# Identification and Analysis of New Mutations that Suppress the Slow Defecation Phenotype of *clk-1(qm30)* Mutants

**Tania Rodrigues**

Department of Biology
McGill University, Montreal, Canada

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**Abstract**

Mutations in the *clk-1* gene of *Caenorhabditis elegans* result in an average slowing down and deregulation of a variety of developmental and physiological processes. In addition, *clk-1* mutants are defective in responding to temperature changes. For example, wild-type worms adjust their defecation cycle length after a temperature shift whereas the defecation cycle length of *clk-1* mutants is unaffected by such a shift. To understand the basis of the *clk-1* phenotype, a number of genetic screens have been carried out to isolate feature-specific suppressor mutations. *dsc-3(qm179)* and *dsc-4(qm182)* were isolated in this manner. It has previously been found that *dsc-3(qm179)* and *dsc-4(qm182)* strongly suppress the slow defecation phenotype of *clk-1* mutants at 20°C, as well as after a temperature shift to 25°C. Molecular analysis of *dsc-4*, which encodes the microsomal triglyceride transfer protein, suggests that *dsc* genes affect lipid metabolism. We carried out a genetic screen for additional mutations that can suppress the slow defecation of *clk-1* mutants after a temperature shift to 25°C and isolated two new suppressor mutations. Complementation tests as well as linkage analysis and mapping indicates that *dsc-6(qm192)* and *dsc-7(qm193)* define new *dsc* genes. We analyzed the phenotype of the new suppressor strains and have found that, like *dsc-4(qm182)*, *dsc-6(qm192)* and *dsc-7(qm193)* can suppress a variety of *clk-1* phenotypes. Based on additional phenotypic analyses of the new suppressor strains, including the determination of their sensitivity to exogenous cholesterol, we believe that, like *dsc-4*, *dsc-6* and *dsc-7* encode activities that affect lipid metabolism in worms.

**Resumé**

Les mutations du gène *clk-1* chez le nématode *Caenorhabditis elegans* est caractérisée par un phénotype pleiotropique qui comprend un ralentissement des comportements rythmiques tel que le mouvement, la défécation et le pompage pharyngien. De plus, les mutants *clk-1* ne réagissent pas normalement aux changements de température. Les vers sauvages ajustent leurs rythmes de défécation en fonction de la température, mais ceci n'est pas le cas dans les vers *clk-1.* Afin de mieux comprendre les causes du phénotype *clk-1*, de nombreux criblages génétiques furent effectués permettant la découverte de plusieurs mutations supprimant le ralentissement du rythme défécatoire de ces mutants. Les mutations *dsc-3(qm179)* et *dsc-4(qm182)* furent isolées de cette manière. De études antérieurs ont montré que ces deux mutants accélèrent le rythme défécatoire à 20°C ainsi qu'à 25°C. L'analyse moléculaire de *dsc-4*, dont le produit est la protéine MTP (microsomal triglyceride transfer protein), suggère que les gènes *dsc* affectent le métabolisme des lipides. Nous avons effectué un nouveau criblage génétique qui nous a permis d'identifier deux nouveaux mutants. Des tests de complémentation ainsi que le positionnement génétique ont établi que *dsc-6(qm192)* et *dsc-7(qm193)* constituent de nouveaux mutants *dsc*. L'analyse phénotypique de ces nouveaux suppresseurs a démontré qu'ils supprimaient de nombreux phénotypes associés à la mutation *clk-1,* ce dont *dsc-4* est également capable. De plus, les résultats basés sur des analyses phénotypiques additionnels tels que la détermination de leur sensibilité au cholestérol exogène, semblent confirmer que, *dsc-6* et *dsc-7* affectent aussi le métabolisme des lipides chez le vers.

**Acknowledgements**

I gratefully acknowledge the Natural Sciences and Engineering Research council of Canada for their financial support and Siegfried Hekimi, my supervisor, for giving me the opportunity to complete my research studies in his laboratory. I owe special thanks to Robyn Branicky for all of her insightful comments, advice and help throughout the course of my degree. For providing clones, I would like to thank A. Coulson and for providing strains, I would like to thank the Caenorhabditis Genetics Centre (which is funded by the NIH NCRR). I would also like to thank Darius Camp for the French translation of my abstract and Phanh Nguyen for critically reading this manuscript. For their friendship, I would like to thank all past and present members of the Hekimi Lab, particularly, Irene, Phanh, Darius, Melissa, San San, Jing Jing, Xihua and Wen. Finally, I am especially grateful to my parents for their continuous help and support.

