findings, together with our previous characterization of *nhr-40* as a suppressor of *eud-1*, and studies in humans and rodents indicating that sulfotransferases are regulated by nuclear hormone receptors (Kodama and Negishi, 2015), are consistent with a model in which *sult-1* is a transcriptional target of NHR-40. Consistent with this model, *sult-1(tu1061)* and *nhr-40(tu505)* mutants have similar Eu phenotypes in *P. pacificus*.

To determine whether *nhr-40* can affect *sult-1* at the transcriptional level, we examined *sult-1* transcription in the *nhr-40(tu505)* mutant (Fig. 7A). Indeed, we observed that *sult-1* is significantly downregulated in *nhr-40(tu505)* mutant animals relative to wild type. Thus, *sult-1* might act downstream of *nhr-40*, further supporting an independent function of SULT-1 and EUD-1. In a most parsimonious model, *eud-1*, *nhr-40* and *sult-1* are part of a linear genetic pathway, in which *eud-1* inhibits *nhr-40*, which acts as a transcriptional activator of *sult-1*. This model for *Ppa*-NHR-40 function would show resemblance to the related human receptor HNF4α, which activates the transcription of the cytosolic sulfotransferase SULT1E1 (Kodama et al., 2011). However, the role of bisphenol A would remain unclear in this model, because bisphenol A is known from other systems to be inactivated by sulfotransferases, and acts as xenobiotic ligand of mammalian nuclear hormone receptors, such as the estrogen receptor during breast cancer formation (Kodama and Negishi, 2015; Sui et al., 2012; Xu et al., 2017).

Table 1. Mutant alleles of cytosolic sulfotransferases in *P. pacificus*

Gene	Allele	Mutation	Type of mutation
sult-1	tu1061	10 bp deletion	frame-shift
sult-1	tu1232	4 bp deletion	frame-shift
sult-1	tu1062	3 bp insertion	in frame
sult-2	tu1063	8 bp deletion	nonsense
sult-3	tu1181	31 bp deletion	nonsense
sult-3	tu1182	11 bp deletion	nonsense
sult-4	tu1183	43 bp insertion+6 bp deletion	nonsense
sult-4	tu1184	5 bp deletion+1 bp insertion	nonsense
sult-5	tu1177	8 bp deletion	nonsense
sult-5	tu1178	7 bp deletion	nonsense

All the mutant alleles generated using the CRISPR/Cas9 system are shown. All alleles are in wild-type (RS2333) background. The length of the genetic lesion and type of mutation for each allele are indicated.

sult-1 and *nhr-40* mutations abolish the effect of bisphenol A on mouth-form plasticity

Given the known biochemical interactions of bisphenol A as targets of sulfotransferases and xenobiotic ligands of nuclear-hormone-receptors in mammals, we wanted to identify the interaction of bisphenol A with the mouth-form regulatory machinery in *P. pacificus*. To this end, we performed pharmacological assays with wild-type and mutant animals. If a sulfotransferase is involved in the inactivation and detoxification of bisphenol A, mutations in the corresponding gene should increase the effect of bisphenol A because it can no longer be inactivated. On the contrary, if bisphenol A acts upstream of a given factor, mutants for the corresponding genes would be unaffected by bisphenol A. Using bisphenol A in assays on agar plates as described above, we found that mutants in *sult-3*, *sult-4* and *sult-5* showed frequencies of St animals similar to wild type, whereas *sult-2(tu1063)* mutant animals showed an even greater increase of St frequencies relative to wild type (Fig. 7B). In contrast, *sult-1(tu1061)* mutant animals remained completely Eu after bisphenol A treatment, indicating that bisphenol A acts through

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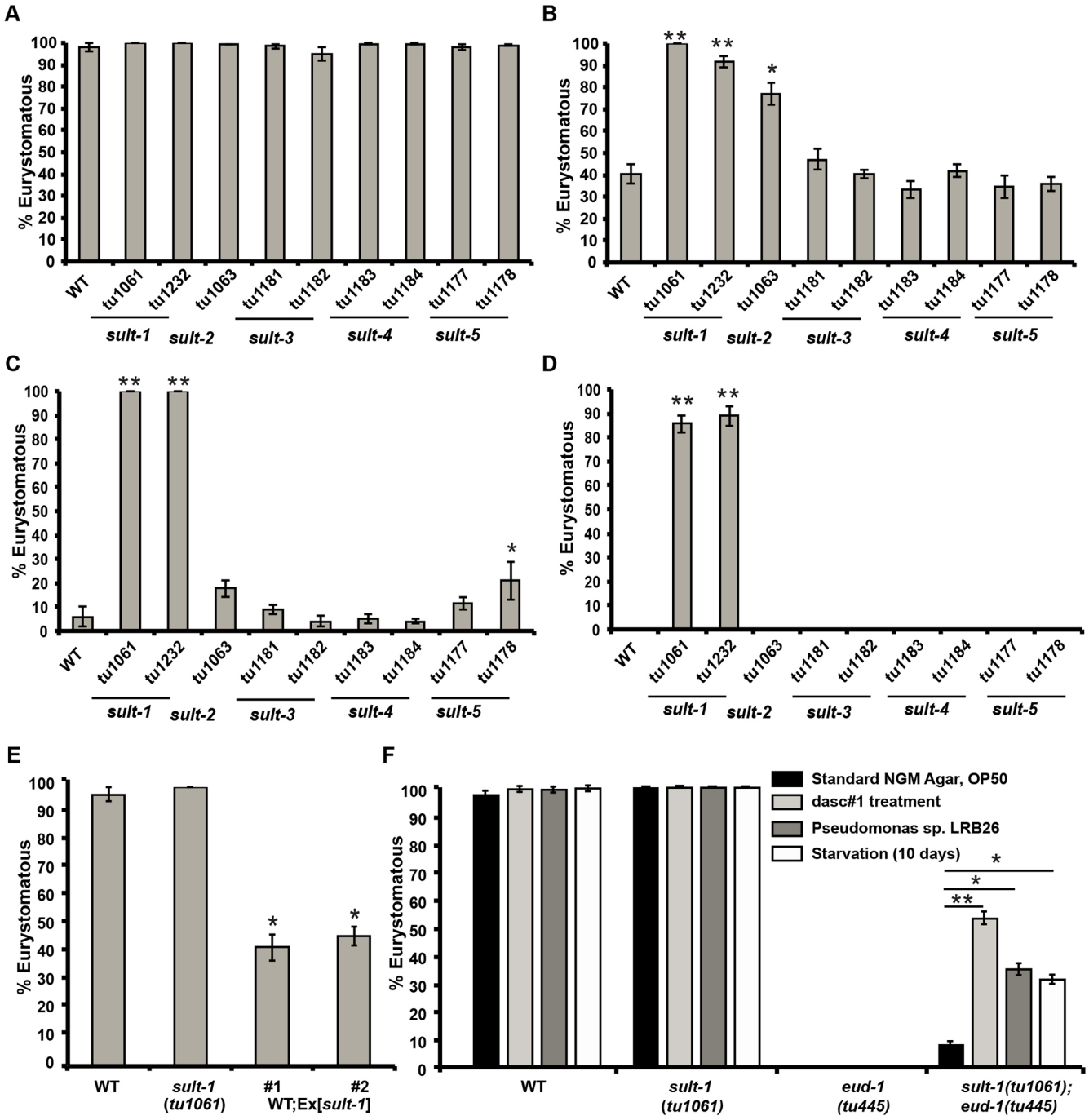

