Genetic Interactions Between Suppressors of the Slow Defecation Phenotype of *clk-1* Mutants

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Table of Contents

List of Figures and Tables

Abstract

Many genes that are involved in nutrient and energy utilization and storage are conserved between humans and *C. elegans*. One of the genes involved in worm mitochondrial respiration is *clk-1*. This gene encodes an enzyme required for ubiquinone biosynthesis. One of the phenotypes of *clk-1* mutants is altered defecation cycle: the slowing down of the defecation cycle and the inability to defecate faster at higher temperatures. these phenotypes are independent of one another. Several lines of evidence suggest that the slowed defecation of *clk-1* mutants is due to alterations in lipoprotein/cholesterol metabolism because the mutant phenotype can be suppressed by the reduction of dietary cholesterol, administration of drugs affecting lipoprotein metabolism, and mutating genes involved in lipoprotein metabolism. These mutations can be separated into two classes: those which suppress the *clk-1* slow defecation at 20 as well as after the shift to 25°C (class I) and those which only suppress it at 20°C (class II). Worm *dsc-4* (lipoprotein assembly) and *dsc-3* (metabolism of bile-like molecules) are class I suppressors. Both of these mutations restore the ability of *clk-1* mutants to react to the temperature shifts. In this study we identified a novel class I suppressor *pgp-2* and classified *sod-4* as a class II suppressor. *pgp-2* encodes a protein essential for the biosynthesis of the worm intestinal lysosome-related organelles. Mutations in both *pgp-2* and *sod-4* are unable to restore the ability of *clk-1* worms to react to the temperature shifts. Combining different class I mutants as well as combining class I with class II mutant *sod-4* revealed that the genetic

interactions between class I mutants as well as genetic interactions between class I and class II mutants are hard to predict.

Resume

Plusieurs des gènes impliqués dans l'utilisation des nutriments et de l'énergie ainsi que pour leur stockage sont conservés de l'humain à *C. elegans*. Un des gènes impliqué dans la respiration mitochondriale du nématode est *clk-1*. Ce gène encode pour une enzyme requise à la synthèse d'ubiquinone. Un des phénotypes des mutants *clk-1* est une altération du cycle de défécation. Chez ces mutants, le cycle de défécation est ralenti et celui-ci ne s'accélère pas à des températures plus élevées, comme observé chez le nématode sauvage. Ces phénotypes sont indépendants l'un de l'autre. Plusieurs résultats expérimentaux suggèrent que le ralentissement du cycle de défécation chez les mutants *clk-1* est du à des altérations du métabolisme lipoprotéines/cholestérol car ce phénotype peut être supprimé par la réduction du cholestérol provenant de la diète, par l'administration de composés affectant le métabolisme des lipoprotéines et par mutation des gènes impliqués dans le métabolisme des lipoprotéines. Ces mutations peuvent êtres divisées en 2 classes : celles qui suppriment le ralentissement du cycle de défécation à 20^{o}C aussi bien qu'à 25^{o}C (classe I) et celles qui suppriment ce phénotypes seulement à 20^{o}C (classe II). La mutation *dsc-4* chez le nématode (impliqué dans l'assemblage des lipoprotéines) ainsi que la mutation *dsc-3* (impliqué dans le métabolisme des molécules de type bile) agissent comme suppresseurs de classe I. Chacune de ces deux mutations restaure la capacité des mutants *clk-1* à réagir à des changements de température. Dans cette étude, nous avons identifié un nouveau suppresseur de classe I, *pgp-2* et classifié *sod-4* comme étant un suppresseur de classe II. *pgp-2* encode une

protéine essentielle à la biosynthèse des organelles intestinales de type lysosome. Les mutations *pgp-2* et *sod-4* combinées ne suffisent pas à restaurer la capacité de *clk-1* à répondre aux changements de température. La combinaison de différentes mutations de classe I ainsi que des mutations de classe I et la mutation de classe II, *sod-4*, nous a démontré que les interactions génétiques entre les différents mutants sont difficiles à prévoir.

Introduction

1.1 *Caenorhabditis elegans* as a model organism

Caenorhabditis elegans is a nematode (roundworm) that reaches a length of 1–2 mm. It is a colonizer of various microbe-rich habitats, in particular—decaying plant matter. When well-fed in the laboratory, at 20°C, *C. elegans* passes through a short embryonic developmental period, followed by four juvenile larval stages (named L1 to L4). These stages are separated by a phase of lethargus, which entails the ceasing of locomotion and pharyngeal pumping, and moulting. *C. elegans*, specifically the reference laboratory strain N2, can develop and reproduce at a wide range of temperatures, but development halts below 8°C and the animals become sterile above 27°C.

C. elegans are easily maintained under laboratory conditions. In the laboratory, the typical source of food for *C. elegans* is a slow-growing strain of *E. coli*, OP50 (Brenner 1974). They have a fast life cycle: embryogenesis occurs in about 12 hours, development to the adult stage takes approximately 2.5 days, and the life span is about 2-4 weeks. This makes them a very convenient model organism.

Although worms show two sexes, males and hermaphrodites, the self-fertilizing hermaphrodite is the predominant sexual form. Each wild-type hermaphrodite produces about 300 progeny. The standard laboratory strain of *C. elegans* has thus been propagated by self-fertilization for so many

generations that individual worms are considered to be virtually genetically identical. Currently, the Wormbase for *C. elegans* genome and biology (www.wormbase.org) provides molecular and phenotypic information regarding individual genes. Conveniently, a large collection of mutants is available to the research community through the *Caenorhabditis* Genetic Center (CGC).

If the animals are starved or crowded during the transition from L1 to L2, they are able to undergo a developmental arrest, known as diapause. Diapause is characterized by reduced metabolism and increased resistance to various environmental stresses as well as to the lack of food. The resultant motile dauers are covered with a thick cuticle, which is a flexible and resilient exoskeleton that protects the worm from environmental adversities (Cassada and Russell 1975). Dauer worms are capable of surviving for several months. When the stressors (lack of food or increased population density) are removed, dauer larvae develop into L4 and then into adults (Cassada and Russell 1975).

The adult hermaphrodite consists of 959 somatic cells. The anatomy of *C. elegans* contains several organ systems. The alimentary system includes the mouth, pharynx, intestine, rectum, and anus. The reproductive system consists of the gonad, uterus, spermatheca, and vulva in the hermaphrodite and the gonad, seminal vesicle, vas deferens, and cloaca in the male. The nervous system contains 302 neurons in the hermaphrodite, with a nerve ring located

in the head. The excretory system is made of a four cells that regulate waste elimination and osmotic pressure. Worms also have striated and non-striated muscle, connective tissue, basement membrane, hypodermis, and a protective cuticle secreted by the hypodermis (Eisenmann, D. M. 2005). *C. elegans* lacks a circulatory system, so worms rely on passive diffusion of oxygen and carbon dioxide through the cuticle and the surface of the intestinal cells for respiration and maintenance of osmotic pressure (Cheung, Cohen et al. 2005).

In general, *C. elegans* are a great experimental organism for understanding the interactions among multiple biological systems and for modeling the pathology of various diseases in intact, multi-organ, multicellular organisms. Even though the worm is a very simple organism that is easy to work with, it shows complex phenotypes common to other multicellular organisms: differentiated cells and organs, ageing, a well-defined lifespan, and a capability for learning and memory formation (Hulme and Whitesides 2010). There is a substantial genetic and biochemical overlap between worms and humans, and despite the fact that the worm genome is much smaller than that of humans (100 Mb as opposed to 3000 Mb), the worm carries almost as many genes (20000 as opposed to 23000, respectively) (Hillier, Coulson et al. 2005). Although only a small amount of worm genes have multiple isoforms as compared to humans (Kim, Magen et al. 2007), 60–80% of the genes found in the worm have human homologs (Kaletta and Hengartner 2006). This presents an excellent opportunity to study various genetic and biochemical

pathways, potentially elucidating their function and creating new venues of mammalian research.

C. elegans are able to modify gene products both post-transcriptionally and post-translationally, and although post-transcriptional modifications are not as common in worms as they are in humans, phosphorylation, acylation, ubiquitination, alkylation, and glycosylation are routinely carried out in *C. elegans* (Forsythe, Love et al. 2006; Carrano, Liu et al. 2009; Morck, Olsen et al. 2009).

With regards to gene expression regulation, worms are different from mammals because they use operons for transcribing about 15% of their proteins (Blumenthal, Evans et al. 2002). Operons are groups of genes that have a common upstream promoter regulating the expression of the entire group. This is a very efficient system because the need for individual promoters is eliminated by transcribing all genes grouped in the operon into a single strand of messenger RNA (Lawrence 2002).

Since many genes that are involved in nutrient utilization and energy storage are conserved between worms and humans, it is likely that these organisms share a lot of similarities in terms of the biochemical processes these genes participate in (Eisenmann 2005). Examples include genes involved in basic pathways of cellular respiration: glycolysis, pyruvate decarboxylation, β-oxidation of fatty acids, the citric acid cycle, the electron transport chain, and

ATP synthesis (Holt and Riddle 2003; Eisenmann 2005; McElwee, Schuster et al. 2006). Just like humans, worms generate glycogen and lipids for energy storage (Cooper and Van Gundy 1970), and use saturated, monounsaturated, polyunsaturated, and branched chain fatty acids (Kuhnl, Bobik et al. 2005; Watts 2009). A feature that separates *C. elegans* from mammals is cholesterol synthesis: mammals are able to synthesize their own sterols, but worms are cholesterol auxotrophs unable to synthesize sterols *de novo* (Kurzchalia and Ward 2003).

1.2.1 Function of CLK-1

As mentioned above, many cellular respiration processes are conserved between worms and humans. One of the genes involved in worm mitochondrial respiration is *clk-1*. This gene encodes an enzyme required for ubiquinone biosynthesis (Felkai, Ewbank et al. 1999). Structurally, ubiquinone (UQ) is a lipid molecule arranged as a redox-active hydrophilic benzoquinone ring and a lipophilic polyisoprenoid side-chain. The side-chain length is different depending on species. For instance, humans and mice produce both UQ_{10} and UQ_9 (the subscripts denote the number of isoprenoid subunits in the side chain), worms produce $UQ_{9,}$ bacteria make UQ_8, and yeast—UQ_6 (Stepanyan, Hughes et al. 2006).

Ubiquinone has an array of functions in the cell. For instance, in mitochondria it transfers electrons from complex I and II to complex III. The leakage of electrons to oxygen can result in the production of reactive oxygen species (ROS). ROS are highly reactive due to the presence of unpaired valence shell electrons. They strive to stabilize their valence by acquiring electrons from the neighboring compounds. Those compounds include essential macromolecules like DNA, amino acids, and lipids.

The mitochondrial electron transport chain uses electrons acquired from the citric acid cycle to generate ATP, by coupling the passage of electrons along the electron transport chain to the pumping of protons into the inter-

membrane space. Complex V uses this proton gradient to drive the production of ATP. Complex I moves electrons from nicotinamide adenine dinucleotide (NADH) to ubiquinone, forming the reduced quinol form of ubiquinone, ubiquinol. Electrons are also transferred to ubiquinone from complex II. Complex III passes electrons from ubiquinol to cytochrome c. Complex IV transfers electrons from cytochrome c to oxygen to generate water (Van Raamsdonk and Hekimi 2010). During electron transfer electrons can also react with oxygen, producing the ROS superoxide at complex I and complex III.

Ubiquinone is also involved in NADH oxidase-dependent transfer of electrons across the plasma membrane (Kishi, Morre et al. 1999; Kishi, Takahashi et al. 1999; Kishi, Takahashi et al. 1999). In its reduced form, ubiquinol is able to act as a soluble lipid antioxidant in cell membranes (Turunen, Olsson et al. 2004). A unique feature of ubiquinone is that it acts as both a pro- and anti-oxidant in biological membranes. Ubiquinone is also present in the lysosome, where it acts as a part of the NADH-dependent ubiquinone reductase proton pump which maintains an acidic luminal pH (Gille and Nohl 2000).

CLK-1 is a nuclear-encoded mitochondrial peripheral inner membrane-associated protein that has homologs in yeast, worms, and mice (Jonassen, Proft et al. 1998; Felkai, Ewbank et al. 1999; Jiang, Levavasseur et al. 2001). In short, CLK-1 hydroxylates 5-demethoxyubiquinone (DMQ) to 5-

hydroxyubiquinone. The latter is then modified into ubiquinone by COQ-3 (Marbois and Clarke 1996). In the absence of CLK-1 activity, DMQ accumulates instead of ubiquinone (Jonassen, Larsen et al. 2001; Levavasseur, Miyadera et al. 2001; Miyadera, Amino et al. 2001). DMQ supports mitochondrial respiration both *in vivo* and *in vitro*, but *clk-1* mutant worms cannot live on bacteria defective in ubiquinone biosynthesis, implying that ubiquinone but not DMQ functions at non-mitochondrial sites (Felkai, Ewbank et al. 1999; Levavasseur, Miyadera et al. 2001; Hihi, Gao et al. 2002).

1.2.2 The Clk-1 phenotype

clk-1 mutants display a multitude of phenotypes referred to as the "abnormal function of biological clocks" (Clk) . This includes slow and desynchronized embryonic and postembryonic development, decreased brood size, slower egg production rate, slower defecation and thrashing rates, and most interestingly, a prolonged lifespan (Wong, Boutis et al. 1995; Kayser, Sedensky et al. 2004). The mechanism by which the impairment of CLK-1 function results in the "Clk" phenotype is still unclear.

Ubiquinone, the product of CLK-1, functions in shuttling electrons to complex III from both complex I and II of the electron transport chain. Although complex I-dependent oxidative phosphorylation is 70-80% lower in *clk-1* mutants as compared to the wild-type worms, complex II activity remains unaffected. All other aspects of the mitochondrial electron transfer machinery function normally in *clk-1* mutants (Kayser, Sedensky et al. 2004). *clk-1* worms show a decrease in energy metabolism as compared to the wild-type worms without displaying reduced oxidative damage (Van Raamsdonk and Hekimi 2010).

C. elegans clk-1 mutants have provided indirect evidence of ROS-mediated signalling in the worm. It was shown that the oxidation of low-density lipoproteins by ROS is important for signal transduction (Shibata, Branicky et al. 2003). It was shown that the altered redox status of the *clk-1* mutants

affects the worm germline development by two pathways: firstly, by the oxidation of worm lipoproteins through the Ack-related tyrosine kinase and inositol trisphosphate signaling, and secondly, through the oncogenic *ras* signaling pathway. Two *clk-1* alleles have been studied extensively: *clk-1(qm30)*, a deletion allele, and *clk-1(e2519)*, a point mutation allele. Interestingly, both of these alleles result in a complete absence of ubiquinone. Yet, most of the phenotypes affected in *clk-1* mutants are slowed down much more severely in the putative null allele *qm30* than they are in the partial loss-of-function allele *e2519* (Wong, Boutis et al. 1995; Felkai, Ewbank et al. 1999).

The defecation cycle length phenotype had been extensively studied as it is can be quantified easily and precisely (the mechanics of defecation will be explained in more detail in the next section). In the presence of adequate food, the normal defecation cycle of wild-type worms is ~56 seconds, with a standard deviation of only a few seconds, while *clk-1* worms show a lengthened defecation cycle of ~85 seconds, with a larger standard deviation (Wong, Boutis et al. 1995; Branicky, Shibata et al. 2001). Another feature that is interesting about *C. elegans* defecation is their ability to adjust to temperature changes: wild-type worms are able to adjust their defecation speed to defecate faster at 20 than at 25°C. However, *clk-1* worms do not exhibit this normal reaction to the temperature shifts – the defecation cycle length remains unchanged in *clk-1*. Therefore, *clk-1* activity is necessary for

readjusting the defecation cycle length in response to changes in temperature (Figure 1). The change in defecation speed in response to changes in temperature is therefore an active process requiring CLK-1, and not merely a consequence of changes to biochemical rates due to the temperature increase. In the absence of *clk-1*, a temperature compensation mechanism that maintains a constant defecation cycle length regardless of changes in temperature is revealed (Branicky, Shibata et al. 2001). The presence of this temperature compensation mechanism is suggested by existence of defecation cycle suppressors of *clk-1 (dsc)* genes. Mutations of these genes (such as *dsc-3* and *dsc-4*, discussed below) are able to suppress the *clk-1* slow defecation phenotype. Some of these suppressor mutations are able to also restore the ability of *clk-1* mutants to respond to the temperature shift, and some are not. These findings demonstrate that *clk-1* is an essential part of the biological mechanism present in *C. elegans* that is responsible for the active adjustment to the temperature changes. After removing this temperature compensation mechanism, a basic passive temperature-dependent adjustment of defecation cycle takes place, where the rates of biochemical reactions passively follow a change in temperature. The presence of two types of suppressors (some do restore the ability to react to temperature shifts and some do not) suggests that the slow defecation and the inability to adjust the defecation cycle length in response to changes in temperature are separable phenotypes in *C. elegans* (Branicky, Shibata et al. 2001).

1.3.1 Worm defecation phenotype

In *C. elegans*, defecation is defined by a defecation motor program consisting of three steps: the posterior body muscle contraction, the anterior body muscle contraction, and expulsion (Thomas 1990). The worm defecation cycle length can be altered by a multitude of environmental variables such as temperature, food, and mechanical stimulation. Defecation cycle length is consistent within and between animals, with the defecation cycle length averaging 56 seconds at 20°C (Branicky, Shibata et al. 2001).

Defecation cycle length can be modulated by introducing a variety of mutations. Mutations in genes that modulate energy metabolism, such as *clk-1*, had been shown to lengthen the defecation cycle length of the worms. However, there are many more mutations that change the defecation cycle length of *C. elegans* (Branicky and Hekimi 2006). The length of the defecation cycle is controlled by calcium oscillations in the intestinal epithelial cells, with the intestinal calcium level peaking just before the initial posterior contraction. Mutations in genes responsible for this oscillation (ex. *itr-1*) alter the worm defecation cycle. *egl-8* mutants are deficient in the production of a phospholipase that is important for carrying out the posterior body contraction through regulating acetylcholine release at neuromuscular junctions. These mutants also show an abnormal egg-laying phenotype. Genes that play a role in modulating the anterior body contraction, such as *abo* and *aex* mutants, are also known to alter defecation cycle length of *C. elegans*.