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# Chapter 1.0 – Introduction

## 1.1 *Caenorhabditis elegans* as a model organism

In 1965, Sydney Brenner selected *Caenorhabditis elegans* as an experimental model to study animal development and behavior. Brenner picked *C. elegans* for reasons that are now well recognized: its rapid life cycle, fecundity, genetic tractability, and anatomical simplicity. At present, *C. elegans* is very widely used by a number of influential research groups to investigate many avenues of biological research, including human disease. In 2002, research on *C. elegans* was awarded a Nobel Prize.

*C. elegans* is a microscopic (~1mm long adult), non-pathogenic nematode that normally lives in a temperate soil environment. The small size of the animal allows large numbers to be cultivated inexpensively on 60-mm agar plates seeded with *Escherichia coli*, in a laboratory setting. Similar to all animals, *C. elegans* starts life as a fertilized egg, which undergoes a rigid pattern of mitotic divisions to produce a reproductively mature adult that develops through four larval stages (L1 through L4) (Figure 1). The complete life cycle of the animal is 3 days at 20°C. The rapid generation time of *C. elegans* permits the completion of many research experiments in a relatively short period of time. The number of cells in the adult hermaphrodite of *C. elegans* is invariable, which has enabled the documentation of its complete cell lineage (Sulston, 1998). There are 959 somatic cells in an adult hermaphrodite, of which 302 are neurons.

*C. elegans* exists as two sexes: self-fertilizing hermaphrodites and males. The somatic cells of hermaphroditic *C. elegans* contain 5 pairs of autosomes and two X chromosomes. Infrequently, nondisjunction occurs during meiosis with the loss of one X chromosome. Animals with 5 pairs of autosomes and a single X chromosome are males. Self-fertilizing hermaphrodites can generate a brood of 300-350 genetically identical animals. If genetic crosses need to be performed, hermaphrodites can be mated with the male sex.

*C. elegans* was the first multicellular organism to have its complete genome sequenced (1998). The *C. elegans* genome is approximately 97 million base pairs in length and contains about 20,000 protein-encoding genes. Several homologs of human genes that are targets for mutation in human disease have been found to function in the worm. Often, entire developmental and signaling pathways are conserved among humans and nematodes (for example, the insulin signaling pathway). This makes *C. elegans* a practical model for the study of several human diseases including diabetes, cancer, obesity, alcoholism and Alzheimer's disease (Boulton et al., 2004; Davies et al., 2003; Hafen, 2004; Link, 1995; McKay, McKay et al. 2003; Smith and Levitan, 2004).

### 1.2 The *clk* genes

Ageing can be defined as a progressive increase in the probability of dying. *C. elegans* is one of the most broadly used model organisms in elucidating the regulatory systems that control ageing. There are a number of clear benefits in using *C. elegans* to investigate the regulation of lifespan in multicellular

organisms. (1) Worms are small and easy to keep. (2) Worms can be frozen indefinitely in liquid nitrogen. Consequently, strains do not have to be actively maintained. (3) Worms are short-lived (the wild-type strain (N2) lives for about two weeks at 20°C). This allows ageing experiments to be concluded within weeks or months of their initiation. (4) A number of genetic and molecular tools have been developed to accelerate *C. elegans* research, including advanced microscopes, an assortment of antibodies, RNA interference (RNAi), microinjection and the use of laser microbeams to ablate individual cells (Kenyon, 1988; Wood, 1988; Fire et al., 1988; Kimble et al., 1982).

In 1995, Wong et al. carried out a genetic screen to identify maternal-effect mutations that influence developmental and behavioral timing in *C. elegans*. The authors identified a group of genes that they called *clk* for abnormal function of biological **clock**s. Through further study, Wong et al. (1995) found that *clk-1*, *clk-2*, and *clk-3* mutants exhibit a comparable phenotype, which is an average slowing down of the rates of embryonic and post-embryonic development, rhythmic adult behaviours, reproduction and ageing. In addition, the authors found that mutants in all three genes are almost completely maternally and zygotically rescued for most phenotypes. Up to now, eight additional *clk* genes have been isolated: *clk-4*, through *clk-10* and *gro-1*. Hodgkin and Doniach isolated the *gro-1* mutation in 1997, on the basis of its slow growth. Subsequent study of *gro-1* mutants revealed a Clk phenotype, including the maternal effect (Wong et al., 1995). In 2000, Meng isolated *clk-4* through *clk-10* in a genetic screen for maternal effect mutants with a Clk phenotype.