Fig. 5. *sult-1* is important for mouth-form determination. (A) Mouth-form ratios presented as % Eu for hermaphrodites of wild type (RS2333) and sulfotransferase mutants in agar (solid medium) culture. The total number of animals examined in three biological replicates (each with at least 100 worms) is greater than 300. (B) Mouth-form ratios for males of RS2333 and sulfotransferase mutants in agar (solid medium) culture. The total number of animals examined in three biological replicates (each with at least 50 worms) is greater than 150. (C) Mouth-form ratios for hermaphrodites of wild type and sulfotransferase mutants in liquid culture condition. The total number of animals examined in three biological replicates (each with at least 60 worms) is greater than 200. (D) Mouth-form ratios for males in liquid culture condition. The total number of animals examined in three biological replicates (each with at least 30 worms) is greater than 100. (E) Mouth-form ratios of wild type, *sult-1* (*tu1061*) and *sult-1* overexpression lines Ex[*sult-1*] #1 (*tuEx266*) and #2 (*tuEx281*). The total number of animals examined in three biological replicates (each with at least 50 worms) is greater than 200. (F) Mouth-form ratios of wild type, *sult-1*, *eud-1* and *sult-1;eud-1* in Eu form-inducing conditions: dasc#1 treatment, starvation and worm culture on *Pseudomonas* sp. LRB26. The total number of animals examined in three biological replicates (each with at least 50 worms) is greater than 200 for each condition. Error bars represent standard deviation. **P*<0.05, ***P*<0.01, two-tailed Student's *t*-test with respect to wild-type values, except in F, where it is with respect to % Eu form of the double mutant in standard NGM agar condition.

SULT-1 (Fig. 7B). This finding suggests that bisphenol A is not a substrate of SULT-1 for mouth-form regulation, which is contrary to our starting assumption and the findings for *Cel-ssu-1* (Hattori et al., 2006). Interestingly, bisphenol A has also no effect on mouth morphology in *nhr-40* mutant animals, which remained all Eu (Fig. 6B). Together, these results suggest that bisphenol A is not a

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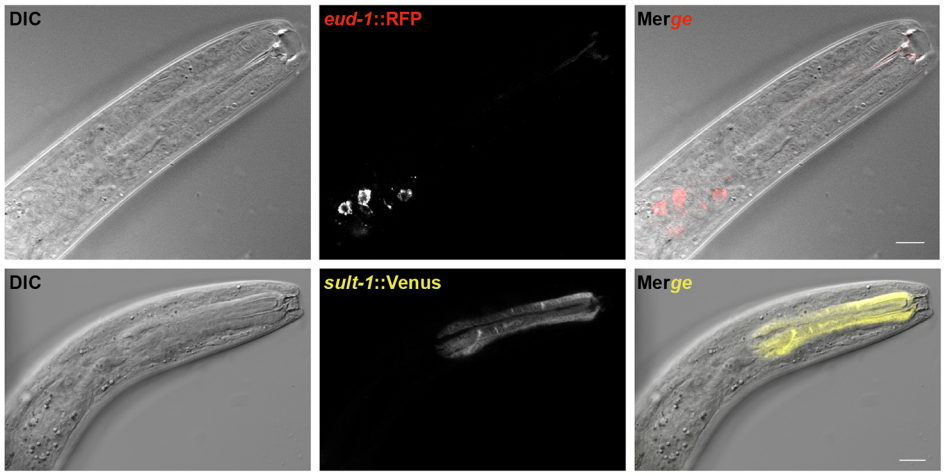

Fig. 6. *sult-1* and *eud-1* exhibit distinct expression profiles. (A,B) Representative images of expression pattern of *eud-1* in *eud1*::RFP reporter line (*tuEx177*) (A), expression pattern of *sult-1* in *sult-1*::Venus reporter line (*tuEx282*) (B). More than 15 animals of different stages were observed and imaged for A and B. Scale bars: 10 µm.

target of SULT-1, and that it acts upstream of, or at the level of, NHR-40 itself. Thus, bisphenol A might act as a xenobiotic ligand of NHR-40 during *P. pacificus* mouth-form regulation.