Mutations in genes involved in the expulsion phase of defecation are able to change worm defecation cycle length and tend to have general defects in neurotransmission (ex. *fat-3*), neurotransmitter γ-aminobutyric acid (ex. *exp-1*), enteric muscle development (ex. *hlh-8*), and semi-dominant gain-of-function mutations (that are also egg-laying defective). Mutations in genes involved in lipid metabolism are also capable of altering the defecation cycle length. For instance, RNAi of *elo-2* (a fatty acid elongation enzyme) and mutations in *dsc-4* (a putative worm microsomal transfer protein, discussed further below) shorten the worm defecation cycle, while mutations in *elo-1* (another fatty acid elongation enzyme), *fat-2* and *fat-3* (fatty acid desaturation enzymes) lengthen defecation cycle and make it less regular (Branicky and Hekimi 2006).

1.3.2 Suppressors of the *clk-1* slow defecation phenotypes

A screen for mutations that suppress the slow defecation phenotype identified two distinct classes of suppressors (Branicky, Shibata et al. 2001). The suppressors of *clk-1* that slow defecation are divided into classes based on their ability to accelerate *clk-1*'s slow defecation at 20°C and after a temperature shift to 25°C (Figure 1).

Class I but not class II mutants are able to restore the ability of *clk-1* to react to temperature shifts, and defecate faster at 25 then at 20°C (Branicky, Shibata et al. 2001). Class II mutants only accelerate *clk-1* defecation rates at 20°C. After a shift to 25°C, defecation rates actually slow to the same rate as un-suppressed *clk-1* mutants.

On the *clk-1* mutant background class I mutants restore the ability of *clk-1* mutants to react to temperature shifting. The proteins that these class I mutants encode might be components of the temperature compensation mechanism present in the worm. These mutations are therefore likely to act by abolishing the mechanism of temperature compensation observed in the absence of *clk-1*, revealing a passive thermodynamic adjustment process (Branicky, Shibata et al. 2001).

Some of the suppressors identified so far include *dec-7 (qm166* and *qm178),dsc-3(qm179, qm180, qm184), dsc-4(qm182),dsc-1(qm133), dsc-2(qm142),* and *dsc-5(qm141)* (Branicky, Shibata et al. 2001).

1.4.1 DSC-4

As mentioned before, worm *dsc-4* is a class I suppressor of the *clk-1* slow defecation phenotype (Branicky, Shibata et al. 2001). This gene is a homolog of the large subunit of the mammalian microsomal triglyceride transfer protein (MTP), which is essential for the assembly of apolipoprotein B (Apo-B)-containing lipoproteins (Shibata, Branicky et al. 2003). Despite molecular similarities between the human MTP and its worm homolog DSC-4, there are also interesting differences between the two: the human MTP transfers both phospholipids and triacylglycerols, while *in vitro* studies show that worm DSC-4 only transfers phospholipids (Rava and Hussain 2007).

In vertebrates, MTP is the large subunit of a heterodimer formed by MTP and disulfide isomerase (Wetterau and Zilversmit 1985). It is proposed to act as a lipid shuttle: MTP extracts lipid molecules from the endoplasmic reticulum membrane, transiently binds and delivers these lipids to the lipoprotein membrane. After this the MTP shuttle becomes available for another cycle of lipid transfer (Atzel and Wetterau 1993). In the worm, DSC-4::GFP fusion protein is expressed in the intestine starting from embryonic elongation and continuing on through adulthood. Just like in vertebrates, the expression of this protein localizes to the endoplasmic reticulum (Rava and Hussain 2007).

It had been suggested that mammalian MTP plays a role in regulating cholesteryl ester biosynthesis in cells that produce lipoproteins, as its

inhibition enhances cellular free cholesterol levels (Iqbal, Rudel et al. 2008). It is possible that DSC-4 performs an analogous function during lipoprotein assembly of *C. elegans*, with DSC-4 regulating the cholesterol uptake instead of lipoprotein assembly, since worms are cholesterol auxotrophs (Shibata, Branicky et al. 2003). *dsc-3*, which is another strong suppressor of *clk-1* slow defecation, had been shown to be involved in the biosynthesis of worm bile-like molecules (Ju-Ling Liu, personal communication). These two mutations were shown to have non-additive effects on the defecation cycle length, suggesting they act in the same genetic pathway (Branicky 2005)).

Lipoproteins are assembled in the endoplasmic reticulum, mature in the Golgi, and secreted by cells. They are composed of a spherical arrangement of lipids and proteins, such as ApoB in vertebrates. This shell encapsulates a neutral lipid core made of triglycerides and cholesterol esters. The bulk of ApoB-containing lipoproteins are assembled in the liver and intestine. The shell of a lipoprotein consists of a phospholipid monolayer. This shell encapsulates a neutral lipid core made of triglycerides and cholesterol esters (Hoofnagle and Heinecke 2009).

Both MTP and ApoB belong to the lipid transfer protein superfamily, which also includes insect apolipoprotein and vitellogenin (Babin, Bogerd et al. 1999). ApoB is an amphipathic secretory integral membrane glycoprotein assembled in the lumen of the endoplasmic reticulum. Vertebrate ApoB is involved in both lipid uptake and lipid redistribution in the body, insect

apolipoprotein functions as a general-purpose lipid transporter (Sappington and Raikhel 1998), and vitellogenin acts as a yolk protein found in females of most egg-laying animals, including *C. elegans* (Romano, Rosanova et al. 2004). Although vitellogenins share structural similarity with ApoB , they are more phospholipid-rich and carry less neutral lipid than the ApoB-lipoproteins (Raag, Appelt et al. 1988; Banaszak, Sharrock et al. 1991; van der Horst, van Hoof et al. 2002).

Vertebrate lipoprotein assembly consists of two steps: first, a precursor Apo-B is formed in the rough endoplasmic reticulum concurrently with Apo-B translation, and then these nascent lipoproteins fuse with triglyceride-rich emulsion particles produced in the smooth endoplasmic reticulum. As mentioned above, MTP facilitates both of these steps, performing the transfer of lipids (triacylglycerols, phospholipids, and cholesteryl esters) (Shelness and Ledford 2005). Vitellogenins are known to transport yolk constituents to the germline for oogenesis in *C. elegans.* DSC-4 may be responsible for secretion of another class of LDL-like lipoproteins, because *dsc-4* mutant worms do not show a decrease in brood size. If *dsc-4* was required for yolk production, mutations of *dsc-4* should drastically reduce brood size, as do mutations in *rme-2*, which encodes a receptor for yolk proteins (Shibata, Branicky et al. 2003; Turunen, Olsson et al. 2004).

1.4.2.1 DSC-3

dsc-3 is another reported class I suppressor of the *clk-1* slow defecation phenotype (Branicky, Shibata et al. 2001). Bile supplementation studies show that *dsc-3* is likely involved in the worm secretion of bile-like molecules (Ju-Ling Liu, personal communication). DSC-3 is a worm homolog of mammalian P-type ATPase ATP8B1.

ATP8B1 transports aminophospholipids from the outside of a bilayer leaflet to the inside, maintaining membrane lipid asymmetry. Bilayer membranes are usually asymmetric in nature, containing higher levels of aminophospholipids on the cytosolic leaflet and higher levels of choline-containing lipids on the extracellular leaflet. This asymmetry is crucial for signal transduction, vesicular trafficking, and membrane stability (Verkleij and Post 2000). The mammalian expression of ATP8B1 is largely specific to the liver, intestinal, pancreatic, and cochlear hair cells.

In vertebrates, mutations in ATP8B1 result in a variety of phenotypes such as the impairment of hepatic secretion and absorption of bile acids and extrahepatic symptoms such as diarrhea, hearing loss, and a lack of microvilli formation in polarized epithelial cells (Verhulst, van der Velden et al. 2010). ATP8B1 deficiency leads to hepatic cholestasis, a clinical condition where bile cannot flow from the liver to the duodenum. This impairment of bile flow is a result of phospholipid randomization and dysfunction of the hepatic

canalicular membrane. As a result, the canalicular membrane of the affected individuals shows a dramatically reduced cholesterol-to-phospholipid ratio and an impairment of bile salt excretory pump (BSEP) activity (Paulusma, de Waart et al. 2009).

Dysfunction of ATP8B1 impairs BSEP function in two ways. Firstly, disrupting lipid leaflet asymmetry leads to a mechanical inability of the BSEP protein to function properly when it is integrated into the compromised membrane. Secondly, ATP8B1 post-translationally activates the Farnesoid X-Receptor, which in turn mediates transcription of BSEP (Makishima, Okamoto et al. 1999; Parks, Blanchard et al. 1999; Sinal, Tohkin et al. 2000; Ananthanarayanan, Balasubramanian et al. 2001). In either case, BSEP dysfunction results in disruptions in bile flow. It is not yet known if *C. elegans* carry a BSEP homolog.

1.4.2.2 ATP cassette-containing genes

As described in the previous section, DSC-3 is a worm homolog of mammalian P-type ATPase ATP8B1. Both worm DSC-3 and human ATP8B1 belong to the family of genes called ATP-binding cassette (ABC) transporter family. The conservation analysis of ABC transporter family across species is minimal, and none of the *pgp* genes in *C. elegans* have a simple orthologous relationship with any of the mammalian *pgp*s (Sheps et al., 2004). However, the basic functional organization of an ABC transporter in the membrane is the same in all subclasses from bacteria to humans.

In humans, genes belonging to the ABC family code for transporter proteins that utilize ATP to move fats, sugars, amino acids, and drugs across cell membranes. The presence of a strongly conserved ATP-binding motif determines membership in the family. Nomenclature of the ABC transporters in literature is not completely uniform and has not yet been fully standardized. Generally, in the human genome ATP transporters are designated by the letters ABC and an additional letter indicating the subgroup to which they belong. The subgroup designation is based on their structure (the number of ATP domains and the number of transporter domains). They also receive a number to designate the specific gene within the subgroup (Sheps et al., 2004; Higgins et al., 1992).

A typical ABC transporter consists of at least one nucleotide-binding domain and a transmembrane domain (half transporter). Full transporters contain two nucleotide-binding domains and two transmembrane domains (Hyde et al. 1990). A functional transporter complex has to contain at least two ATP-binding domains coupled to two blocks of membrane-spanning helices. Depending on a transporter, these domains can be found either within a single molecule or distributed across separate proteins that assemble into a complex (Croop et al., 1998, Zhao et al., 2007).

In *C. elegans* there are 60 known ABC genes. They are broken down into eight subfamilies, A to H. Subfamily A contains *abt* and *ced* genes, subfamily B—*pgp* and *haf* genes, subfamily C—*mrp* genes, and subfamily D—*pmp* genes. Subfamilies E-H contain previously unidentified genes (Zhao et al., 2006). The present work shows that out of all 14 functional *pgp* (p-glycoprotein) genes present in the worms, only mutations in *pgp-2* suppress the slow defecation of *clk-1*.

PGP-2 is expressed in the intestinal cells, specifically— in the membrane of lysosome-like organelles (LROs). LROs were previously proposed to store lipid in the worm intestine because these compartments are stained by a lipophilic vital dye Nile red. However, it has recently been shown that *pgp-2* mutants have relatively normal triacylglyceride levels, while displaying very low levels of Nile red fluorescence in the intestine. It was suggested that the loss of LROs does not eliminate lipid storage. Nile red had been shown to co-

localize with the PGP-2::GFP, but not with a fixative lipophilic dye Oil Red O. Since LROs also display many of the endocytic markers, it was concluded that they are terminal endocytic compartments, and Nile red gathers in LROs as the dye is being degraded (O'Rourke et al., 2009). Research suggests that LROs and lysosomes carry out separate functions in the worms (Ashrafi et al., 2003; Nunes et al., 2005; Schroeder et al., 2007). The precise function of LROs has not yet been elucidated and the role PGP-2 plays in the functioning of this organelle also remains unclear.

1.4.3 SOD-4

A superoxide dismutase-4 *(sod-4)* knockout had been shown to partially suppress most *clk-1* phenotypes (Stepanyan, Hughes et al. 2006). It was hypothesized that *clk-1* worms have less lipoprotein oxidation because of decreased ROS. Although ROS are responsible for much of the damage done to cell structures, including lipoproteins, they are also involved in many redox signaling mechanisms (such as apoptosis and mobilization of ion transport). Cells are normally able to defend themselves against ROS damage by using antioxidant molecules like ascorbic acid, tocopherol, glutathione and enzymes like catalases, lactoperoxidases, glutathione peroxidases, peroxiredoxins, and superoxide dismutases (Van Raamsdonk and Hekimi 2010).

Removal of SOD-4 may result in an increase in oxidative stress and, subsequently, an increase in the amount of damage sustained by worm lipoproteins (Stepanyan, Hughes et al. 2006). It was shown that mammalian oxidized low density lipoproteins are no longer recognized by their respective receptors. This happens because the lipoprotein particle undergoes morphological modifications when exposed to ROS (Steinberg 1997). These oxidized lipoproteins are cleared by scavenger receptors and then degraded (Murphy, Tedbury et al. 2005). It is possible that the *sod-4* suppression effects are due to a decrease in circulating lipoprotein due to a similar mechanism.

SODs are a class of enzymes that catalyze the conversion of superoxide into oxygen and hydrogen peroxide. Superoxide is a type of reactive oxygen species (ROS) (Van Raamsdonk and Hekimi 2010). In worms there are five *sod* genes. *sod-1* encodes the primary cytoplasmic SOD, *sod-2* encodes the primary mitochondrial SOD, and *sod-4* encodes extracellular SOD, while *sod-3* and *sod-5* encode secondary SODs (Van Raamsdonk and Hekimi 2010). SODs are the first line of defense against ROS: they convert superoxide to H_2O_2, which can be then converted to water.

sod-4 has been shown to suppress the *clk-1* slow defecation phenotype but not slow germline development in *C. elegans* (Jing-Jing Li, personal communication). *sod-4* had not yet been characterized as either a class I or II *clk-1* slow defecation suppressors, as it had previously only been scored at 20°C (Stepanyan, Hughes et al. 2006).

1.4.4 PGP-2 and LROs

As it will become apparent in subsequent sections of this thesis, mutations in the worm p-glycoprotein gene (*pgp-2*) suppress the *clk-1* slow defecation phenotype. *pgp-2* encodes a protein essential for the biosynthesis of the worm lysosome-related organelles (LROs). These organelles are located in the worm intestine (Hermann, Schroeder et al. 2005; Schroeder, Kremer et al. 2007). Generally, LROs re a group of cell-type specific compartments that share many characteristics with conventional lysosomes: they are acidified and contain lysosomal proteins, but have distinct functions and morphology (Raposo et al., 2007). The precise physiological function of these structures in the worm intestine has not yet been identified.

1.5 Link between *clk-1* and worm lipoprotein metabolism

Several lines of evidence suggest that the slowed defecation of *clk-1* mutants is due to alterations in lipoprotein/cholesterol metabolism. Indeed, the mutant phenotype can be suppressed by the reduction of dietary cholesterol (as described above, *C. elegans* are sterol auxotrophs). Drugs affecting lipoprotein metabolism (e.g. that alter HMG-CoA reductase activity, reverse cholesterol transport, or high density lipoprotein uptake) also rescue the *clk-1* slow defecation phenotype (Hihi, Beauchamp et al. 2008). Mutations in the *dsc-4* and *dsc-3* genes have been also shown to suppress *clk-1* slow defecation (Branicky, Shibata et al. 2001).

Our current understanding of the *clk-1* slow defecation phenotype is far from complete. Current results are consistent with the notion that *clk-1* mutant worms contain an increased amount of lipoproteins. A knockdown of genes such as *dsc-3* (putatively responsible for the production of bile-like molecules), *dsc-4* (likely involved in lipoprotein assembly), and *sod-4* (putatively increasing oxidative damage leading to degradation of lipoproteins) can suppress this phenotype.

1.6 Objectives of this project

This work is meant to fulfill several objectives. First, it is important to identify other suppressors of the *clk-1* slow defecation phenotype to build a better understanding of the biological mechanisms responsible for the *clk-1* slow phenotype. Since worm *dsc-3* is a homolog of human *atp8b1*, and BSEP is located downstream of ATP8B1, the next logical step is to check if BSEP has any worm homologs. If worm BSEP homolog(s) exist, the next step is to check if these homologs are suppressors of the *clk-1* slow defecation phenotype.

Secondly, it is important to classify the novel *sod-4* suppressor and check for additive effects with other known suppressors such as *dsc-3* and *dsc-4*. There is evidence that *dsc-3* and *dsc-4* are involved in the same genetic pathway. Checking for additive effects of the novel *sod-4* suppressor would indicate whether or not it acts in the same genetic pathway as *dsc-3* and *dsc-4*.

Furthermore, the significance of the two classes of *clk-1* slow defecation suppressors needs to be investigated. One way to do this is to construct strains containing combinations of various mutations of the same class as well as strains containing mutations from both classes. The defecation length of mutants containing these mutations needs to be assessed on the wild-type and *clk-1* backgrounds. Patterns that may emerge from these experiments may elucidate the significance of the two classes of *clk-1* suppressors.

Materials and Methods

2.1 Nematode strains and general cultivation methods

Animals used as a wild-type control belong to the N2 Bristol strain. All mutants were obtained from the Caenorhabditis Genetics Center. Table 1 contains mutant alleles used in all experiments.