*gro-1*, *clk-1*, *clk-2*, and *clk-8* have been characterized in great detail, including molecularly. The *gro-1* gene encodes a highly conserved cellular enzyme, isopentenylpyrophosphate tRNA transferase (IPT), which modifies a subset of tRNAs (Lemieux et al., 2001). *clk-1*, encodes a mitochondrial protein required for the biosynthesis of ubiquinone (UQ) (Ewbank et al. 1997; Miyadera et al. 2001). *clk-2* encodes a protein homologous to *S. cerevisiae* Tel2p (Benard et al., 2001). In yeast, the gene *TEL2* regulates telomere length and participates in gene silencing in subtelomeric regions (Runge and Zakian 1996). In *C. elegans*, *clk- 2* mutants have elongated telomeres, and *clk-2* overexpression can lead to telomere shortening (Benard et al., 2001). Furthermore, in 2001, Ahmed et al. showed that *clk-2* is a DNA damage checkpoint protein, which may play a role in oncogenesis. *clk-8* encodes thiamine pyrophosphokinase, which forms thiamine pyrophosphate from thiamine at the expense of ATP (de Jong et al., 2004). Intriguingly, *gro-1*, *clk-1*, *clk-2* and *clk-8* encode proteins that appear to be molecularly unrelated regardless of the Clk phenotype common to mutants in all four genes. This has made it difficult for ageing researchers to identify a communal mechanism responsible for the Clk phenotype.

### 1.3 The *clk-1* phenotype

The *clk-1* gene of the nematode *C. elegans* is the best understood and characterized *clk* gene. Mutations in the *clk-1* gene are highly pleiotropic, resulting in an average slowing down and deregulation of a variety of developmental and physiological processes including the cell cycle,

embryogenesis and postembryonic development. The period of a number of rhythmic adult behaviors such as swimming, defecation and pharyngeal pumping are also retarded (Wong et al. 1995). Moreover, mutations in *clk-1* lengthen lifespan. While this effect is only moderate in the wild-type background, *clk-1* mutations can synergise with other lifespan extending mutations to increase lifespan nearly five fold (Lakowski and Hekimi, 1996).

In addition to being slower on average, many of the physiological traits affected by mutations in *clk-1* are more irregular (Wong et al. 1995). For example, even though the duration of embryonic development of *clk-1* mutants is several times greater than that of wild-type animals, some mutant embryos can develop faster than wild type embryos. Also, a number of observations suggest that *clk-1* mutants are defective in responding to changes in temperature (Wong et al. 1995; Branicky et al. 2001). For example, when wild-type worms are grown at 20°C and shifted to a new temperature, the defecation rate speeds up or slows down, depending on that new temperature. In contrast, when *clk-1* mutants are shifted to a new temperature, the defecation cycle length is unaffected (Branicky et al. 2001). Finally, all *clk-1* phenotypes can be maternally rescued; that is, homozygous mutant progeny that originate from a heterozygous hermaphrodite are phenotypically wild-type (Wong et al. 1995). The mutant phenotype can only be observed in the second homozygous generation.

The *C. elegans* hermaphrodite adult reproductive system normally consists of two U-shaped gonad arms (an anterior and a posterior). The two U shaped arms are joined centrally by two spermathecae and a uterus, which

stores fertilized eggs. *C. elegans* hermaphrodites first produce sperm, then switch permanently to oocyte production. It has previously been found that the switch from sperm production to oocyte production is considerably delayed in *clk-1* mutants (Shibata et al., 2003). To illustrate, in wild-type hermaphrodites, primary spermatocytes can be detected at the L4 stage and oocytes can be detected shortly after the adult molt. In comparison, the majority of *clk-1* mutants are either before or in the process of spermatogenesis at the adult molt (Shibata et al., 2003).