DISCUSSION

We have identified the influence of bisphenol A and other phenolic compounds on mouth-form plasticity in *P. pacificus* and subsequently delineated the role of the sulfotransferase *sult-1* in mouth-form specification. Although the investigation of a potential role of sulfotransferases in mouth-form regulation was initiated under the assumption that a SULT enzyme is required for the inactivation of bisphenol A, the subsequent experiments involving the characterization of the *sult-1* mutant and the treatment of various mutants with bisphenol A resulted in several unexpected findings. Together, our work highlights the importance of sulfation processes for the regulation of developmental processes independent of disease contexts and results in three major conclusions.

First, pharmacological screens remain a powerful tool to investigate biological processes such as phenotypic plasticity. With regard to mouth-form plasticity in *P. pacificus*, the effects of bisphenol A, tyramine and dopamine extend the number and type of bioactive compounds involved in mouth-form regulation. Previous pharmacological and genetic analyses had indicated that bioactive compounds are involved in regulating mouth-form plasticity at various levels of the molecular network. While the hormone dafachronic acid and the small molecule dasc#1 were shown to act genetically upstream of *eud-1*, sulfate and phosphate molecules most likely directly inhibit the sulfatase EUD-1, similar to effects described for vertebrate sulfatases (Glössl et al., 1979; Ragsdale et al., 2013). The effects of bisphenol A and tyramine resulted in the identification of the sulfotransferase *sult-1* and its role in mouth-form regulation. It should be noted, however, that the effects of dopamine might rely on a different mechanism. Although dopamine is known as a substrate of sulfotransferases (Yasuda et al., 2007), it is also a known neurotransmitter, and as such it might be involved in neuronal environmental perception. Interestingly, recent studies have shown that in *P. pacificus* animals, serotonin, but not dopamine, is involved in predatory feeding, the physiological and behavioral consequence of mouth-form plasticity (Wilecki et al., 2015; Okumura et al., 2017). Thus, neurotransmitters generated by related enzymatic pathways, such as serotonin and dopamine, have distinct functions in the context of mouth-form plasticity and predation.

Second, our analysis of sulfotransferases indicated that *P. pacificus* has undergone a massive expansion of cytosolic sulfotransferases relative to *C. elegans*. By the use of the CRISPR/Cas9 technology we have identified *sult-1* to be involved in mouth-form regulation, demonstrating how lineage-specific duplications can generate genes that can be incorporated into networks regulating novel phenotypes. *sult-1* mutants are highly Eu under most tested conditions. Only liquid culture conditions in males did not result in all-Eu *sult-1* mutant animals, but still in very high Eu frequencies (85% in *tu1061* and 89% in *tu1232*). This male trait could either be caused by partial redundancy with other sulfotransferases under certain growth conditions, or the incomplete penetrance of the reduction-of-function alleles available for *sult-1*. Thus, the *sult-1* gene encodes a sulfotransferase regulating mouth-form plasticity. This finding highlights the role of sulfation-desulfation for *P. pacificus* plasticity.

It should be noted that *sult-2* mutants also showed altered mouth-form ratios relative to wild type; however, with much smaller effects than *sult-1*. On the one hand, *sult-2* mutant males are highly Eu on agar plate. On the other hand, the effect of bisphenol A on mouth-form regulation is enhanced in *sult-2* mutants, resulting in less than 10% of animals being Eu (Figs 5 and 7). These observations would be consistent with a role of SULT-2 in the sulfation and inactivation of bisphenol A. However, given the fact that *sult-2* mutants did not show any effect in other test conditions, i.e. growth of hermaphrodites and particularly males in liquid culture, the specific role of this sulfotransferase might simply be minor.

In contrast to our original assumption, we did not obtain any evidence for EUD-1 and SULT-1 forming a sulfation module that acts in the same tissues or cells. First, *sult-1* is expressed in pharyngeal muscles cells, whereas *eud-1* is expressed in sensory neurons. Second, epistasis analysis indicates that while *sult-1;eud-1* double mutants are largely St under agar growth conditions, other conditions cause double mutants to adopt more Eu mouth forms. Such a pattern was never seen in *eud-1* single mutants. Together, these observations result in the third major conclusion of our study, suggesting that two independent sulfation processes regulate mouth-form plasticity. In general, *P. pacificus* mouth-form regulation represents a rare example of sulfation processes in normal developmental processes in invertebrates. Although sulfation processes are commonly seen in disease contexts, such as breast cancer (Martinez et al., 2013; Jamil et al., 2017), little is known about the role of sulfation in organismal development. Many studies in invertebrate model systems such as *D. melanogaster* and