Worms were maintained on Nematode Growth Medium (NGM) seeded with *Escherichia coli* strain OP50 as described (Brenner 1974). NGM contains sodium chloride, Bacto-peptone, agar, and cholesterol. The petri dishes were obtained from Fisher Scientific. Animals were cultured at 20°C, and subsequently scored at two temperatures: 20 and 25°C.

2.2 Defecation cycle scoring

For this project, scoring of both RNAi-treated worms and genetic mutants was performed in the same way. Fifteen L4 stage worms per plate were picked 24 hours prior to scoring. Water-filled isolator plates were used to prevent the heating of the worm-containing plates by the microscope lamp. Each plate was scored for a maximum of 15 minutes at a time. Worms were scored at two temperatures: 20 and 25°C. A temperature- and humidity-controlled environmental chamber was used for scoring the defecation cycle length at 25°C, while 20°C scoring was performed in the lab. Worms were placed in the 25°C environmental chamber 2 hours before scoring to allow them to adjust to the temperature shift.

Defecation cycle length was measured in seconds with a standard timer. A defecation cycle was defined as the time between two consecutive posterior contractions. A minimum of three consecutive defecation cycles per worm were recorded, and an average of the defecation cycle length was calculated for each worm. At least 12 worms of each genotype were assessed. All mutants were scored on the same day as their respective controls. The scoring results were plotted and analyzed in GraphPad Prism4 (GraphPad) software. Defecation cycle lengths of worms of different genotypes were compared using Student's t-test.

Differences were considered statistically significant at $p<0.05$. Visually, statistical differences were represented in figures by asterisks:

* depicts p=<0.05

** depicts p=<0.001

*** depicts p=<0.0001

2.3 RNAi screen for novel suppressors of *clk-1* slow defecation

The screen for novel suppressors was performed via RNAi feeding against worm p-glycoprotein genes (*pgp*s) to observe the effect of silencing each *pgp* on the defecation cycle length of both wild-type and *clk-1(qm30)* worms. RNAi delivery technique has been described in detail (Mello and Conte 2004). In short, RNAi clones were obtained from the GeneService RNAi library. RNAi plates were prepared by adding 1 mL of 50 mg/mL Amp and 1M IPTG per per 1L NGM medium to the NGM solution. The bacteria were grown overnight, diluted (1mL bacterial culture into 8 mL of LB-Amp), and seeded on the plates once the culture reached the absorbance of 0.6. These plates were allowed to dry overnight. Worms in the L4 stage of development were then transferred onto those plates. The effects of knocking down each *pgp* were assessed in the F1 generation. In all RNAi experiments negative and positive controls were used:

Negative control: *E. coli* strain HT115(DE3) (L4440), an empty vector with no insert

Positive control: *E. coli* strain HT115(DE3) with *dsc-4* insert , as *dsc-4* has been previously identified as a class I suppressor of *clk-1* slow defecation phenotype (Branicky, Shibata et al. 2001).

The RNAi screen was performed on both N2 and *clk-1(qm30)* worms at 20 and 25°C. After isolating a novel *clk-1* slow defecation suppressor *pgp-2*, a

secondary screen was performed using both available *clk-1* alleles: *qm30* and *e2519* at 20 and 25°C.

The *C. elegans* genome contains 14 *pgp* genes for which the constructs are available from the RNAi library (GeneService, Source BioScience LifeSciences). The sequences of these constructs were blasted against the *C. elegans* genome to see if any other genes apart from the target *pgp*'s were going to be affected by the RNAi feeding. Any constructs targeting more than 25 consecutive base pairs of multiple genes were noted (Table 2).

2.4 Testing the genetic mutant *pgp-2*

Since RNAi can vary in its efficiency, it is important to test if the genetic mutant *pgp-2* acts as a suppressor of *clk-1* slow defecation. This was done by crossing the novel suppressor *pgp-2(gk114)* to multiple *clk-1* alleles: a more severe *qm30* and a less severe *e2519* (Ewbank, Barnes et al. 1997). Once *pgp-2* was identified as a suppressor, it was classified by scoring the double mutants at both 20 and 25°C.

2.5.1 Construction of worm strains containing multiple mutations

To test for epistasis between the known suppressors of *clk-1* slow defecation phenotype, multiple mutations were introduced on the wild-type and *clk-1(qm30)* backgrounds. Males were generated by picking two plates containing L4 hermaphrodites to several plates, 20 worms to each plate. Those plates were mildly heat shocked at 30°C for 6 hours. Once several males were obtained, they were mated back into the homozygous mutant strain. Matings were always set up on an approximately 1 cm diameter bacterial spot located in the center of the plate. Males were always mated with virgin hermaphrodites (L4 stage).

After the initial cross, the F1 worms were picked to fresh plates and allowed to lay eggs. After laying eggs, those worms were genotyped. Plates that contained heterozygotes were kept. Eighteen progeny of each heterozygote were isolated to fresh plates again. These worms were genotyped to reveal homozygous mutants of interest.

The following mutant strains were constructed:

pgp-2(gk114); clk-1(qm30)

clk-1(qm30);dsc-3(tm1634)

pgp-2(gk114);dsc-4(qm182)

pgp-2(gk114; sod-4(gk101)

pgp-2(gk114);dsc-3(tm1634)

dsc-4(qm182);sod-4(gk101)

pgp-2(gk114);dsc-4(qm182); sod-4(gk101)

pgp-2(gk114);clk-1(qm30); sod-4(gk101)

pgp-2(gk114);clk-1(qm30); dsc-4(qm182)

pgp-2(gk114);clk-1(qm30); dsc-3(tm1634)

clk-1(qm30);dsc-3(tm1634);dsc-4(qm182)

clk-1(qm30); dsc-4(qm182);sod-4(gk101)

clk-1(qm30); dsc-3(tm1634);sod-4(gk101)

pgp-2(gk114);clk-1(qm30);dsc-4(qm182);sod-4(gk101)

pgp-2(gk114);clk-1(qm30);dsc-3(tm1634);dsc-4(qm182)

2.5.2 Genotyping

Worm Lysis: 22 µL of water were mixed with 2.5 µL of PCR buffer (Qiagen) and 0.5 µL of 20mg/L proteinase K solution (BioWorld). The mixture was votrexed and allocated to PCR strip tubes. Individual worms were picked to those tubes. Run at 65°C for 1 hour and for 95°C for 5 minutes. The resultant mixture was used immediately for PCR as a DNA template to avoid DNA degradation. PCR was performed with HotStarTaq polymerase according to product protocol (Qiagen). *clk-1(e2519)* and *dsc-4(qm182)* are point mutations, so the PCR product was restriction cut to identify the mutants.

Genotype	Forward primer	Reverse primer	Restriction enzyme
clk-1 (qm30)	gtg tgg ctg ctt atg ctc tcg	gcc tga ttg aag caa ctt taa g	-
clk-1 (e2519)	cga cga tcc tga aac aca c	cca ttt gag agc cga gta g	Hind III
dsc-3 (tm1634)	gaa caa ttc cag gcg gat agc	ctt cca gtt tga tat tcc ca	-
dsc-4 (qm182)	gac cgg cgc tta ctc gag aaa c	cgc att tct gca caa tgt ctg gac	Hinf I
sod-4 (gk101)	gat tgt agg ggc tca aac ttc	gaa acc gat ccg tta agc tg	-
pgp-2 (gk114)	ccg ttg att gtg ctg ctc tct g	ggg gac aga aac ggt act c	-

2.6 Nile Red fluorescence: checking for the presence of gut granules

Nile red is a vital dye that stains for specific intestinal organelles called lysosome related organelles (LROs). Nile red staining was carried out to confirm the absence of functional LROs in *pgp-2* mutants. Although LROs are autofluorescent, the Nile Red feeding makes the lack/reduction of LRO fluorescence easier to view. The protocol has previously been described in detail (Mak, Nelson et al. 2006). In short, Nile Red (Invitrogen) was dissolved in acetone to the solution of , 0.5 µg/mL. This stock solution was diluted in 1X PBS to 1µg/mL. Five hundred microliters of this solution was added to NGM plates on top of the fresh *E. coli* OP50 lawn. Those plates were dried overnight. L4 stage worms were added to the plates containing Nile red, and allowed to lay eggs for 5 hours. F1 progeny was picked for imaging. Worms were anaesthetized with 250 µM sodium nitrate and viewed with the 10X objective. The microscope used was a Leica Leitz DMRBE equipped with a Leica DC 300F camera. All Nile red images were acquired using identical settings and exposure times with ImagePro software (MediaCybernetics). The excitation filter wavelength was ~545 nm and the emission filter wavelength was ~630 nm.

Results

3.1 *pgp* RNAi on wild-type and *clk-1* worms at 20 and after the shift to 25°C

Since worm *dsc-3* has been previously shown to suppress the *clk-1* slow defecation phenotype, the worm homologs of BSEP were tested for candidate suppressors of this phenotype. Upon blasting the human BSEP protein sequence against the worm proteome, one family of genes containing 15 members stood out: the worm *pgp* (p-glycoprotein) gene family. Fourteen *pgp*s were listed as functional genes, and one (*pgp-15*) was listed as a pseudo-gene. The pseudo-gene was discarded from the candidate list. Table 4 lists the protein similarities between the known worm PGPs and human BSEP. To screen for potential suppressors of *clk-1* slow defecation, RNAi experiments against all 14 *pgp*s were performed on wild-type and *clk-1* worms. Performing RNAi of all fourteen *pgp* genes on wild-type worms revealed that knockdown *pgps* did not affect the defecation cycle length of the wild-type worms at either 20 or after the shift to 25°C (Figure 2). However, RNAi of *pgp-2* was capable of suppressing the *clk-1* slow defecation phenotype at both 20 and 25°C. RNAi of the other *pgp* genes had no effect (Figure 3).

3.2 Characterization and classification of *pgp-2*

RNAi against *pgp-2* was performed on the two *clk-1* alleles to determine if both alleles were affected in a similar fashion. It is suspected that *clk-1(e2519)* still retains some of the *clk-1* activity, since the magnitude of its effect on defecation is smaller than that of *clk-1(qm30)*. The *clk-1 alleles* used were the weaker *e2519* point mutation and the stronger deletion allele *qm30*. The experiment confirmed that *pgp-2* RNAi did not affect the wild type worms, but suppressed the slow defecation of both *clk-1* alleles (Figure 4). This suggests that the *pgp-2* suppression effect is not allele-specific. However, the suppression effect of *pgp-2* seemed to be proportional to the severity of the *clk-1* allele, suppressing *clk-1(e2519)* to a greater extent than *clk-1(qm30)*. Therefore, although *pgp-2* suppresses slow defecation of both *clk-1* alleles, the magnitude of suppression varies. *dsc-4* suppresses the long defecation of both *clk-1* alleles to the same extent. In both mutant backgrounds worms on *dsc-4* RNAi were able to respond to the temperature shift, but the same worms on *pgp-2* RNAi were not.

RNAi does not always eliminate a hundred percent of the gene product. In this study it can be demonstrated by comparing positive controls of different groups (Figures 2 and 3), it is evident that the RNAi feeding was not very consistent between trials. To make more concrete conclusions, a *pgp-2(gk114)* mutant was acquired from CGC. This is a deletion mutant that is thought to be a complete knockout. Mutations in *pgp-2* had no effect on the wild-type background at either temperature, but had a suppression effect on the *clk-1(qm30)* slow defecation

phenotype at both temperatures (Figure 5). *pgp-2* is therefore a class I suppressor of the *clk-1* slow defecation phenotype. Although *pgp-2* was found to be a strong suppressor of *clk-1* slow defecation phenotype, it was not able to completely restore *clk-1*'s defecation cycle back to wild-type length. Mutations in *pgp-2* were also unable to restore the ability of *clk-1* to react to the temperature shift (Figure 5).

As mentioned previously, PGP-2 is involved in the biosynthesis of worm intestinal lysosome-related organelles (LROs). To determine if it was purely the absence of LROs that suppressed the *clk-1* slow defecation phenotype, other mutants that result in LRO loss were assessed. For instance, *glo-1, apd-3,* and *glo-3* mutants are LRO-deficient. Mutations in *glo-1* result in malfunction of a Rab GTPase that plays a key role in worm LRO biogenesis (Hermann, Schroeder et al. 2005). On the wild-type background at both temperatures *glo-1* and *apd-3* increased the defecation cycle length of the worms, while *glo-3* decreased it (Figure 6). On the *clk-1* background, *glo-1* also increased the defecation cycle length of the *clk-1(qm30)* worms (Figure 7). Therefore it is not simply the absence of LROs that is responsible for *pgp-2*'s suppression of the *clk-1* defecation phenotype.

To visualize the LROs of the wild-type worms and see if they are visibly different from the LROs of the *clk-1(qm30)* worms vital dye Nile red staining was performed. Nile red accumulates more in the *clk-1(qm30)* mutants than in the wild-type worms (Figure 18).

3.3 Classification of *clk-1* slow defecation suppressors

pgp-2(gk114) suppressed the *clk-1* slow defecation phenotype at both 20 and 25°C, putting *pgp-2* in the class I suppressor category (Figure 8). *pgp-2* mutation was unable to suppress the inability of *clk-1* worms to adjust to the temperature shift, while *dsc-3* and *dsc-4* mutations were able to do so (P=<0.001 and P=0.08 respectively). *sod-4* was previously identified as a suppressor of *clk-1* slow defecation, but was never placed in a suppressor class. This study shows that *sod-4* is a class II suppressor because it shortens the slow defecation of *clk-1(qm30)* at 20°C, but has no effect at 25°C (Figure 9).

3.4 Interactions between class I suppressors

The two suppressor classes had been identified, but their significance had not been elucidated. One approach is to combine class I mutants and see if there is a pattern to their interactions. In this experiment, various combinations of class I mutants were tested on both wild-type and *clk-1(qm30)* backgrounds.

On the wild-type background at 20°C the effects of *dsc-4* and *pgp-2* mutations were not additive, with the double mutant defecating at the same speed as the *dsc-4* mutant (Figure 10 (a)). However, after shifting the temperature to 25°C, there appeared to be a weak additive effect (Figure 10 (a)). All mutants at both temperatures were able to adjust to the temperature shift. The same combination of *pgp-2* and *dsc-4* mutations on the *clk-1* background did not show any additivity at 20°C (the double mutant defecates at the speed of the *dsc-4* mutant), but did show interference at 25°C (*pgp-2;clk-1;dsc-4* worms showed defecation phenotype intermediate between *pgp-2;clk-1* and *clk-1;dsc-4* worms) (Figure 10 (b)). *dsc-4* but not *pgp-2* restored the ability of *clk-1* worms to react to the temperature shift. When these mutations are combined, *pgp-2* has a dominant effect over *dsc-4* where *pgp-2;clk-1;dsc-4* worms are not able to adjust to the temperature shift (Figure 10 (b)).

On the wild-type background the combination of *pgp-2* and *dsc-3* showed interference at both temperatures (Figure 11 (a)). All mutants on wild-type background were able to adjust to the temperature shift. On the *clk-1* background

dsc-3 is a much stronger suppressor of the *clk-1* slow defecation phenotype than is *pgp-2* (Figure 11 (b)). At both temperatures on the *clk-1* background the effects of *pgp-2* and *dsc-3* mutations were additive, with the triple mutant defecating faster than either of the double mutants (Figure 11(b)). In contrast to *dsc-4, dsc-3* is dominant over *pgp-2*, and the worms containing the combination of these two mutations on *clk-1* background are able to react to the temperature shift (Figure 11 (b)).

Previously the effects of *dsc-3* and *dsc-4* were shown to be non-additive (Branicky, Shibata et al. 2001). In their work, Branicky et al. used a point mutation allele *dsc-3(qm179),* which has not been proven to be a complete knockout. In this experiment, *dsc-3(tm1634)* deletion allele was used. This allele is more likely to result in DSC-3 loss of function than is *qm179.* After using the putative deletion allele (*tm1634*) as opposed to a point-mutant (*qm179*), the effects of *dsc-3* and *dsc-4* were shown to be additive at 25°C, but not 20^{o}C on *clk-1* background (Figure 12).With regards to restoring the ability of *clk-1* worms to react to the temperature shift, mutations in both *dsc-3* and *dsc-4* restored it mildly. When combined, these mutations show a much stronger reaction to the temperature shift (Figure 12).

To see the effect combining all class I suppressors together on the *clk-1* background, a quadruple mutant strain was constructed. This combination of all class I suppressors resulted in complex interactions that are hard to interpret (Figure 13). In short, it seems that mutations in *dsc-3* always make *clk-1* worms

defecate faster after shift to 25, regardless of any other mutations. Although the biochemical pathways governing the defecation cycle length of these mutants cannot be unraveled by this method, it is interesting that *dsc-3* is dominant to all other mutations tested with regards to the restoration of the *clk-1* ability to react to the temperature shift. This suggests that *dsc-3* plays an essential part in the mechanism involved in temperature compensation.

3.5 Interactions between class I and class II suppressors

To attempt understanding the significance of the two suppressor classes, class I and class II mutations were combined on the wild-type and *clk-1* backgrounds. On the wild-type background at 20°C the effects of *sod-4* and *pgp-2* were additive, with the double mutant defecating faster than either *pgp-2* or *sod-4* single mutants. However, after shifting the temperature to 25°C, this additive effect disappeared (Figure 14). On *clk-1* background the same combination of mutations acts very differently*: sod-4* is a stronger suppressor of *clk-1(qm30)* slow defecation than is *pgp-2* at 20°C. At 20°C the combination of the two mutations showed interference: the triple mutant defecated slower than either of the double mutants. At 25°C the combination of *pgp-2* and *sod-4* on *clk-1* background showed additive effects (Figure 14). With regards to the ability of these mutants to adjust to the temperature shift, neither *pgp-2* or *sod-4* single mutants defecate faster at 25°C than at 20°C. However, when combined on the *clk-1* background, the *pgp-2;clk-1;sod-4* mutant was able to adjust to the temperature shift.