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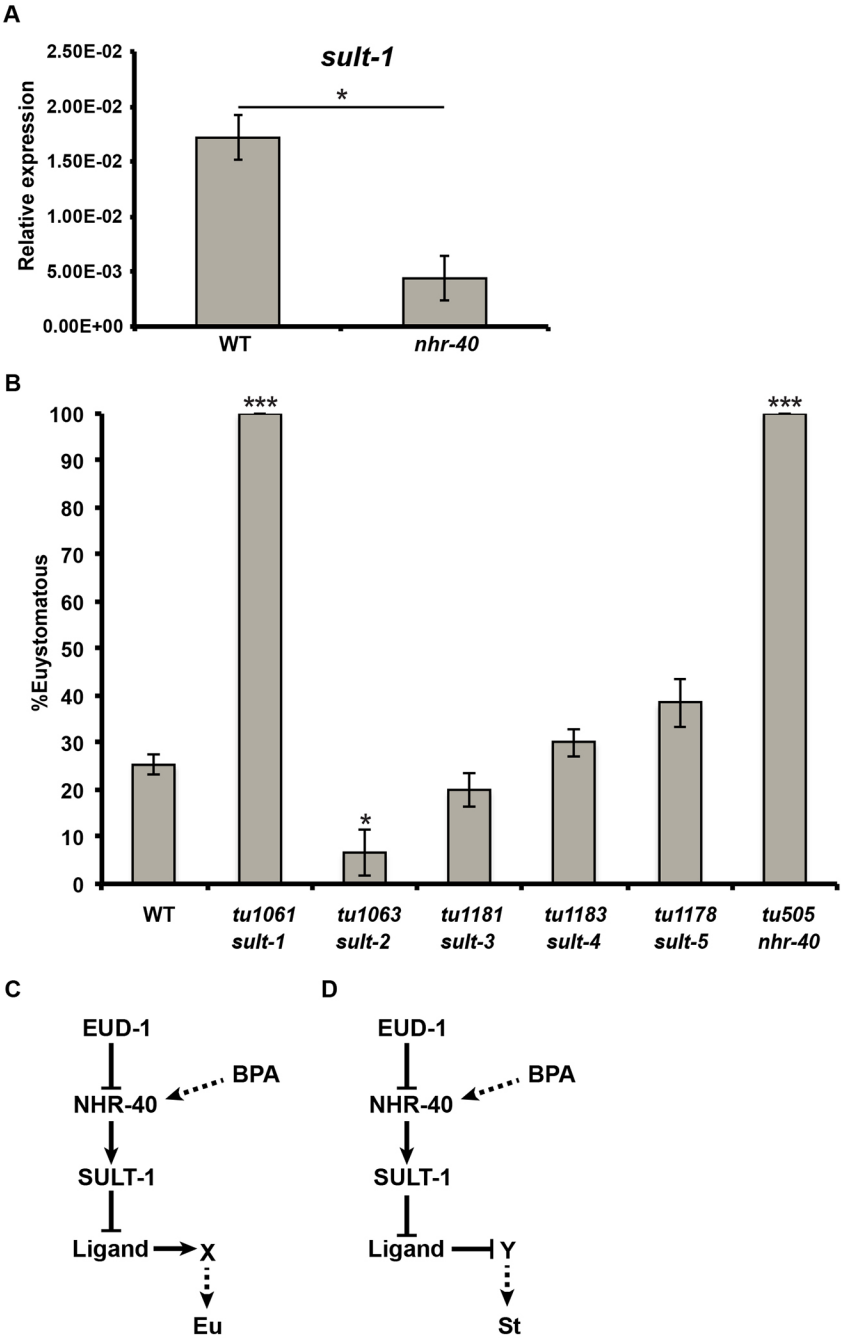

Fig. 7. Bisphenol A acts through NHR-40 to influence mouth morphology. (A) Expression levels of *sult-1* in *nhr-40* mutant line. (B) Bisphenol A treatment was performed on wild type (RS2333), sulfotransferase mutants and *nhr-40* animals. Mouth-form ratios presented as % Eu. The total number of animals phenotyped is greater than 200. Three biological replicates were performed, each having at least 50 animals. (C,D) Representations of the two most parsimonious models, showing the genetic factors involved in mouth morph determination in *P. pacificus*. **P*<0.05, ****P*<0.001, two-tailed Student's *t*-test with respect to wild-type values.

C. elegans provided examples for the role of phosphorylation in development with strong phenotypes of mutants in specific kinases and phosphatases (Zielinska et al., 2009; Chen and Jiang, 2013). In contrast, enzymes regulating sulfation have rarely been found in model system approaches (Lin et al., 1999). This is surprising because sulfation is a common modification in biological systems and, in principle, the inactivation of bioactive compounds through sulfation would be an obvious target for regulatory processes in development and physiology.

Many mechanisms have been identified to control developmentally plastic traits in different organisms. For example, in *C. elegans*, sensing of the environment using the calcium-dependent kinase CMK-1 can regulate DAF-7/TGF-β and insulin-like protein DAF-28/ILP, which control dauer entry (Ren et al., 1996; Neal et al., 2015). In the brown planthopper *Nilaparvata lunges*, the insulin receptors are linked to a developmental plastic decision between short-winged and long-winged morphs (Xu et al., 2015). This study, in combination with the earlier studies on sulfatase, EUD-1, establishes sulfation of biomolecules as a mechanism that can regulate developmentally plastic traits.

Future studies will address the targets of SULT-1 during the regulation of mouth-form plasticity in *P. pacificus*. Currently, the available genetic data and the *sult-1* expression in pharyngeal muscles cells place *sult-1* downstream of, or in parallel to, other mouth-form regulators. Therefore, we speculate that SULT-1 targets a bioactive compound that interacts with a downstream or parallel regulator of mouth-form plasticity acting in the pharyngeal muscle cells. Given the known roles of sulfotransferases in the inactivation of xenobiotic or endobiotic bioactive compounds, two different models are similarly possible given the current data. SULT-1 either

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inhibits or inactivates a ligand of a mouth-form regulator X that promotes the Eu mouth form (Fig. 7C). In the absence of *sult-1*, this ligand is overactivated resulting in all-Eu mouth forms. Alternatively, *sult-1* might control a mouth-form regulator Y that promotes St development by inhibiting or deactivating the ligand of factor Y (Fig. 7D). Although the distinction between these two models is currently impossible, the identification of the molecular nature of the factor(s) acting downstream of *sult-1* might be elucidated, given the powerful genetic tools available in *P. pacificus*. Thus, this nematode model organisms and its unusual example of phenotypic plasticity represent a promising system to investigate sulfation processes in invertebrate model organisms.

In summary, our findings add an additional layer of regulation of mouth-form plasticity that points towards the role of a second sulfation process. Most importantly, *sult-1* is the first gene to be identified in mouth-form regulation that acts in the pharyngeal muscle cells. This is a striking finding because the pharyngeal muscles cells pm1, pm2 and pm3 are known to be involved in the secretion of the extracellular matrix that eventually forms the teeth-like structures in *P. pacificus* and other diplogastrid nematodes (Baldwin et al., 1997; Jay Burr and Baldwin, 2016). Thus, mouth-form regulation involves multiple regulatory levels and cell types, from neurons involved in environmental perception to pharyngeal muscle cells involved in mouth-form specification.

MATERIALS AND METHODS

Culture conditions

For culturing worms on agar plates, all *P. pacificus* strains, including the wild-type strain RS2333, all mutants generated in this study and transgenic worms were grown on 6 cm plates containing NGM agar. Worms were fed on a 300 µl bacterial lawn containing *E. coli* OP50 strain grown in LB broth. All cultures were maintained at 20°C.

For culturing worms in liquid we used S medium, a standard protocol to obtain high frequency of St animals as reported earlier (Werner et al., 2017). In short, for each sample, three agar plates in which worms had eaten all the OP50 on plates were washed with M9 buffer into 15 ml conical tubes. We added bleach (5 M NaOH in a 2:1 ratio) to a final volume of 30%. This mix was left on a rotor for 9 min with gentle rotation at room temperature. Carcasses were filtered through a 120 µm nylon net (Millipore) fixed between two rubber gaskets in a plastic funnel, washed by applying 3 ml M9 drop-wise on the filter, then pelleted at 500 ***g*** for 1 min at room temperature. Remaining eggs-J1 larvae were washed again with 3 ml M9 and centrifuged at 500 ***g*** for 1 min at room temperature. The pelleted eggs-J1 larvae were then suspended in 50 ml volume autoclaved Erlenmeyer flasks containing 10 ml S medium. To this bacterial pellet (centrifuged at 3000 ***g*** for 30 min at 4°C), an empirically determined amount of 100 ml, grown overnight on OP50 in LB medium (at an optimal density at 600 nm of 0.5), was added. Nystatin (20 µg/ml, final concentration) was added to prevent fungal contamination. Liquid cultures were incubated at 20°C and shaken at 180 rpm (INFORS HT Multitron standard) for 4 days.

Because mouth-form ratios are influenced by several environmental conditions (Bose et al., 2012; Werner et al., 2017), all experiments included their own wild-type control for Eu frequency.

Phenotypic scoring

Mouth-form phenotypes were scored in agar cultures using a method described earlier (Bento et al., 2010). For liquid culture, worms were phenotyped for mouth form by filtering using a 20 µm filter. Adults were then gently pelleted and transferred to 4% agar pads (containing 10 mM sodium azide) with 5-8 µl M9 and observed under a differential interference contrast (DIC) microscope (Zeiss Axioskop) at 40-100× magnifications. Discrete characters were used to discriminate between Eu and St individuals: the presence versus absence of a subventral tooth, and a claw-like versus flint-like or triangular dorsal tooth, respectively, which were together sufficient to distinguish the two forms.

Pharmacological screening

To test the effect of different bioactive compounds on mouth morphology, pharmacological screening was performed on RS2333 and RSC019 worms and mutants of sulfotransferases and *nhr-40*. All compounds tested and described in Fig. 2 were separately dissolved in either ethanol or water and thereafter mixed with melted NGM agar to bring chemicals to a final concentration of 10 µM. Control treatments consisted of agar mixed with the corresponding volumes of ethanol or water, and they did not show any significant difference compared with non-treated worms. Six-centimeter plates containing 10 ml agar were seeded with 300 µl OP50 and 10 µM of the test chemical, and were then incubated overnight at room temperature to allow bacterial growth. Three J3-J4 hermaphrodites were picked to each plate from the same well-fed source plate. Plates were kept at 20°C for 1 week. Adult animals in the next generation were screened for the mouth-form phenotype. Experiments were conducted in at least three replicates for each treatment type.

Phylogenetic analysis

Sulfotransferase domains (type I PF00685 and type II PF03567) of *C. elegans*, *C. briggsae* and *P. pacificus* were identified using the hmmsearch program of the HMMER package (version 3.1b2, e-value <0.001). *D. melanogaster* sequences were taken from the sequence alignments of the corresponding Pfam profiles. Manual curation, multiple sequence alignment and tree reconstruction were performed as previously described (Baskaran et al., 2015). The final tree (Fig. 3) represents a maximum-likelihood tree under the LG substitution model, with a correction for invariant sites and four rate classes that follow a gamma distribution. The robustness of internal nodes was measured as the number of 100 bootstrap replicates that would support a given topology.

Generation of CRISPR-induced mutants for sulfotransferases

We generated mutant alleles for the five closest paralogs of the *C. elegans* cytosolic sulfotransferase *ssu-1* using the CRISPR/Cas9 technique following the protocol described previously (Witte et al., 2014). The gene structures are based on earlier published RNA sequencing results and gene annotation (Ragsdale et al., 2013; Rödelsperger et al., 2017). Single guide RNAs (sgRNAs) for five sulfotransferases were ordered from Integrated DNA Technologies (see Table S1 for sequences). For inducing mutations, sgRNAs were co-injected with Cas9 nuclease (M0386M, NEB). Heterozygous mutant carriers were identified and singled out by analyzing high-resolution melting curves using a quantitative PCR system (Lightcycler 480 II, Roche) with separate primers for each gene (Table S2).