When *dsc-4* and *sod-4* mutations were combined on *clk-1* background, the double mutants defecated at the speed of *sod-4* mutants at both 20 and 25°C (Figure 15). On the *clk-1* background at both 20 and 25°C *sod-4* partially over-rides the suppressive effect of *dsc-4*. On the *clk-1* background, the combination of *dsc-4* and *sod-4* mutations does not restore the ability of the worms to react to the temperature shift (Figure 15).

On *clk-1* background worms containing the *sod-4* and *dsc-3* mutations defecated at the same speed as *dsc-3* single mutants at 20°C. However, these mutations showed an additive effect at 25°C (Figure 16). It is also interesting that mutations in *dsc-3* rescued the ability of *clk-1;sod-4* worms to adjust to the temperature shift.

When two class I mutants, *pgp-2 and dsc-4*, were combined with a class II mutant *sod-4* on the wild-type background, the interactions between classes became complex. The triple mutant containing all three mutations defecated at the same speed as *pgp-2;sod-4* double mutant, but significantly slower than *pgp-2;dsc-4* or *dsc-4;sod-4* double mutants (Figure 17). The same mutations on *clk-1* background showed a different suppression pattern. At 20°C all mutations containing *dsc-4* suppressed the *clk-1* slow defecation phenotype to the same degree. At 25°C the quadruple mutant defecated faster than any of the triple mutants (Figure 17). It seems that the combination of class II *sod-4* mutation with any class I mutation results in faster defecation at 25 than the class I alone. It is mutant combinations containing *pgp-2* and *sod-4* were able to respond to the temperature shift. This is interesting because neither mutant is capable of suppressing the *clk-1* slow defecation phenotype on its own after shifting.

Figures and Tables

Table 1: Mutant alleles used in this project. The molecular and phenotypic information regarding individual genes was obtained from the Wormbase for *C. elegans* genome and biology (www.wormbase.org). Some strains were obtained from the National Bioresource Project for the nematode (http://www.shigen.nig.ac.jp/c.elegans/index.jsp).

Gene (allele)	Mutation type	WormBase gene reference ID/strain name
clk-1(qm30)	426 bp deletion	WBGene00000536/MQ130
clk-1(e2519)	G to A mutation at 444bp	WBGene00000536/CB4876
dsc-3(qm179)	G to A mutation at 1993 bp	Constructed in this laboratory
dsc-3(tm1634)	751 bp deletion	WBGene00001098/National Bioresource Project for the nematode
dsc-4(qm182)	G to A mutation at 501 bp	WBGene00001099/ MQ920
sod-4(gk101)	551 bp deletion	WBGene00004933/ VC175
pgp-2(gk114)	1674 bp deletion	WBGene00003996/VC134

Table 2: RNAi constructs targeting more than 25 consecutive base pairs of more than one *pgp*. Some *pgp* constructs found in the RNAi library have the potential to target more than one gene of interest.

RNAi library construct available	**Genes potentially targeted by this RNAi treatment**
pgp-1	*pgp-1*
pgp-2	*pgp-2*
pgp-3	*pgp-3 and pgp-4*
pgp-4	*pgp-3 and pgp-4*
pgp-5	*pgp-5*
pgp-6	*pgp-6 and pgp-7*
pgp-7	*pgp-5, pgp-6, and pgp-8*
pgp-8	*pgp-8*
pgp-9	*pgp-9*
pgp-10	*pgp-10*
pgp-11	*pgp-11*
pgp-12	*pgp-12, pgp-13, pgp-14, and haf-7*
pgp-13	*pgp-12, pgp-13, and pgp-14*
pgp-14	*pgp-12, pgp-13, and pgp-14*

Table 3: Function and expression patterns of worm PGPs as well as protein similarity of each worm PGP to human BSEP. No single worm PGP is significantly more similar to BSEP then the next.

Worm PGP	Expression pattern	Function	BSEP similarity (%)
1	Intestine, intestinal valve	Defense against pathogens	57
2	Intestine	Lysosome-related organelle formation	58
3	Excretory cell, intestine	Defense against pathogens	55
4	Excretory cell	Unidentified	55
5	Intestine	Predicted to export exogenous toxins	53
6	Amphids, pharynx, intestine	Unidentified	52
7	Male tail	Unidentified	52
8	Head neurons	Unidentified	51
9	Pharynx, intestine	Unidentified	56
10	Intestine, hypodermis	Unidentified	47
11	Excretory cell, intestine	Unidentified	52
12	Excretory cell	Unidentified	54
13	Intestine, amphids	Unidentified	53
14	Pharynx	Unidentified	53

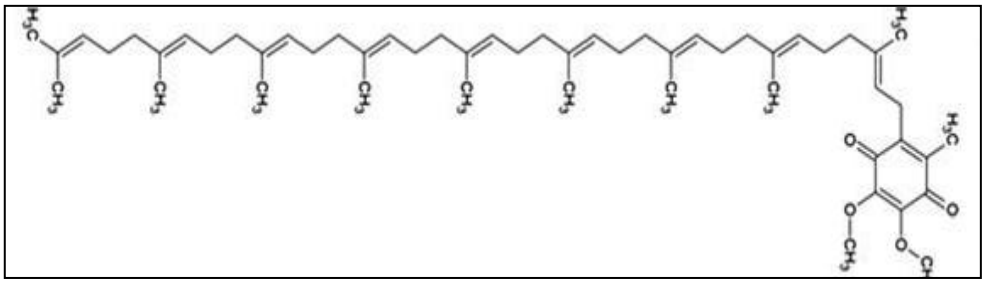

Figure 1(a): Ubiquinone chemical structure (adapted from Branicky et al., 2010)**.**

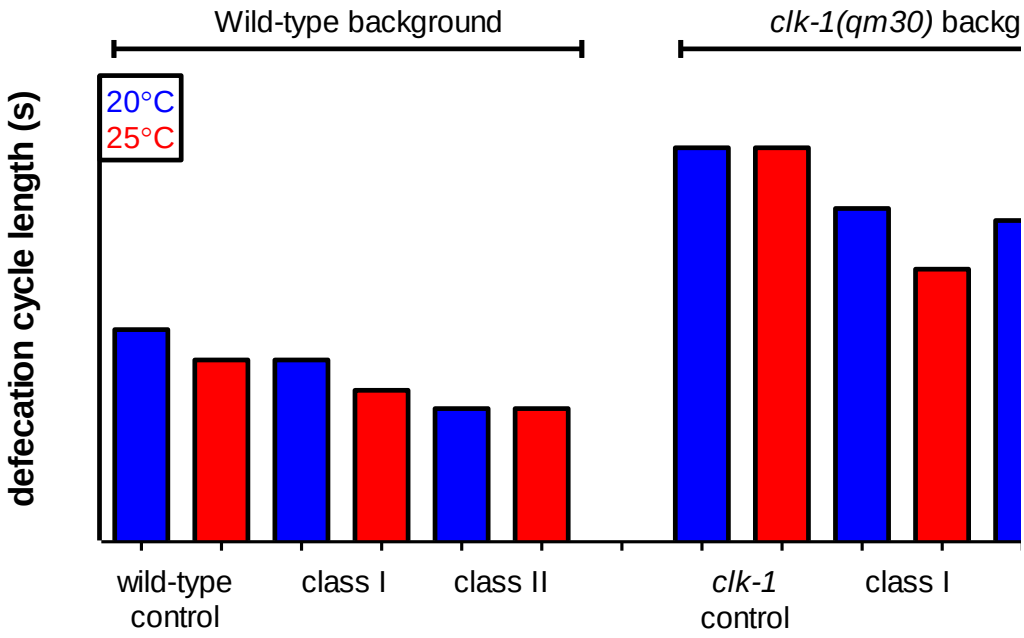

Figure 1(b): representative depiction of how class I and class II mutants behave compared to the controls (wild-type and clk-1(qm30)) (adapted from Branicky, Shibata et al. 2001).

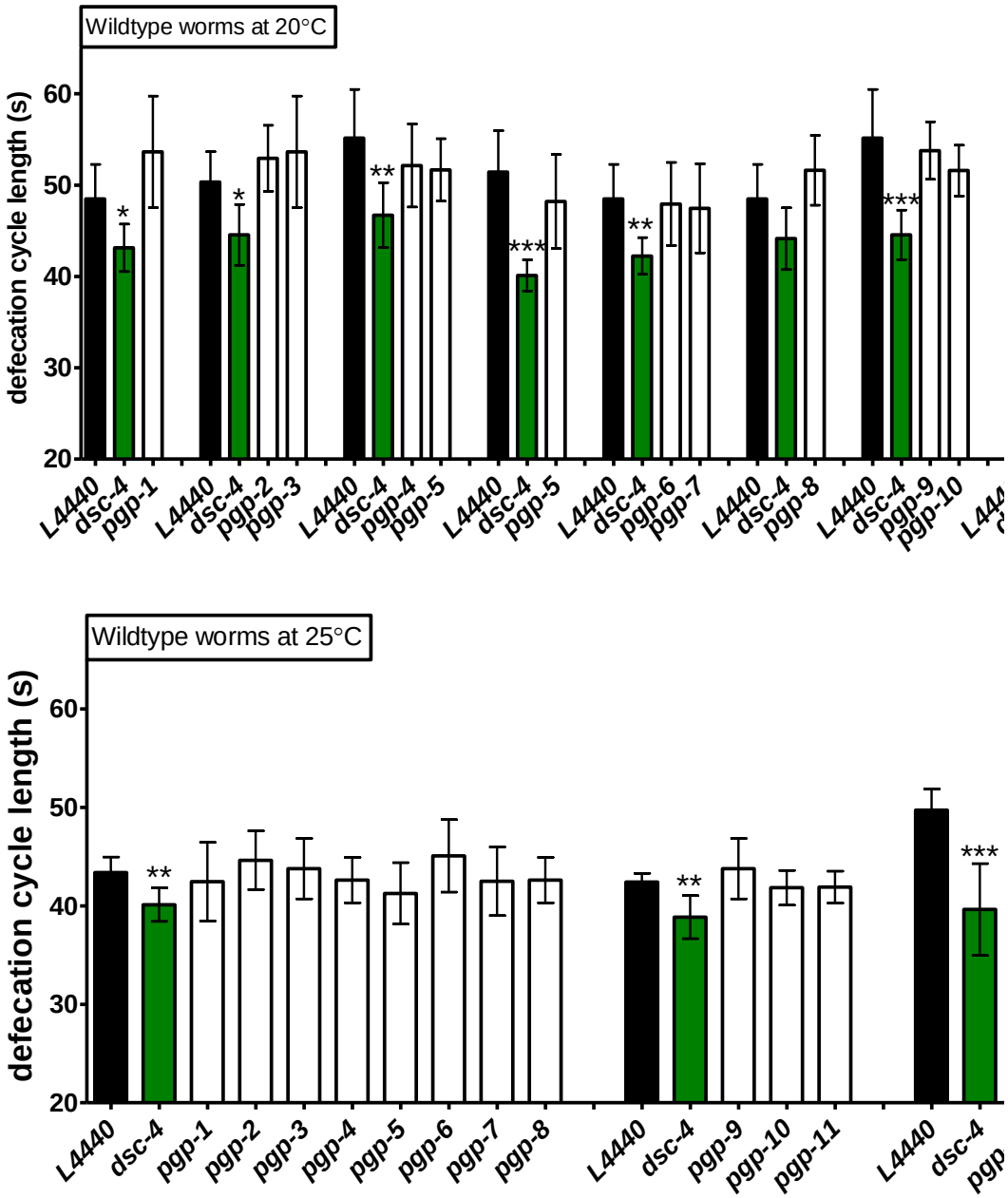

Figure 2: **at 20 and 25°C pgp RNAi did not shorten defecation cycle of wild-type worms.** Black bars represent negative (L4440) controls and green bars represent positive (dsc-4) controls. RNAi were grouped for scoring on different days. Each bar represents the mean defecation cycle length of >12 worms scored at 3 consecutive defecation cycles; the error bars represent the 95% confidence interval.

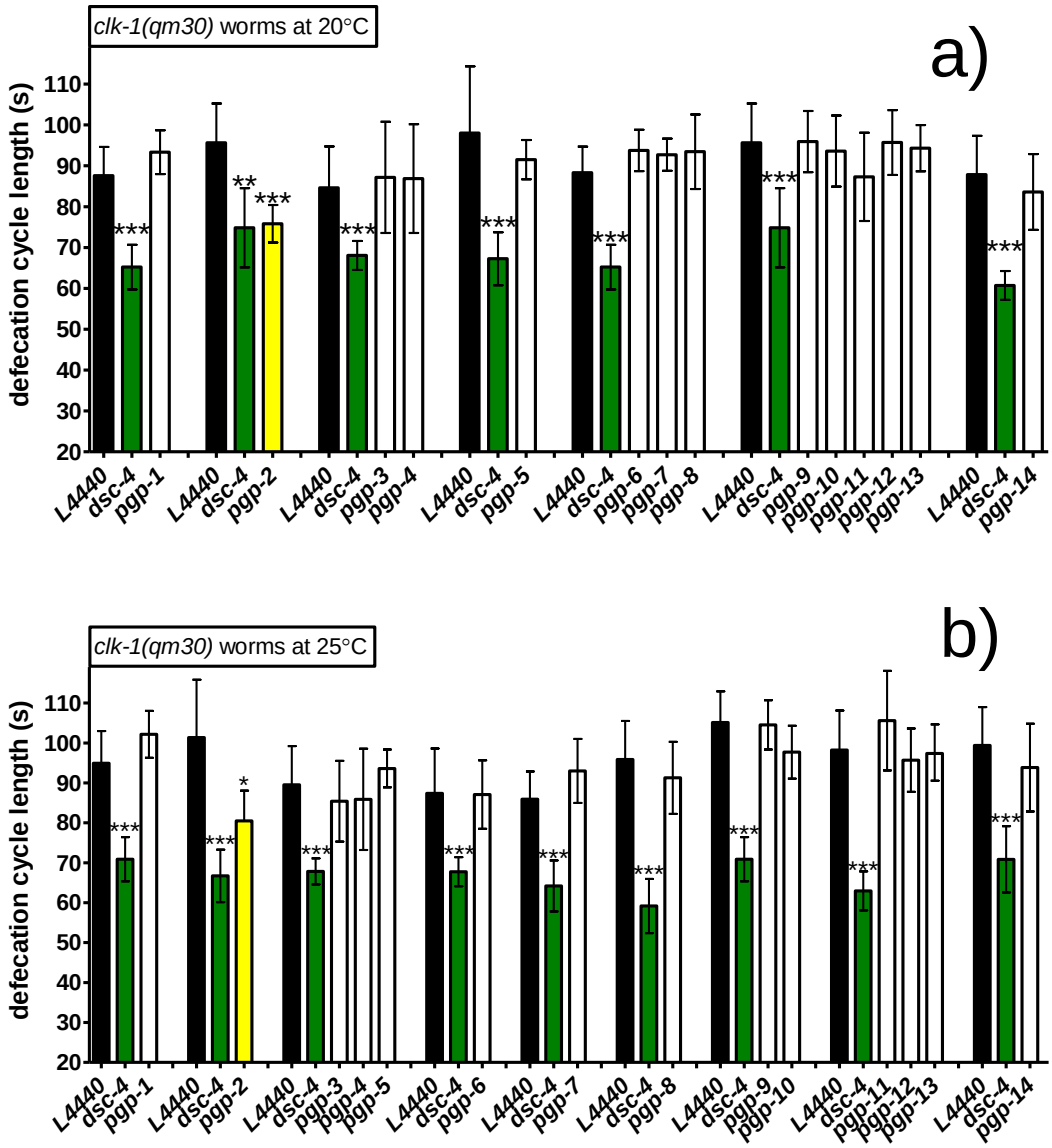

Figure 3: at 20 and 25°C *pgp-2* RNAi shortened defecation cycle of *clk-1* worms. Black bars represent negative (*L4440*) controls, and green bars represent positive (*dsc-4*) controls. RNAi were grouped for scoring on different days. Yellow bar represents the novel suppressor of *clk-1* slow defecation phenotype. Each bar represents the mean defecation cycle length of >12 worms scored at 3 consecutive defecation cycles; the error bars represent the 95% confidence interval.

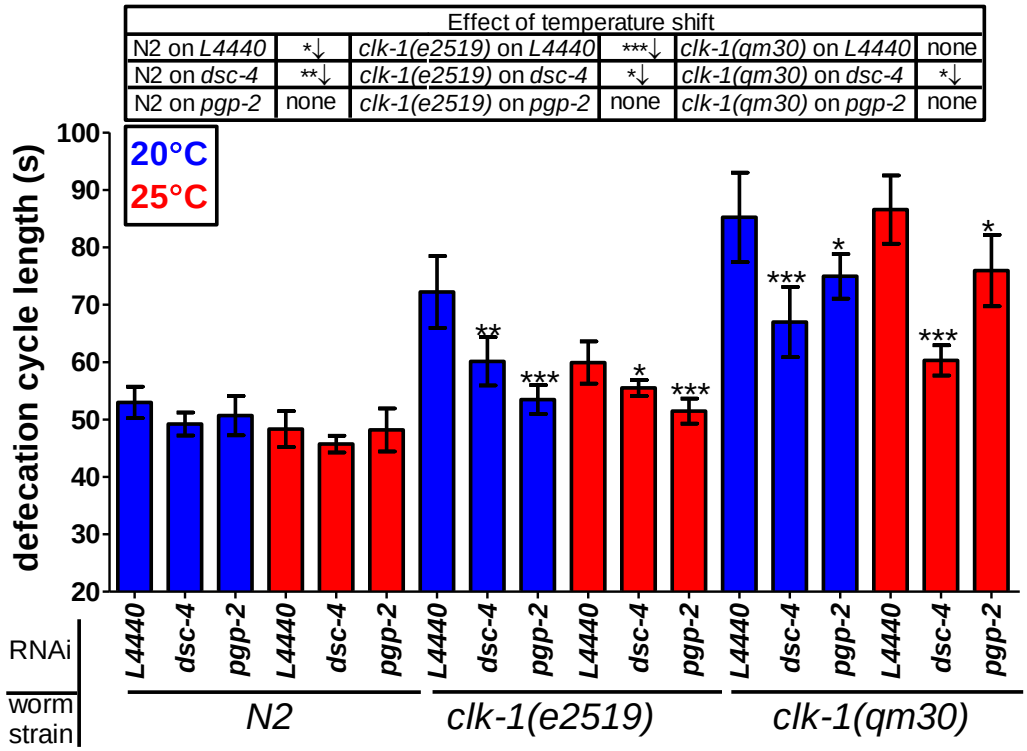

Figure 4: RNAi of *pgp-2* suppresses the *clk-1* slow defecation phenotype of both *clk-1* alleles: *qm30* and *e2519*. The wild-type, *clk-1(e2519)*, and *clk-1(qm30)* genotypes were scored on different days. Each bar represents the mean defecation cycle length of >12 worms scored for 3 consecutive defecation cycles; the error bars represent the 95% confidence interval. Stars indicate significant differences between the RNAi treatment and the corresponding *L4440* negative control.

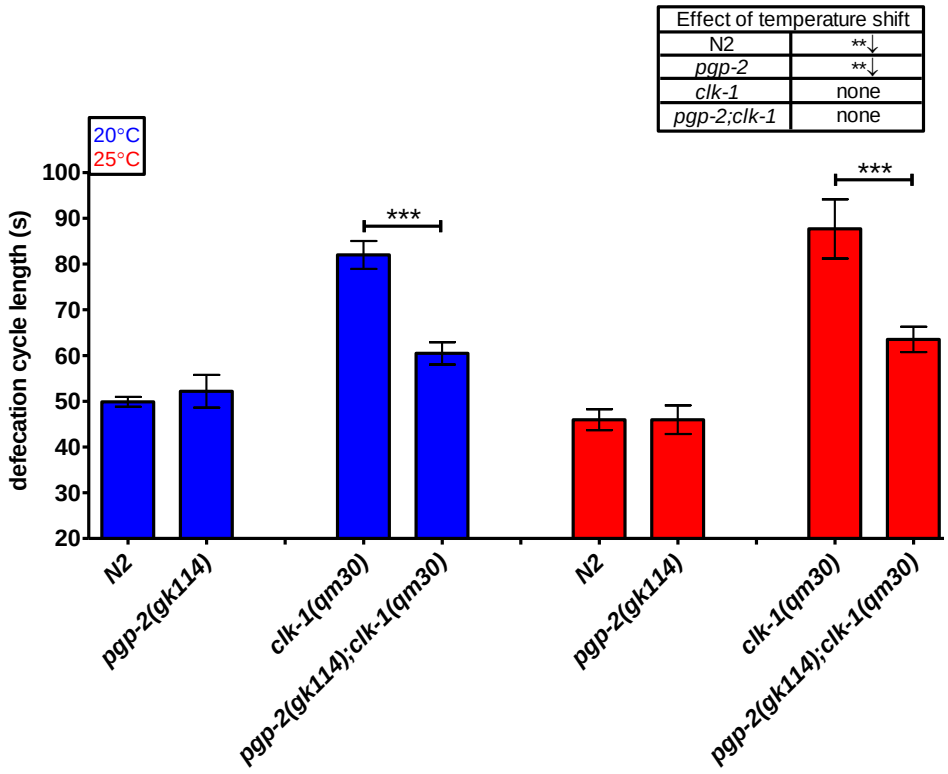

Figure 5: *pgp-2* is a class I suppressor. Each bar represents the mean defecation cycle length of >12 worms scored at 3 consecutive defecation cycles; the error bars represent the 95% confidence interval.

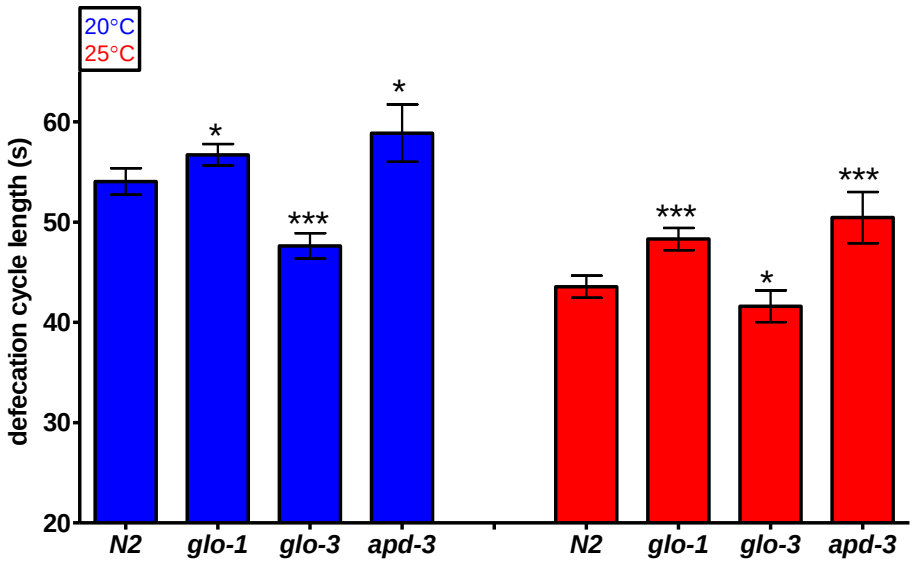

Figure 6: The gut granule loss mutants, *glo-1* and *apd-3*, defecate slower than the wild-type strain, and *glo-3* defecates faster than wild-type strain at both 20 and 25°C. Each bar represents the mean defecation cycle length of >12 worms scored for 3 consecutive defecation cycles; the error bars represent the 95% confidence interval.

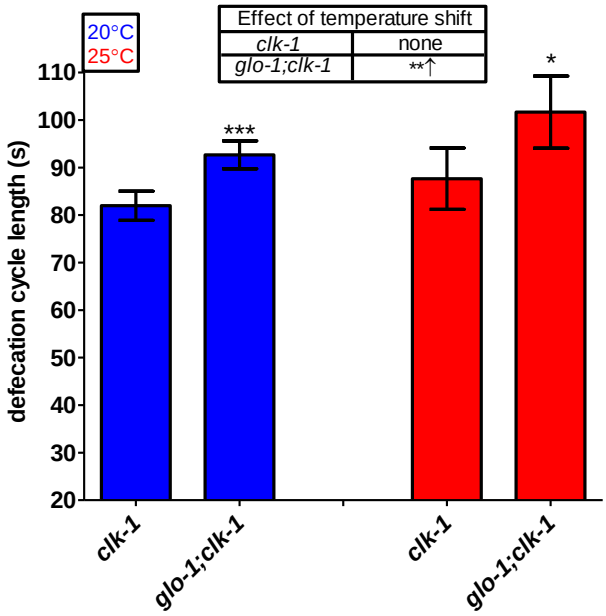

Figure 7: *glo-1* lengthens defecation cycle of the *clk-1(qm30)* worms. On the *clk-1(qm30)* background at both 20 and 25°C gut granule loss mutant *glo-1* defecates slower than the *clk-1(qm30)* strain. Each bar represents the mean defecation cycle length of >12 worms scored at 3 consecutive defecation cycles; the error bars represent the 95% confidence interval.

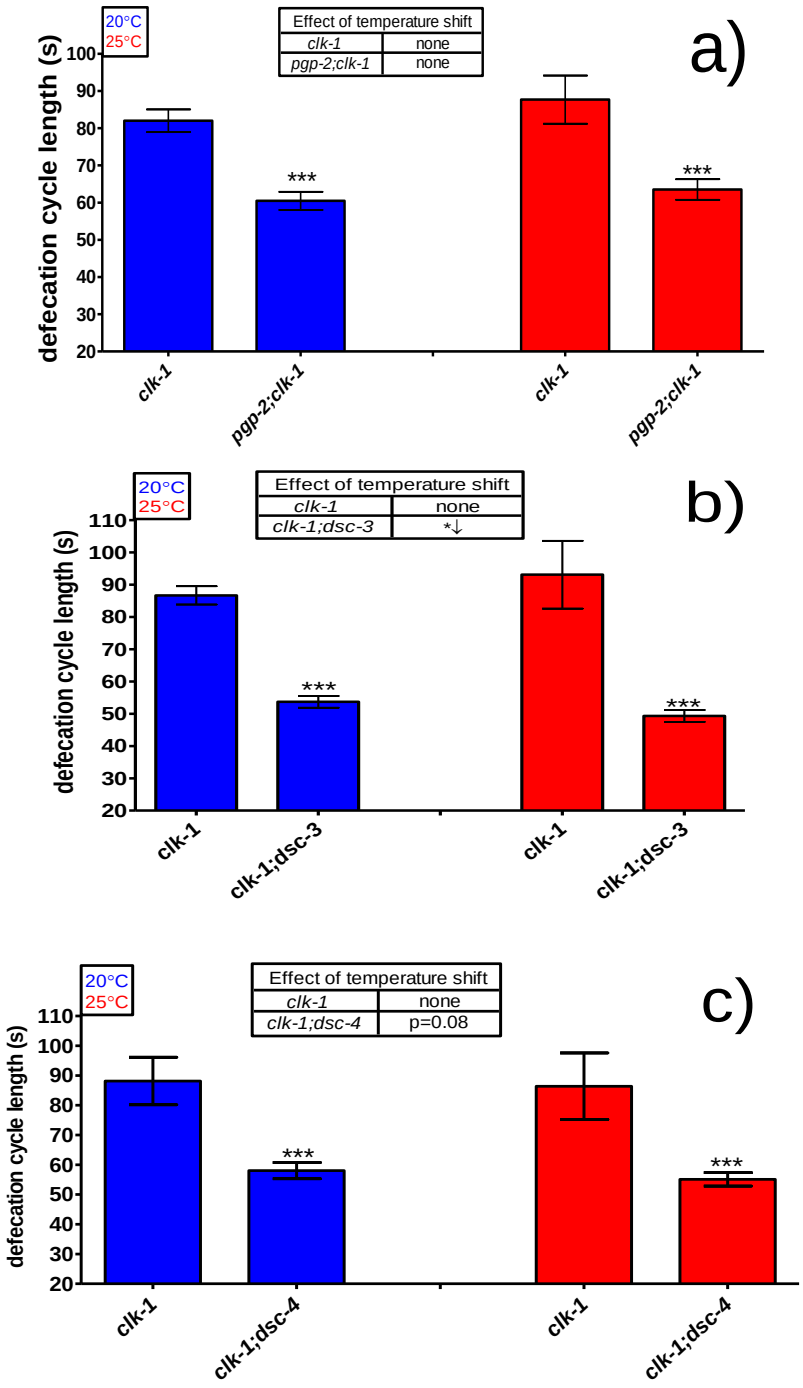

Figure 8: *pgp-2* (a), *dsc-3* (b), and *dsc-4* (c) are class I *clk-1* slow defecation suppressors. Alleles used were *clk-1(qm30), pgp-2(gk114), dsc-3(tm1634)* and *dsc-4(qm182).* Each bar represents the mean defecation cycle length of >12 worms scored at 3 consecutive defecation cycles; error bars represent the 95% confidence intervals.

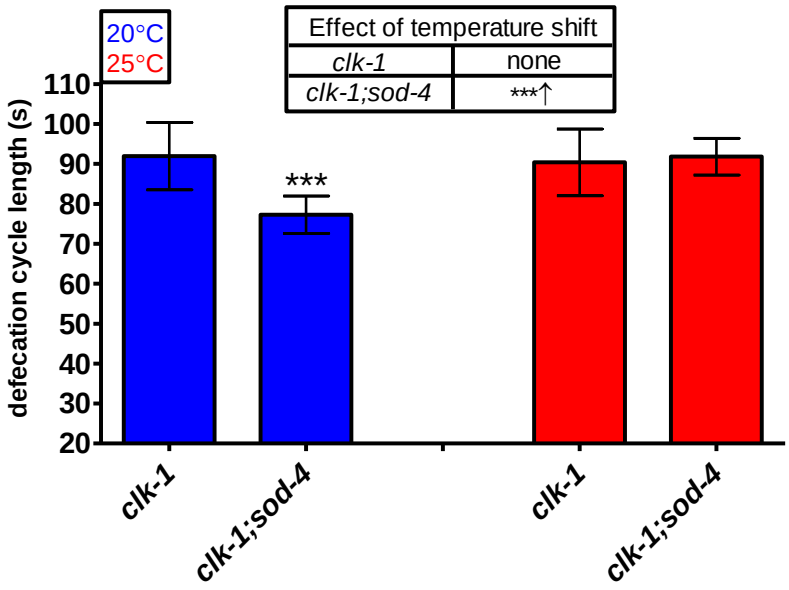

Figure 9: *sod-4* is a class II *clk-1* slow defecation suppressor. Alleles used were *clk-1(qm30)* and *sod-4(gk101).* Each bar represents the mean defecation cycle length of >12 worms scored at 3 consecutive defecation cycles at either 20 or 25°C; the error bars represent the 95% confidence intervals.

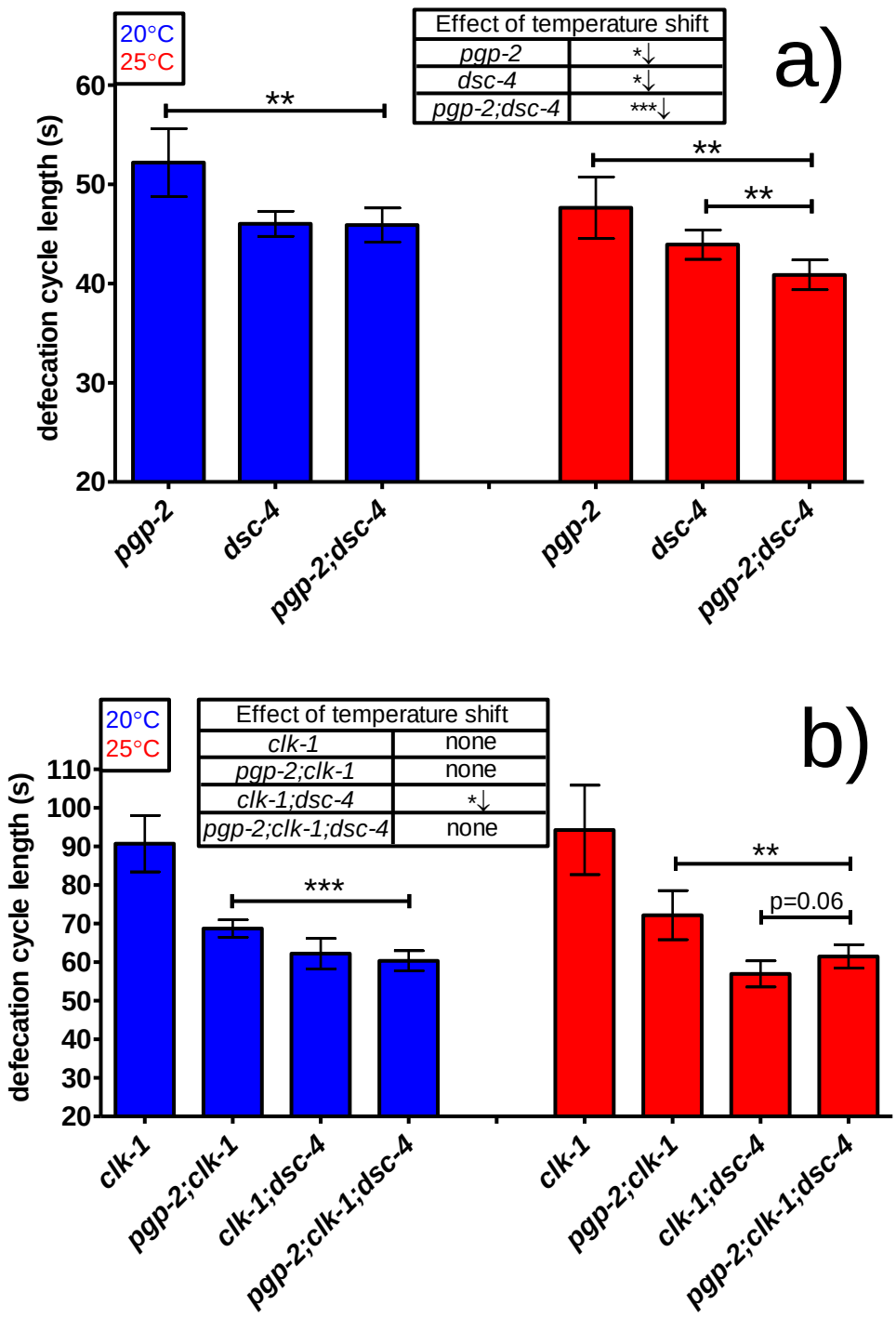

Figure 10: combination of two class I mutants, *dsc-4(qm182)* and *pgp-2(gk114)* on wild-type (a) and *clk-1(qm30)* (b) background. Each bar represents the mean defecation cycle length of >12 worms scored at 3 consecutive defecation cycles at either 20 or 25°C; the error bars represent the 95% confidence intervals.