Conditional epistasis of *sult-1* and *eud-1*

Worms were first treated with the pheromone dasc#1 using a method described previously (Bose et al., 2012). In short, dasc#1 was first dissolved in ethanol and then added to 10 ml NGM to obtain 1 µM final concentration. The melted NGM was added to 6 cm plates, which were then seeded with 300 µl OP50 culture in LB medium. Control treatments consisted of agar mixed with the corresponding volumes of ethanol. These plates were incubated overnight at 20°C and the next day, three J4 hermaphrodites were picked to each plate from the same well-fed source plate. Adult offspring of these worms were phenotyped in 1 week for mouth morphology. Experiments were conducted in at least three replicates.

Pseudomonas sp. LRB26 was isolated from the scarab beetle *Oryctes borbonicus* found in La Réunion Island. To examine the effect of growth on *Pseudomonas* sp. LRB26, we seeded a 6 cm NGM plate with 300 µl *Pseudomonas* sp. LRB26 in LB medium. These plates were incubated overnight at 20°C and the next day, three J4 hermaphrodites were transferred to each plate from the same well-fed source plate. Offspring of these worms were phenotyped in 1 week for mouth-form morphology. Experiments were conducted in at least three replicates.

To examine the effects of starvation, we transferred three J4 hermaphrodites from a well-fed source plate to 6 cm NGM plates seeded with 300 µl OP50 in LB. Plates were kept at 20°C for 10 days. Adults were phenotyped for mouth morphology from these 10-days-starved plates. Experiments were conducted in three replicates.

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Genetic transformation

Transgenic animals were generated as previously described (Schlager et al., 2009). To obtain overexpression lines for *sult-1*, germ lines of adult hermaphrodites were injected with a mix of genomic construct of *Ppa-sult-1* (10 ng/µl), the marker *Ppa-egl-20*::*TurboRFP* (10 ng/µl), and genomic carrier DNA (60 ng/µl) from the RS2333 strain. The *Ppa-sult-1* genomic construct had a 2.2 kb promoter for the generation of the first line Ex[*sult-1*]#1 (*tuEx266*) and an ∼8 kb promoter for the second Ex[*sult-1*]#2 (*tuEx281*). Transgenic animals were scored over multiple generations involving at least 200 transgenic animals per line.

To generate reporter lines for *sult-1*, cDNA of fluorescence protein Venus was transcriptionally fused to a ∼9 kb long *sult-1* promoter and 3′ UTR sequence of the gene *rpl-23* to create a 12 kb long *sult-1*::Venus construct. Fragments were then fused and amplified by overlapping extension PCR. All amplified fragments were verified by sequencing. The *sult-1*::Venus construct (10 ng/µl) was injected along with the co-injection marker *egl-20*::Venus (10 ng/µl), and genomic carrier DNA (60 ng/µl) from the wild-type strain. Two independent transgenic lines Ex[*sult-1*::Venus]#1 (*tuEx282*) and Ex[*sult-1*::Venus]#2 (*tuEx283*) were generated. For *eud-1*, a reporter line (*tuEx177*) with TurboRFP generated in an earlier study (Ragsdale et al., 2013) was used.

In all cases, we used the restriction enzymes *Pst*I (Thermo Fisher Scientific) for digestion of the respective construct and genomic host DNA. For both experiments, two independent transgenic lines were generated.

Quantitative reverse transcription PCR experiments

Mixed-stage worms were washed from at least five crowded plates and filtered using 20 µm nylon filter (Millipore) to collect J2-stage worms. Worms were pelleted (20,817 ***g*** for 1 min at room temperature) and re-suspended in Trizol. Total RNA was isolated using a PureLink (Invitrogen) RNA micro kit following the manufacturer's protocol. Reverse transcription was performed with 1 µg total RNA using superscript II reverse transcriptase (18064, Invitrogen) following the manufacturer's instructions. The quantitative reverse transcription PCR experiments were performed using SyberGreen I mastermix (Roche Diagnostics) following a previously described method (Schuster and Sommer, 2012), on a Roche Lightcycler 480 system. *cdc-42* and *β-tubulin* were used as reference genes to calculate ΔCt values. The sequences of the primers used are listed in Table S3. Expression levels were analyzed with advanced relative quantification on the Roche Lightcycler 480 system according to the manufacturer's instructions. At least three biological replicates were performed for each experiment.

Imaging

Image acquisition was performed on a Leica SP8 confocal system using settings to maximize the detection of fluorescent protein tags TurboRFP and Venus. At least 15 animals were imaged for each sample type. Image analysis was performed using Fiji (ImageJ) software (Schindelin et al., 2012).

Statistical analyses

All phenotypic data show percentage Eu frequency calculated from total individuals screened in three biological replicates. Total sample size is illustrated on graphs. Significant differences were tested by two-tailed Student's *t*-test.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.N., R.J.S.; Methodology: S.N., E.M., C.R., P.B., H.W.; Investigation: S.N., E.M.; Resources: C.R., P.B.; Data curation: C.R.; Writing - original draft: E.M., C.R., R.J.S.; Writing - review & editing: S.N., R.J.S.; Supervision: R.J.S.; Funding acquisition: R.J.S.