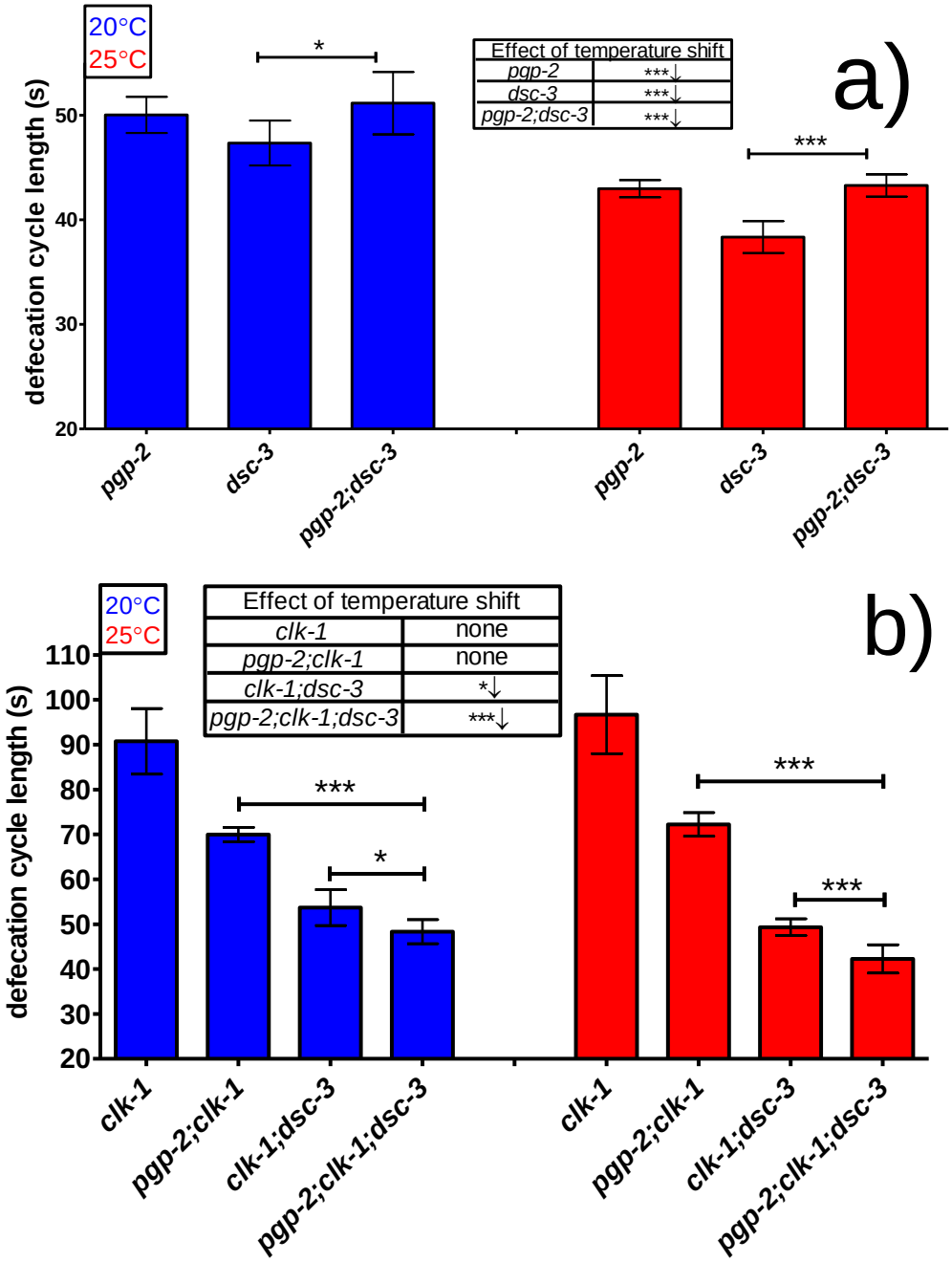

Figure 11: combination of class I mutants *pgp-2(gk114)* and *dsc-3(tm1634)* on wild-type (a) and *clk-1(qm30)* (b) background. Each bar represents the mean defecation cycle length of >12 worms scored at 3 consecutive defecation cycles at either 20 or 25°C; the error bars represent the 95% confidence intervals.

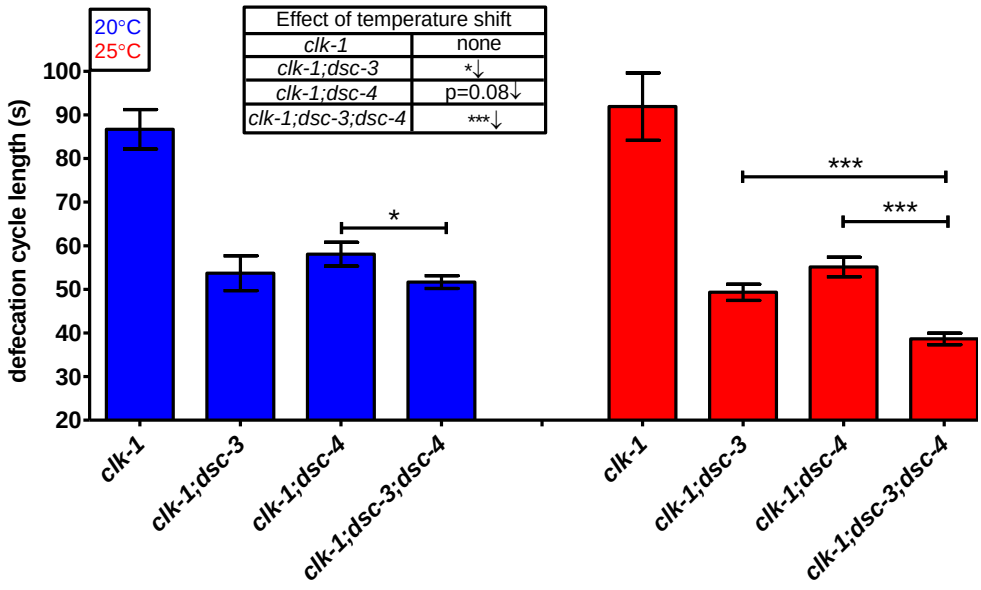

Figure 12: combination of class I mutants *dsc-3(tm1634)* and *dsc-4(qm182)* on *clk-1(qm30)* background. Each bar represents the mean defecation cycle length of >12 worms scored at 3 consecutive defecation cycles at either 20 or 25°C; the error bars represent the 95% confidence intervals.

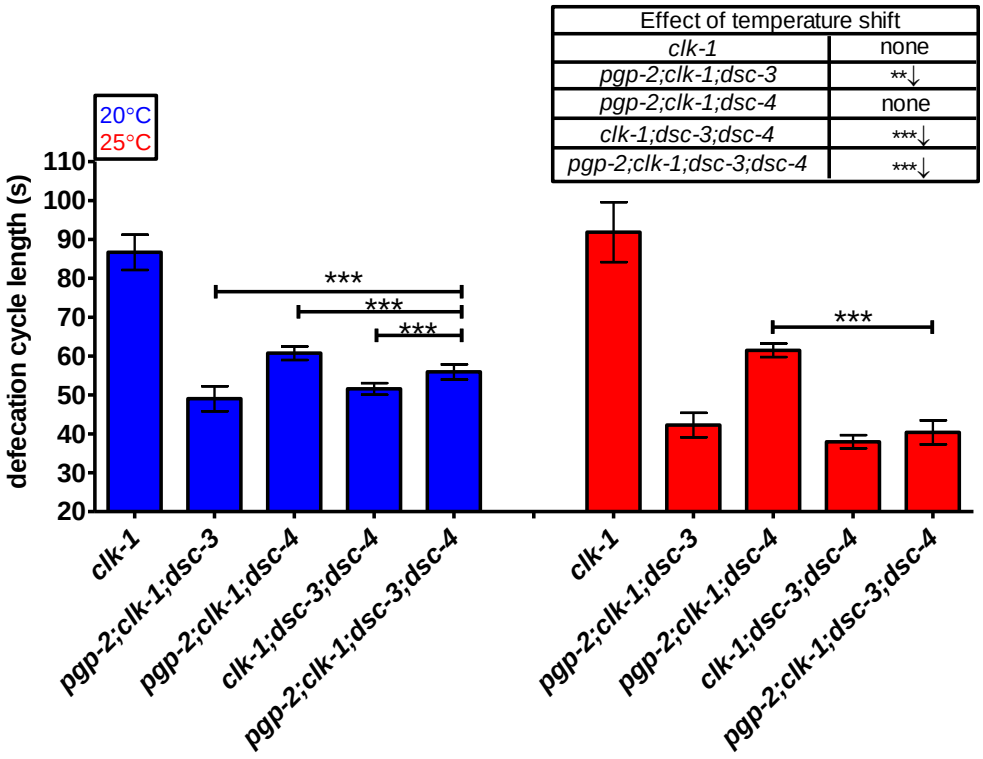

Figure 13: combination of class I mutations *dsc-3(tm1634), dsc-4(qm182)* and *pgp-2(*gk114*)* on *clk-1(qm30)* background. Each bar represents the mean defecation cycle length of >12 worms scored at 3 consecutive defecation cycles at either 20 or 25°C; the error bars represent the 95% confidence intervals.

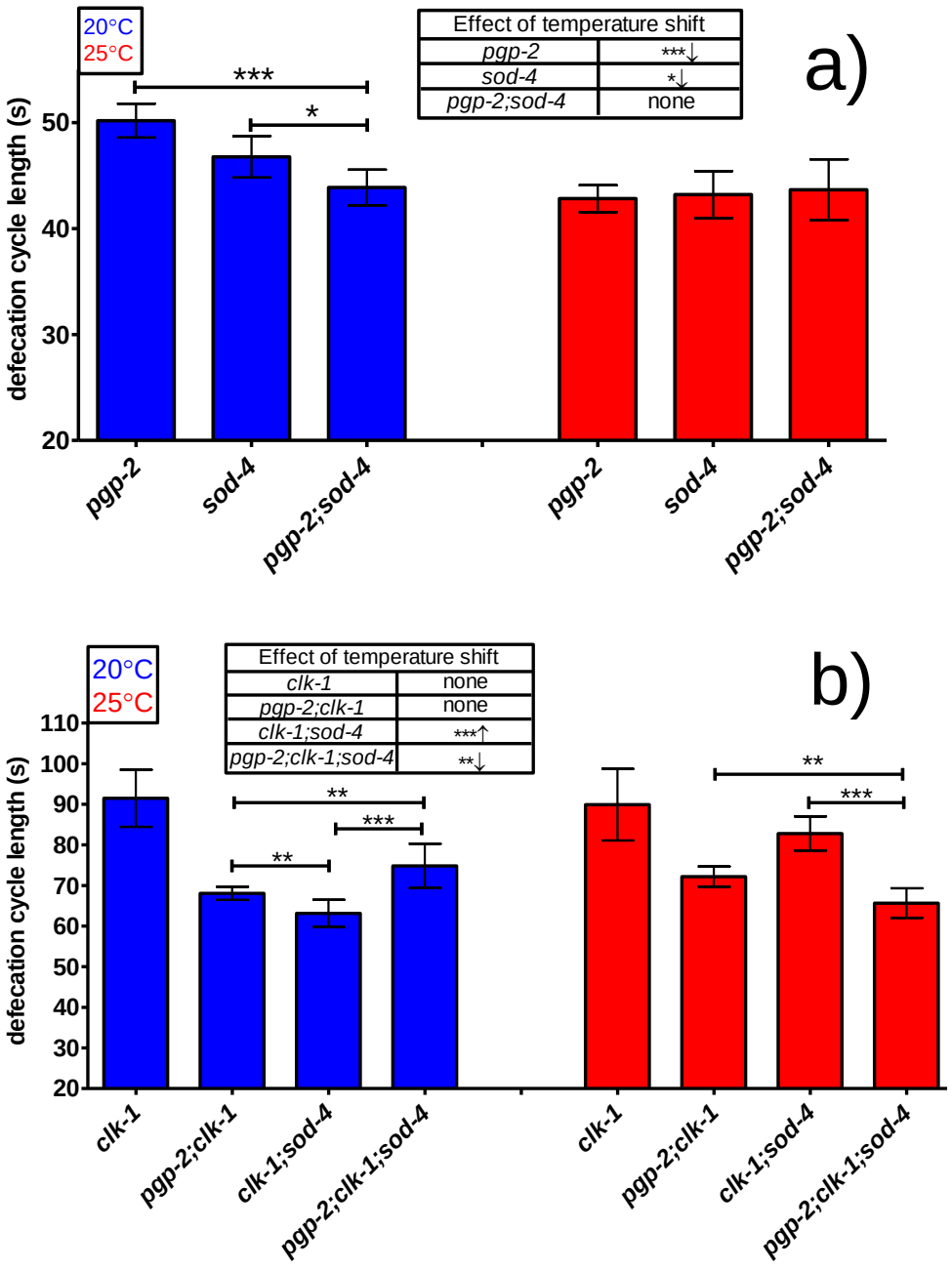

Figure 14: combination of class I mutation *pgp-2(gk114)* with class II mutation *sod-4(gk101)* on wild-type (a) and *clk-1(qm30)* (b) background. Each bar represents the mean defecation cycle length of >12 worms scored at 3 consecutive defecation cycles at either 20 or 25°C; the error bars represent the 95% confidence intervals.

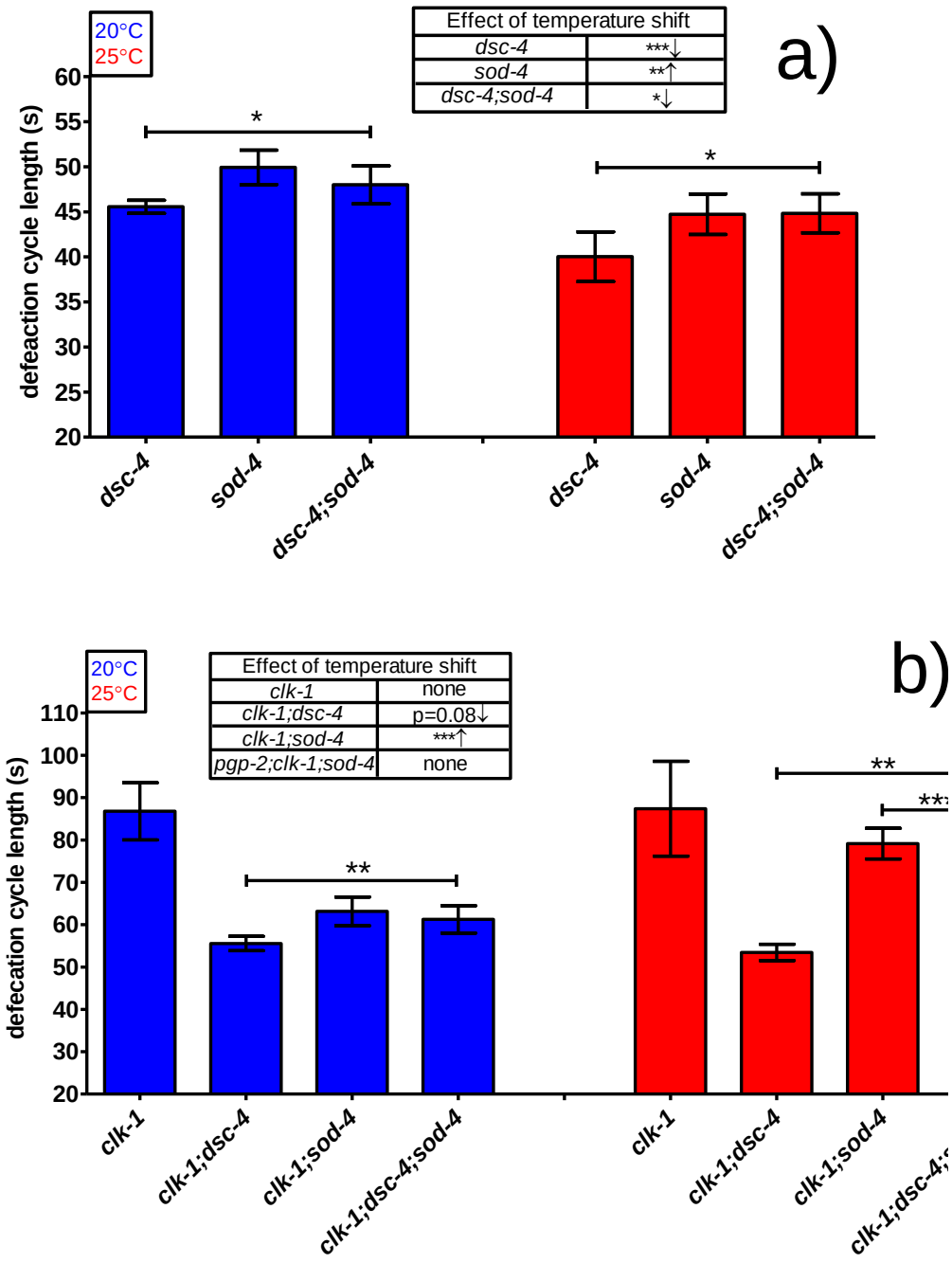

Figure 15: combination of class I mutant *dsc-4(qm182)* and class II mutant *sod-4(gk101)* on wild-type (a) and *clk-1(qm30)* (b) background. Each bar represents the mean defecation cycle length of >12 worms scored at 3 consecutive defecation cycles at either 20 or 25°C; the error bars represent the 95% confidence intervals.