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Supplementary information

Supplementary information available online at
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Fig S1

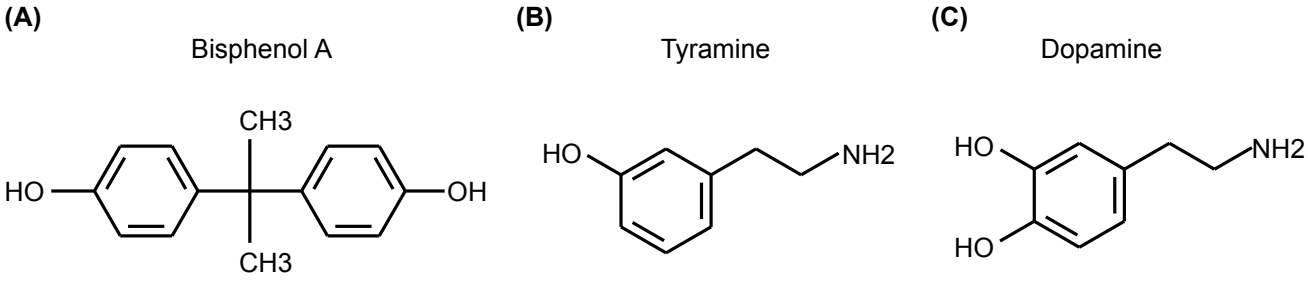
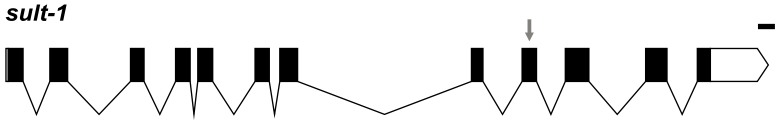


Fig. S1: Molecular structures of St form inducing chemicals; Bisphenol A, Tyramine and Dopamine.

Fig S2

sult-1



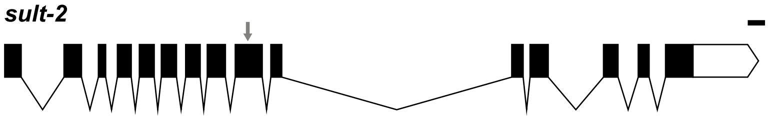
RS2333 CCATCGTGGCGAGTTCATCCGCAAGGGCGGATCGAGAGATTGG - - - AAGAATCAGTTCGGATACGAGC

tu1061 CCATCGTGGCGAGTTCATCCGCAAGGGCG - - - - - - - - - - TTGG - - - AAGAATCAGTTCGGATACGAGC (-10 bp)

tu1062 CCATCGTGGCGAGTTCATCCGCAAGGGCGGATCGAGAGATTGGCAAAAGAATCAGTTCGGATACGAGC (+3 bp)

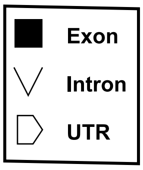
tu1232 CCATCGTGGCGAGTTCATCCGCAAGGGCGGATC - - - - GATTGGCAAAAGAATCAGTTCGGATACGAGC (-4 bp)

sult-2

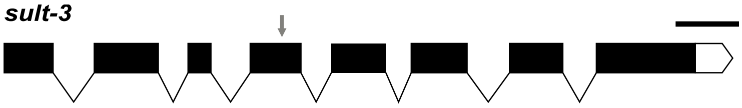


RS2333 TCGGAAAGATGTCTTCGTGCGAAAAGGAGGATCAAGAGATTGGAAGAATTTCTTCTCGCATGAAC

tu1063 TCGGAAAGATGTCTTCGTGCGAAAAGGAG - - - - - - - - GATTGGAAGAATTTCTTCTCGCATGAAC (-8 bp)



sult-3

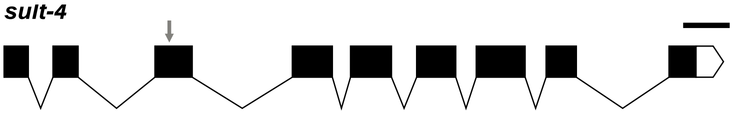


RS2333 CTCGAGCGATTCGGTGCCGAATATGCGGAGACTTTACCATCGCCTCGCATACTCAAGGTTCGCAC

tu1181 CTCGAGCGATTCGGT - GCATACTCAAGGTTCGCAC (-31 bp)

tu1182 CTCGAGCGATTCGGTGCC - - - - - - - - - - - GACTTTACCATCGCCTCGCATACTCAAGGTTCGCAC (-11 bp)

sult-4

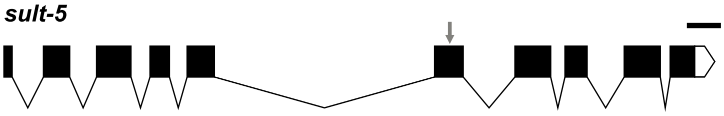


RS2333 TCACCTATGATCGAGCAGATCGGC - - GCGG - ACTTC

tu1183 TCACCTATGATCGAGCAGATCG - - - - - - - - - TTAACGCCACTTCATCATTAACGCTTCATTAACGCTTCATTAACTTC (+37 bp net)

tu1184 TCACCTATGATCGAGCAG - - - - GC - AGCGG - ACTTC (-4 bp net)

sult-5



RS2333 GTCGGTTGCGGCGAGTATTTCGTCCATTTGACATCCTGGTTAGAAGCCGTGAAAAAGATGGAAGA

tu1177 GTCGGTTGCGGCGAGTATTTCGT - - - - - - - - CATCCTGGTTAGAAGCCGTGAAAAAGATGGAAGA (-8 bp)

tu1178 GTCGGTTGCGGCGAGTATTTCGTCCAT - - - - - - - CCTGGTTAGAAGCCGTGAAAAAGATGGAAGA (-7 bp)

Fig. S2: Mutant alleles of sult-1, sult-2, sult-3, sult-4 and sult-5 produced using CRISPR/Cas9 technique. Arrows and underlined texts indicate sgRNA sequence including PAM of respective genes. Induced genetic lesions are highlighted in grey. Scale bar represents 100 bp.