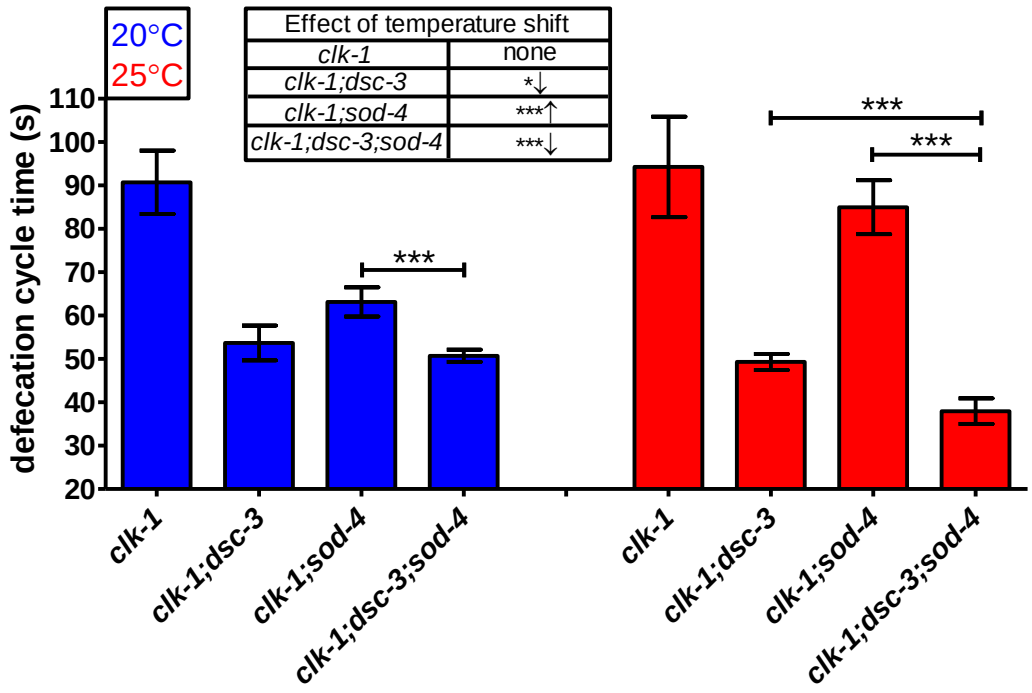

Figure 16: combination of class I mutant *dsc-3(tm1634)* and class II mutant *sod-4(gk101)* on *clk-1(qm30)* background. Each bar represents the mean defecation cycle length of >12 worms scored at 3 consecutive defecation cycles at either 20 or 25°C; the error bars represent the 95% confidence intervals.

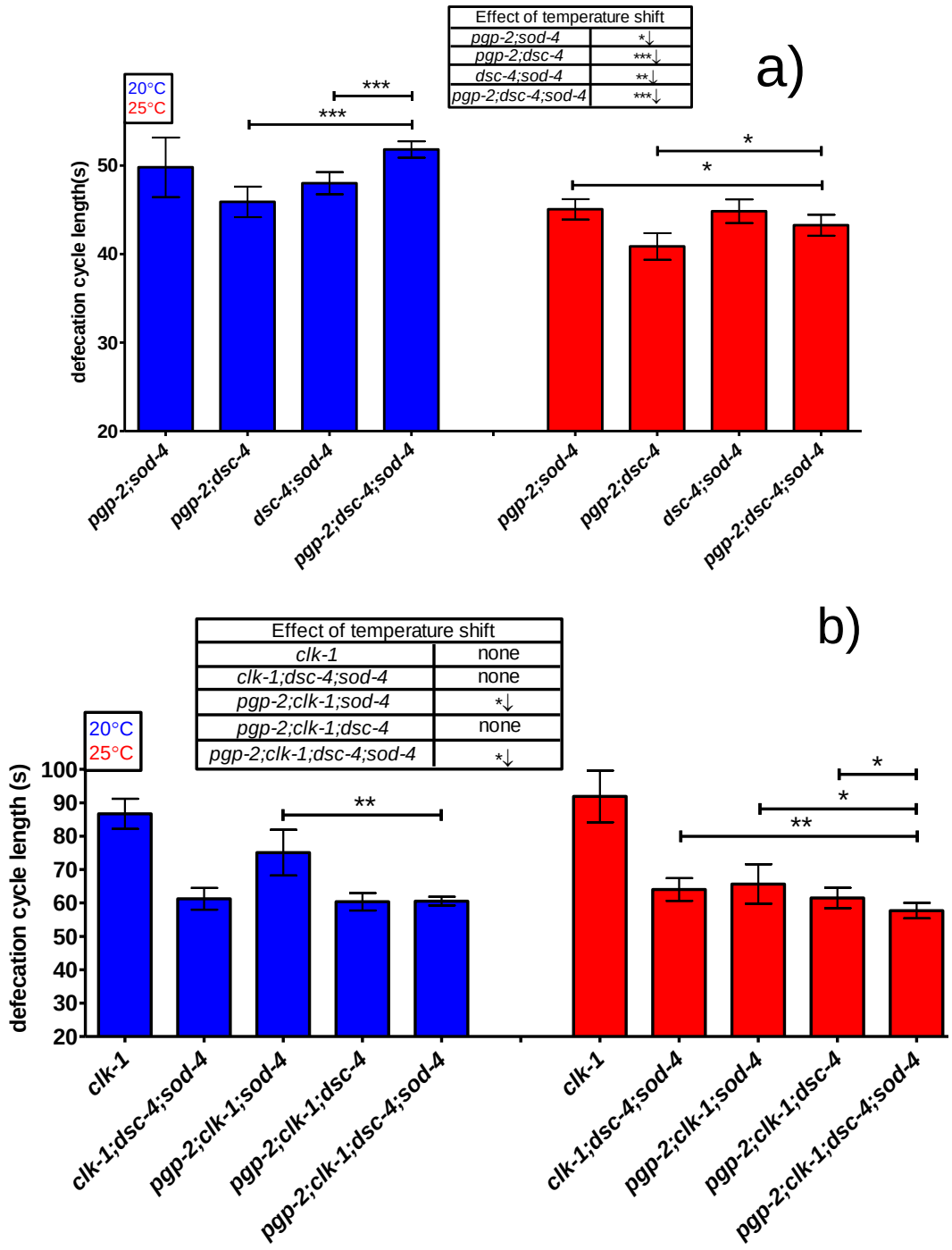

Figure 17: combination of class I mutants *pgp-2(gk114)* and *dsc-4(qm182)* with class II mutant *sod-4(gk101)* on the wild-type and *clk-1(qm30)* backgrounds. Each bar represents the mean defecation cycle length of >12 worms scored at 3 consecutive defecation cycles at either 20 or 25°C; the error bars represent the 95% confidence intervals.

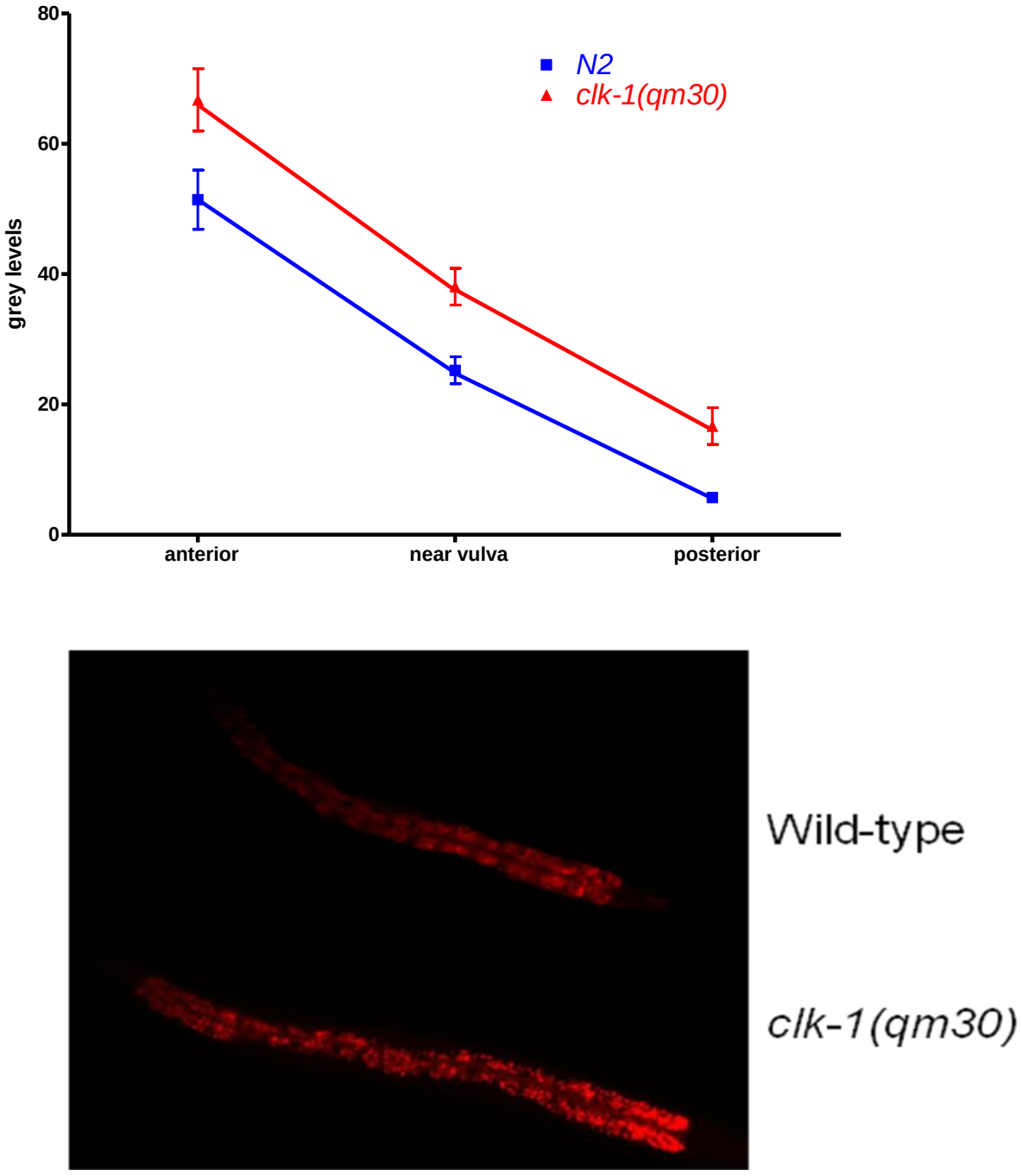

Figure 18: Nile Red fluorescence detected in the LROs of wild-type and *clk-1(qm30)* worms. Each point represents the mean LRO fluorescence of >25 worms; the error bars represent the 95% confidence intervals.

Discussion

4.1.1 Worm PGPs are homologous to human BSEP

C. elegans dsc-3 is a class I suppressor of the *clk-1* slow defecation phenotype (Branicky, Shibata et al. 2001). The addition of this mutation to *clk-1* background not only suppresses the slow defecation of the *clk-1* worms, but also their inability to adjust to temperature changes. Worm *dsc-3* belongs to the ABC gene family, and its human homolog is *atp8b1*. The ABC transporter family possessed a very adaptable mechanism for coupling substrate binding to ATP hydrolysis and extrusion. ABC transporters tend to recognize substrate directly within the cytoplasmic leaflet of the plasma membrane. Their binding sites can operate at relatively low affinity, meaning that these transporters are able to recognize more than one substrate. This recognition flexibility is responsible for the fact that ABC transporters are capable of carrying out multiple tasks.

Mammalian *atp8b1* encodes a transmembrane flippase p-glycoprotein (pgp) responsible for maintaining membrane lipid asymmetry (Bull, van Eijk et al. 1998). One of the phenotypes associated with ATP8B1 dysfunction is hepatic cholestasis, a condition where bile accumulates in the liver resulting in cellular damage (Paulusma, de Waart et al. 2009). Worm DSC-3 had also been shown to function in the metabolism of bile-like molecules (Ju-Ling Liu, personal communication).

In humans, the absence of ATP8B1 down-regulates the activity of the bile salt export pump (BSEP) (Chen, Ellis et al., 2010). This happens simultaneously by two mechanisms: a) transcriptional down-regulation via Farnesoid X-Receptor as well as b) creating a mechanical disruption of lipid leaflet asymmetry that impairs BSEP protein function (Makishima, Okamoto et al. 1999; Parks, Blanchard et al. 1999; Sinal, Tohkin et al. 2000; Ananthanarayanan, Balasubramanian et al. 2001). The latter also affects the function of other transmembrane proteins apart from BSEP. Similarly to ATP8B1, BSEP is also a mammalian p-glycoprotein. The expression of this protein localizes to many epithelial cell types, but the bulk of it occurs in the liver canalicular membrane. In the canalicular membrane BSEP takes part in bile acid recycling by secreting the re-absorbed bile acids from the intestine back into bile. Disruptions in the function of BSEP lead to hepatic cholestasis in the same way as disruptions in ATP8B1 do (Kubitz, Brinkmeyer et al. ; Strautnieks, Bull et al. 1998).

Since BSEP is downstream of and is regulated by ATP8B1, and *dsc-3* is an *atp8b1* homolog, it was our goal to assess if a homolog of *bsep* was present in the worm system. Potential *bsep* homologs may contain suppressors of *clk-1* slow defecation. After searching for worm homologs of *bsep* we found that the worm p-glycoprotein (*pgp*) gene family was the closest match. This gene family contains 15 members, but only 14 of them code for functional proteins.

Proteins of the PGP group found across species are generally known to transport hydrophobic compounds. In the mammalian system PGPs function in transporting lipids and bile from the liver, defending the body from ingested toxins, and serving as a component of the blood-brain barrier (Sheps, Ralph et al. 2004). Only four genes from the worm *pgp* gene family have been assigned a function: *pgp-1, 3,* and *5* were shown to be a part of the worm defense system against the ingested pathogens and toxins. These genes are expressed in the intestinal and excretory cells of *C. elegans* (Broeks, Janssen et al. 1995; Kamath, Fraser et al. 2003). *pgp-2* has been shown to be involved in the biogenesis of intestinal lysosome-related organelles (LROs) (Schroeder, Kremer et al. 2007). The majority of the *pgp* genes are expressed in the intestine and the excretory cell, apart from *pgp-7* and *8* which are expressed in the male tail and the head neurons, respectively (Table 4).

It is noteworthy that no single *pgp* stood out as a clear close match to BSEP, with protein similarity values ranging from 47 to 58 percent. This likely indicates that there is no single functional BSEP homolog in the worm system. The regions of similarity observed between BSEP and worm PGPs are likely due to the presence of characteristic ATP-binding cassette (ABC) domains that are conserved across species by the members of the ABC transporter family. This was not surprising because even though many metabolic pathways are conserved between humans and worms, there are still significant differences in the physiology and biochemistry of these organisms.

Since no single homolog was identified, all *pgp*s were assessed. All fourteen genes had constructs available from the RNAi library, the RNAi against all *pgp*s was performed on wild-type (control) and *clk-1(qm30)* worms at 20 and 25°C.

4.1.2 *pgp-2* is a novel class I suppressor of *clk-1* slow defecation phenotype

The RNAi against all *pgp*s revealed that none of the genes tested had any effect on the defecation of the wild-type worms at either 20°C or after the shift to 25°C. After performing the same RNAi feeding experiment on *clk-1(qm30)* worms, the results indicated that *pgp-2* has a strong suppressive effect on the *clk-1* slow defecation phenotype at both temperatures (Figure 3 and 4). RNAi against other *pgp*s had no effect on the *clk-1* slow defecation cycle. As a result of this RNAi experiment, *pgp-2* was identified as a novel class I suppressor of the *clk-1* slow defecation phenotype.

clk-1 alleles *qm30* and *e2519* are a deletion and a point mutation respectively (see Materials and Methods section for details on these alleles). The *e2519* allele shows a less severe slow defecation phenotype, indicating that some of the CLK-1 activity is retained in this mutant (Wong, Boutis et al. 1995; Felkai, Ewbank et al. 1999). Defecation cycle length of both of these *clk-1* alleles was shortened by RNAi against *pgp-2* (Figure 5). However, it is noteworthy that the magnitude of suppression of the *clk-1* slow defecation phenotype was allele-specific: *pgp-2* RNAi suppressed the slow defecation of *clk-1(e2519)* mutant to a greater extent than the slow defecation *clk-1(qm30)* mutant, practically restoring the defecation of the *e2519* worms back to the wild-type speed (Figure 5). This suggests that *clk-1(e2519)* indeed retains some of the important CLK-1 activity that is involved in the regulation of

defecation cycle length, and this activity is necessary for the ability of *pgp-2* RNAi to suppress the slow defecation of this allele. Even though genetic *clk-1(e2519)* mutants are able to react to temperature shifts, both *clk-1(qm30)* and *(e2519)* worms lack this ability on *pgp-2* RNAi. These experiments suggest that although *pgp-2* is a suppressor of the *clk-1* slow defecation phenotype, it is unable to restore the ability of *clk-1* worms to react to temperature changes (Figure 5). In contrast, *dsc-4* RNAi was used as a positive control because *dsc-4(qm182)* mutation was previously shown to restore the ability of *clk-1(qm30)* worms to adjust to temperature shifts (Branicky, Shibata et al. 2001). Both *clk-1(qm30)* and *(e2519)* worms on *dsc-4* RNAi were able to adjust to the temperature shifts.

The technique of RNAi does not always eliminate a hundred percent of the gene product, and sometimes yields inconsistent results (Grishok 2005). More certainty can be achieved by using a true genetic mutant, preferably a complete knockout. Alleles that include deletions in the exon regions and are missing a significant portion of the coding sequence are more likely to be complete knockouts than the point-mutation alleles. Our experiments showed that, similar to the effects of *pgp-2* RNAi on wild-type worms, a deletion mutation in *pgp-2* gene had no effect on the defecation of the wild-type worms, but shortened the defecation cycle length of *clk-1(qm30)* worms. *pgp-2* mutant on the wild-type background was able to react to the temperature shift, while *pgp-2* mutant on clk-1*(qm30)* background was not (Figures 5 and

6). This suggests that *pgp-2* is not a part of temperature compensation mechanism that is revealed in *clk-1* worms.

The discovery of *pgp-2* as a suppressor of the *clk-1* slow defecation led to a re-definition of the parameters by which suppressors are placed in classes I and II. Previously, Branicky et al. described the two classes of suppressors in the following way: on *clk-1* background class I but not class II mutants are able to restore the ability of *clk-1* to react to temperature shifts, class II mutants only accelerate *clk-1* defecation rates at 20°C, and on the wild-type background class I mutants generally have slower defecation rates than class II mutants (Branicky, Shibata et al. 2001). *pgp-2* does not fit the requirement of class I mutants to restore the ability of *clk-1* to adjust to temperature shifts. As a result, a re-classification of *clk-1* slow defecation suppressors had to take place. Class I suppressors are now simply defined as suppressing slow defecation of *clk-1* worms at 20°C as well as after a shift to 25°C, and class II suppressors suppress *clk-1* slow defecation at 20°C, but at 25° suppress very little or not at all.

4.1.3 Biological function and possible involvement of PGP-2 in worm lipid metabolism

As it was mentioned before, mutations in worm *pgp-2* result in the absence of LROs from the worm intestine. LROs are intestinal terminal endocytic compartments. The precise function of these compartments had not yet been identified (Hermann, Schroeder et al. 2005). Previously they were proposed to work as lipid storage organelles due to the fact that they accumulated lipophilic vital dye Nile red, but it had recently been shown that in fact the Nile red accumulation simply indicates the degradative function of this compartment (O'Rourke, Soukas et al. 2009).

pgp-2 expression starts soon after the specification of intestinal precursors during early intestinal differentiation (Hermann, Schroeder et al. 2005; Schroeder, Kremer et al. 2007). PGP-2 localizes to the LRO membrane, consistent with its predicted role in the transport of molecules that compose and/or facilitate the accumulation of LRO contents. *pgp-2(−)* embryos display improper formation of LROs, where mislocalization of the contents from the LROs into the intestinal lumen takes place (Currie, King et al. 2007).

It had been proposed that PGP-2 functions as a membrane transporter. This conclusion was made based on the fact that many of the sequences that are specific to the ABC transporter family are conserved in PGP-2. These sequences are essential for the active transport of substrates across the

biological membranes (Sheps, Ralph et al. 2004). However, due to the lack of clear orthology between PGP-2 and mammalian ABC transporters that have characterized substrates, it is not possible to accurately predict the type of molecules transported by PGP-2 (Sheps, Ralph et al. 2004).

LROs are filled with auto-fluorescent birefringent (i.e., a ray of light is broken down into two rays when it passes through it) material. This material probably assembles into a crystalline or liquid crystalline structure. The ability to form crystals *in vivo* is exhibited by many different biological molecules including proteins, lipids, cholesterol, amino acids, and heme. As LROs stain with markers that accumulate in hydrophobic environments (O'Rourke, Soukas et al. 2009), the material forming birefringent crystals is likely to be very concentrated and therefore among the most abundant molecules in the lumen of an LRO. More precise characteristics of this material have not been studied.

LROs appear in the intestinal primordium at the same time as yolk, which is a complex of lipids and lipoproteins (Sharrock, Sutherlin et al. 1990; Bossinger and Schierenberg 1992; Kubagawa, Watts et al. 2006). It had been proposed that the coincidence of yolk accumulation and the appearance of birefringent material in the intestinal primordium suggests that yolk-derived components such as lipoproteins, lipids, or cholesterol could accumulate and form birefringent crystals in the LROs (Entchev and Kurzchalia 2005). This theory

has not yet been tested, but it is in accordance with the proposed role of *clk-1* suppressors in worm lipoprotein metabolism.

It is possible that LROs may play a role in the endocytic recycling of lipoprotein receptors. For instance, the insect low-density lipoprotein receptor homologue mediates endocytosis of an insect lipoprotein that is only distantly structurally related to mammalian low density lipoprotein. The insect lipoprotein-like particles follow distinct intracellular routes upon endocytosis that differ from the intracellular routes of mammalian lipoproteins. These routes are guided by insect lipoprotein-like receptors. The mammalian low density lipoproteins are taken up and completely degraded in lysosomes, but the insect lipoprotein-like particles are able to release their cargo into endocytic terminal compartments and get recycled without being completely degraded in these endocytic compartments (Dantuma, Potters et al. 1999; Van Hoof, Rodenburg et al. 2002). In the mammalian lipoprotein degradation pathway lipoproteins are transported to the endosomes where their dissociation from the lipoprotein receptor takes place (Mukherjee, Ghosh et al. 1997). Subsequently, the lipoprotein receptor is recycled for further use via the trans-Golgi network, and the lipoprotein proceeds to the lysosomes for complete degradation (Stoorvogel, Strous et al. 1991). The insect system is different: when targeted to the degradation pathway, the insect lipoprotein-like particle with its associated receptors evacuates the endosome. The lipoprotein-like receptor does not dissociate from the lipoprotein-like particle,

but instead is transported to the non-lysosomal juxtanuclear compartment (Van Hoof, Rodenburg et al. 2002). This organelle was identified as the endocytic recycling compartment that recycles the insect lipoprotein-like particles. It is possible that a similar mechanism may exist in the nematodes, with LROs acting as a compartment responsible for recycling either the lipoprotein receptors alone or in conjunction with empty lipoprotein vesicles.

Logically, since *pgp-2* results in the loss of LROs and in the suppression of the *clk-1* slow defecation phenotype, the absence of *clk-1* should directly affect LROs. It is unclear how *clk-1* product works in the worm LROs, but it has been previously shown that ubiquinone functions in mammalian lysosomes (Gille and Nohl 2000). In the lysosome ubiquinone acts as a part of the NADH-dependent ubiquinone reductase proton pump. This pump is responsible for the maintenance of an acidic luminal pH necessary for the proper function of lysosomal enzymes that are only active in extremely acidic environments (Gille and Nohl 2000). It remains to be seen if indeed the LROs of *C. elegans* contain ubiquinone as a part of their proton pump to maintain acidic luminal pH.

Staining with lipophilic dye Nile red can be used as a crude visual method of indicating the activity of LROs in the worm intestine. The dye is taken up by LROs for degradation, and can be readily viewed in live worms (O'Rourke, Soukas et al. 2009). Since PGP-2 is essential for LRO biogenesis, *pgp-2(-)* mutants show absence of Nile red- stained LROs. It is interesting that *clk-*

1(qm30) worms show an increase in the amount of Nile red fluorescence as compared to the wild-type worms when assessed as young adults, indicating that *clk-1* mutants either are able to take up more dye, or, more likely, are less able to process the dye they pick up (Figure 18). This finding indirectly supports the hypothesis that ubiquinone may serve as a part of the LRO proton pump, and the absence of *clk-1* activity compromises the luminal pH of these organelles, as low luminal pH is essential for the function of endocytic degradative enzymes.

4.1.4 Effects of other LRO-deficient *glo* mutants on defecation cycle length

As mentioned before, LROs comprise a class of cell-type restricted compartments with specialized functions. The genes controlling the biogenesis of LROs are conserved in metazoa and include, among others, subunits of the AP-3 and HOPS complexes as well as Rab32/38 (Hermann, Schroeder et al. 2005). Since *pgp-2* is a suppressor of the *clk-1* slow defecation, the question was posed: is it simply the lack of LROs that is responsible for the suppression of *clk-1* slow defecation? To find out, we acquired other mutants deficient in LRO biogenesis.

Both *glo-1* and *apd-3* play a role in the general endocytic processes, while *glo-3* is LRO-specific. In worms *apd-3* codes for a unit of an AP-3 complex, which is known to participate in endocytic trafficking, and is primarily associated with endosomes (Berger, Salazar et al. 2007). Generally, the adaptor protein (AP) complexes are involved in membrane transport of many proteins. There are 3 AP complexes in *C. elegans*.

Worm GLO-1 is a homologue of mammalian Rab32, and is required for the formation of *C. elegans* LROs. *glo-1* embryos completely lack LROs filled with birefringent material from their intestinal cells. These mutants mislocalize this material into the embryonic intestinal lumen, much like *pgp-2* mutants. Rabs normally actively interact with effector molecules once they

bind GTP. Generally, Rab proteins function as molecular switches that modulate vesicle and membrane dynamics. The human homolog of *glo-1* is Ras oncogene *Rab32*, which, among other things, regulates one of the critical steps in the trafficking of melanogenic enzymes (tyrosinase in particular) from the trans-Golgi network to melanosomes, as well as biogenesis of melanosomes and other lysosome-related organelles (Wasmeier, Romao et al. 2006). Generally, mammalian Rab protein family members are known to be involved in lipid transport from early to late endosomes and regulation of transport of the recovered lipids and cholesterol from the late endosomes and lysosomes to the trans-Golgi network (Hermann, Schroeder et al. 2005).

Worm *glo-3* mutations lead to mislocalization of LRO contents into the intestinal lumen, consistent with a defect in intracellular trafficking (Rabbitts et al., 2008). *glo-3* acts in parallel with or downstream of the AP-3 complex and the PGP-2 ABC transporter in gut granule biogenesis. *glo-3* encodes a predicted membrane-associated protein that lacks obvious sequence homologs outside of the nematodes (Hermann, Schroeder et al. 2005). The expression of this gene starts with the embryonic intestinal precursors and persists almost exclusively in intestinal cells throughout adulthood. GLO-3::GFP localizes to the gut granule membrane, suggesting it could play a direct role in the trafficking events of the gut granule (Rabbitts et al., 2008).

On the wild-type background, the effects of presence of different *glo* mutations were not consistent. Both *glo-1* and *apd-3* increased the defecation

cycle length, while *glo-3* shortened it (Figure 7). Due to time constraints, only *glo-1* mutation was assessed in *clk-1* background, where it lengthened the long *clk-1(qm30)* defecation cycle even further (Figure 8). These results indicate that it is not simply the lack of LROs that is responsible for the shortening of the slow defecation of *clk-1*. Instead, it seems likely that there is a special interaction between *pgp-2* and *clk-1*. It is noteworthy that *pgp-2* does not affect the defecation length of the wild-type worms, while other LRO mutants are capable of both shortening it (*glo-3*) and lengthening it (*glo-1* and *apd-3*) (Figures 6 and 7). This suggests that genes responsible for LRO formation may have other important roles in the endolysosomal system of *C. elegans*. A possibility that the LRO function is linked to the lipoprotein metabolism should be explored in more detail.

4.2 Classification of *sod-4* suppressor of *clk-1* slow defecation phenotype

Normally, when wild-type worms are grown at 20°C and shifted to 25°C, the defecation cycle length shortens. This reaction to the temperature shift in the wild-type worms is an active process that requires *clk-1* activity. In the absence of *clk-1*, defecation rates are unchanged in response to the temperature shift, revealing a temperature compensation mechanism. Branicky et al. (2001) suggest that *clk-1* is necessary for readjusting the defecation cycle length as well as for the general re-setting of the physiological rates in response to changes in temperature. Several suppressors of the *clk-1* long defecation cycle had been previously identified and placed into two suppressor classes (Branicky, Shibata et al. 2001).

As mentioned before, the discovery of the novel class I suppressor *pgp-2* which does not restore the ability of *clk-1* worms to react to temperature shifts, lead to a re-evaluation and simplification of the classification parameters. *dsc-4* and *dsc-3* suppress at both 20 and 25°C, so they fall within the class I category (Branicky, Shibata et al. 2001). *sod-4* was previously identified as a suppressor of *clk-1* slow defecation phenotype at 20°C, but it was not classified (Stepanyan, Hughes et al. 2006). According to our experiments, *sod-4* can be placed in the class II category, as it does not suppress the *clk-1* slow defecation phenotype at 25 °C (Figure 9). These

results suggest that *sod-4* does not play a role in the active temperature compensation mechanism that is revealed in *clk-1* mutants.

4.3.1 Combining different *clk-1* slow defecation suppressors

To understand the mechanisms behind the suppression of *clk-1* slow defecation and the restoration of the ability of *clk-1* worms to react to temperature shifts by introducing various suppressor mutations, we decided to study the combinations of different suppressors. It is important to consider the biological functions of each suppressor. In short, *dsc-4* is a class I suppressor that is likely involved in lipoprotein assembly in the endoplasmic reticulum, *dsc-3* is a class I suppressor which may be involved in bile biology, *pgp-2* is a class I suppressor that is responsible for the biogenesis of terminal endocytic LROs, and *sod-4* is a class II suppressor believed to be involved in extracellular detoxification of reactive oxygen species. The significance of the classes is unclear, but their presence suggests that the genes of the different classes interact with *clk-1* in different ways. One way to approach this issue is to observe interactions within and between the two classes. This was achieved by constructing strains containing multiple suppressor mutations on wild-type and *clk-1* backgrounds, and testing the resultant mutants for epistasis.

It is important to keep in mind that the defecation cycle length of *clk-1* worms is affected in two ways: these worms are not only slow defecators, but also are unable to react to the temperature shift. These two defecation phenotypes should be assessed separately. To establish possible epistasis, only 20°C scoring data was considered because the temperature adjustment phenotype may interact/interfere with the suppressive effects these mutations have at

25°C. The ability of the worms to adjust to the temperature shift was assessed separately and presented in a table above each figure.

4.3.2 Interactions within class I are hard to predict

Interactions between class I suppressors could reveal if these suppressors are involved in the same genetic pathway, and how they may be involved in active temperature regulation in the worm system. Various mutant combinations were assessed on both wild-type and *clk-1(qm30)* backgrounds. When *dsc-4(qm182)* and *pgp-2(gk114)* were combined on the wild-type background, their suppressive effects were not additive at 20°C, but were at 25°C (Figure 10). On both wild-type and *clk-1(qm30)* backgrounds at 20°C the combination of *pgp-2* and *dsc-4* results in the phenotype that is the same as *dsc-4* alone. This suggests that *dsc-4* has a dominant effect over *pgp-2* at 20°C and sets the defecation cycle length. All mutants on wild-type background were able to respond to the temperature shift. However, on *clk-1* background only *dsc-4* was able to restore the ability of the worms to react to the temperature shift. When both *pgp-2* and *dsc-4* were simultaneously placed on *clk-1* background, the worms were no longer able to adjust to the temperature shift. This indicates that on *clk-1* background *pgp-2* is dominant over *dsc-4* in determining the ability to adjust to temperature changes. These genes may represent the beginning and the tail end of worm lipoprotein metabolism: *dsc-4* is likely involved in lipoprotein assembly (Shibata, Branicky et al.), and *pgp-2* is likely a part of the endolysosomal system (Hermann, Schroeder et al. 2005; Schroeder, Kremer et al. 2007). These genes are possibly in the same pathway because at 20°C there was no additivity.

Previously the effects of *dsc-3(qm179)* and *dsc-4(am182)* were shown to be non-additive. Therefore, it was concluded that these genes likely belong to the same genetic pathway (Branicky PhD thesis, 2005). Before, a point mutation allele (*qm179)* was used. After using a deletion allele *dsc-3*(*tm1634)*, the effects of *dsc-3* and *dsc-4* were shown to be additive after shifting to 25°C but not at 20 °C on the *clk-1(qm30)* background (Figure 12). Both *dsc-3(tm1634),* and *dsc-4(qm182)* suppress the inability of *clk-1* worms to react to temperature shifts. When these mutations are combined on *clk-1* background, the difference between the defecation cycle length at two temperatures became much greater. These results suggest that *dsc-3* and *dsc-4* restore the ability of *clk-1* to react to the temperature shift via different pathways.

dsc-3(tm1634) is capable of restoring the ability of *clk-1(qm30)* to react to temperature shifts, while *pgp-2(gk114)* is not. When *dsc-3* was combined with *pgp-2* on the wild-type background, at both temperatures the presence of the *pgp-2* mutation interfered with the ability of the *dsc-3* mutation to shorten defecation cycle length of the wild-type worms (Figure 11). Since the presence of *pgp-2* interferes with the ability of *dsc-3* to shorten the defecation cycle of wild-type worms at both temperatures, but on the *clk-1* background the suppressive effects of these mutations become additive at both temperatures (Figure 11). These results suggest that *clk-1* function is essential for *pgp-2*'s ability to interfere with *dsc-3* suppression. On wild-type background *pgp-2* is dominant over *dsc-3* in its ability to set the defecation

speed. Also, on wild-type background all mutants were able to react to the temperature shift. On *clk-1* background these mutations show an additive effect at both temperatures. This suggests that the absence of *clk-1* leads to a change in PGP-2 function, where it no longer is a dominant mutation.

Combining all class I suppressors together on *clk-1(qm30)* mutant background showed complex interactions that are difficult to interpret (Figure 13). Interactions between these mutations are likely dependent on the specific functions of each gene. It is interesting that all genetic combinations that included *dsc-3(tm1634)* were able to rescue the *clk-1*'s ability to react to the temperature shift—these strains defecated faster at 25 as compared to 20°C. This suggests that *dsc-3* is heavily involved in the temperature compensation mechanism of *C. elegans* that gets revealed with mutations in *clk-1*. In conclusion, no solid understanding of how the class I suppressor family functions in terms of their genetic pathways could be gathered from this study.

4.3.3 Interactions between class I and class II suppressor categories are hard to predict

On the wild-type background the combination of *sod-4(gk101)* and *pgp-2(gk114)* mutations showed additive effects at 20°C, but not after shifting the temperature to 25°C (Figure 14). The double mutant on the wild-type background does not react to the temperature shift. On the *clk-1* background the combination of the same mutations showed interference at 20°C and additivity at 25°C. When *dsc-4* and *sod-4* mutations were combined on the wild-type background, the double mutant defecated at the speed of *sod-4* at both temperatures (Figure 15), suggesting that there is a complicated interaction between these mutations in absence of *clk-1*. When we look at the ability of the worms to adjust to the temperature shift and defecate faster at 25 than at 20°C, on *clk-1* background only the worms having the combination of *pgp-2* and *sod-4* were able to adapt to the temperature shift.

On the *clk-1(qm30)* background the combination of *sod-4(gk101)* and *dsc-3(tm1634)* mutations showed an additive effect at 25°C (Figure 16), and defecated at the speed of *clk-1;dsc-3* at 20°C. The addition of *dsc-3* mutation is able to restore the *clk-1;sod-4* ability to adjust to the temperature shift, suggesting that *dsc-3* is essential for the maintenance of the temperature compensation mechanism in *C. elegans*. When two class I mutants, *pgp-2 and dsc-4*, were combined with a class II mutant *sod-4* on the wild-type background, the interactions between classes were very complex. When these

mutations are combined on the *clk-1* background, the defecation suppression becomes too complex. It is, however, noteworthy that the combination of *pgp-2* and *sod-4* of wild-type ground seems to remove the temperature compensation mechanism resulting from mutations in *clk-1*.

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