Fig S3

```
WT                  MVPRADDVFICTYPKCGTTWIQHIVHQLLGKTEYETAVDDDENDNVACQPSSSSHEKSEE        60
sult-1(tu1061)      MVPRADDVFICTYPKCGTTWIQHIVHQLLGKTEYETAVDDDENDNVACQPSSSSHEKSEE        60
sult-1(tu1232)      MVPRADDVFICTYPKCGTTWIQHIVHQLLGKTEYETAVDDDENDNVACQPSSSSHEKSEE        60
                    ************************************************************

WT                  DEKKAMCFVSPMIERMGAAYSDTIKTPRVLKSHFTYKNIPKGGGAKYIFAVRNPKDCLTS        120
sult-1(tu1061)      DEKKAMCFVSPMIERMGAAYSDTIKTPRVLKSHFTYKNIPKGGGAKYIFAVRNPKDCLTS        120
sult-1(tu1232)      DEKKAMCFVSPMIERMGAAYSDTIKTPRVLKSHFTYKNIPKGGGAKYIFAVRNPKDCLTS        120
                    ************************************************************

WT                  YFHHNRNFKIYDYEHGEFDVFFKLFMDGKVGFGDYFDHLTSWLEGIEKAEERILFLKYED        180
sult-1(tu1061)      YFHHNRNFKIYDYEHGEFDVFFKLFMDGKVGFGDYFDHLTSWLEGIEKAEERILFLKYED        180
sult-1(tu1232)      YFHHNRNFKIYDYEHGEFDVFFKLFMDGKVGFGDYFDHLTSWLEGIEKAEERILFLKYED        180
                    ************************************************************

WT                  MVADLHSAVVQIASFLGGKAAEIIENDQKLAQIVEASTLASMKKNQQRWFPNKQLHRGEF        240
sult-1(tu1061)      MVADLHSAVVQIASFLGGKAAEIIENDQKLAQIVEASTLASMKKNQQRWFPNKQLHRGEF        240
sult-1(tu1232)      MVADLHSAVVQIASFLGGKAAEIIENDQKLAQIVEASTLASMKKNQQRWFPNKQLHRGEF        240
                    ************************************************************

WT                  IRKGGSRDWKNQFGYEQS-FEMD--KKFRER-CAGTAAAEWWHSEMA-WNVSRPVVAVEP        295
sult-1(tu1061)      IRKG--VGRISSDTSNPSKWTRSSGNAARAQPLPSGGTARWPGTSVDPSSLSNPSAKCLP        298
sult-1(tu1232)      IRKGGSIGRISSDTSNPSKWTRSSGNAARAQPLPSGGTARWPGTSVDPSSLSNPSAKCLP        300
                    ****   .  ..   : * :  .  :  * :   . .:*.*  :.:   .:*.* .   *

WT                  ISEVSSYSSSGFCSASPLSFTSSSLDLSSSLSSH-LRLPSVNPESDLCYSPAVEGPLDRD        354
sult-1(tu1061)      IRRRA----------------SAPPRLSPSLHHHWISLPHSH---RISDSPQ--------        331
sult-1(tu1232)      IRRRA----------------SAPPRLSPSLHHHWISLPHSH---RISDSPQ--------        333
                    * . :               *:   ** **  * : **  :    :. **

WT                  RVDSLQFPFDSLKIDDATEEQD      376
sult-1(tu1061)      ----------------------      331
sult-1(tu1232)      ----------------------      333
```

Fig. S3: Alignment of WT and mutant versions of SULT-1.
Multiple sequence alignment SULT-1 amino acid sequences in WT and mutant alleles tu1061 and tu1232 using clustal Omega.

Table S1: Sites for single guide RNA (sg-RNA) sequences

sult-1	GCAAGGGCGGATCGAGAGATTGG
sult-2	GAAAAGGAGGATCAAGAGATTGG
sult-3	TGGTAAAGTCTCCGCATATTCGG
sult-4	TATGATCGAGCAGATCGGCGCGG
sult-5	ATTTCGTCCATTTGACATCCTGG

Table S2: Primer sequences for qPCR of sulfotransferase genes after CRISPR

Gene	Forward primer	Reverse primer
sult-1	CAAAGATGGTTCCCTAACAAGCA	GATGAAGTGAAGGAGAGAGGCGA
sult-2	GGTTGGACTTGGTGATTACTTCG	GCTAGTTCTCATCTTCCAAGAACCC
sult-3	AGCGGTGGTGAAGCAAATGG	CCGGGAACTAAAGATTGTG
sult-4	GACGGCGATTCTTGTTGC	GGCCGTTTAGCCTGTATTGT
sult-5	CAGAATCTTACGTACCTT	GTAACTAGTGGAATTCTTGAA

Table S3: Primer sequences for examining *sult-1* expression levels with qRT PCR

Gene	Forward primer	Reverse primer
Ppa-sult-1	GTTCATGGATGGAAAGGTCGGATTCG	TGAGGCTTCGACTATCTGAGCCAG
Ppa-cdc-42	CTCTCTTATCCACAGACGGAC	GAAGGGAGTGCGTGAGCAGTG
Ppa-β-tubulin	CTCGGAGGAGGAACTGGATC	GACCGTGTCAGAGACCTTAG