### 1.4 The molecular identity of *clk-1*

*clk-1* encodes a mitochondrial protein required for the biosynthesis of ubiquinone (UQ) (Ewbank et al. 1997; Miyadera et al. 2001), a prenylated benzoquinone lipid that functions as an electron carrier in the respiratory chain of the mitochondria (Trumpower 1981). The mitochondria of *clk-1* mutants do not contain detectable levels of UQ, but instead accumulate demethoxyubiquinone (DMQ), a UQ biosynthetic intermediate (Miyadera et al. 2001; Jonassen et al. 2002) (Figure 2). The quinone phenotype is identical for all three *clk-1* alleles (*e2519*: a partial loss of function allele, *qm30*: a putative null allele, and *qm51*: a putative null allele). As expected, most of the developmental and physiological features affected in *clk-1* mutants are retarded more severely in the two putative null alleles (*qm30* and *qm51*) than they are in the partial loss of function allele (*e2519*). Hence, there is no direct correlation between the quinone phenotype and the severity of the overall phenotype of *clk-1(qm30)*, *clk-1(qm51)* and *clk-*

*1(e2519)* animals. Also, strains harboring mutant alleles of the *clk-1* gene have been shown to have a comparable energy production and consumption to the wild-type strain (Braeckman et al., 1999). Collectively, these observations suggest that the absence of UQ alone is unable to explain completely the pleiotropic phenotype of *clk-1* mutants.

In *clk-1* mutants, DMQ can functionally replace UQ for its role as an electron carrier, such that the mutant mitochondria can maintain respiration despite the complete absence of UQ (Miyadera et al. 2001). However, it is important to note that *clk-1* mutants depend on the UQ they get from their bacterial food source. When *clk-1* mutants are fed strains of *Escherichia coli* that do not produce UQ, they cannot complete development or become fertile adults (Hihi et al. 2002; Jonassen et al. 2001) demonstrating that DMQ cannot entirely functionally substitute for UQ.

### 1.5 The role of ubiquinone

UQ is a redox-active lipid located in all cellular membranes. The point to highlight is that UQ has numerous biochemical functions. In particular, UQ acts in the mitochondrial electron transport chain as an electron shuttle between complexes I, and II to complex III. In this role, UQ is one the main sites of ROS production (Raha and Robinson, 2000). (At low levels, ROS can act as intracellular messengers in signal transduction pathways. At higher levels, ROS can damage a number of cellular macromolecules (including nucleic acids, proteins and lipids) and participate in apoptosis.) In addition, UQ is believed to

function as an electron transporter at the plasma membrane (Santos-Ocana et al., 1998; Sun et al., 1992) and in lysosomes (Gille and Nohl, 2000). In its reduced form, UQ is a potent antioxidant, which prevents the initiation and/or propagation of lipid peroxidation in biological membranes (Ernster and Forsmark-Andree, 1993).

### 1.6 Defecation suppressors of *clk-1*

To help understand how a mutation in the *clk-1* gene of *Caenorhabditis elegans* affects such diverse physiological processes, several genetic screens have been carried out to identify feature-specific suppressor mutations. One of the physiological features affected in *clk-1* mutants is the defecation cycle. In summary, defecation is carried out by three distinct motor steps: the posterior body muscle contraction (pBoc), the anterior body muscle contraction (aBoc), and the expulsion muscle contraction (Exp) (Thomas 1990) (Figure 3). In wild-type worms, the defecation cycle length is 56 ± 3.2 seconds (at 20°C). The defecation cycle is both increased in length and more variable in *clk-1* mutants (Wong et al., 1995). For example, in *clk-1(qm30)* animals the defecation cycle length is 88 ± 14 seconds (at 20°C; Wong et al., 1995; Branicky et al., 2001). Furthermore, when wild-type animals are grown at 20°C and shifted to a new temperature, that new temperature significantly affects the defecation cycle length. In contrast, when *clk-1* mutants are temperature shifted, the defecation cycle length is unaffected (Figure 4). This suggests that *clk-1 (+)* activity is

required for the adjustment of the rate of defecation in response to changes in temperature (Branicky et al. 2001).

To identify genes that interact with *clk-1* to regulate the defecation cycle length, Branicky et al. (2001) carried out a genetic screen at 20°C for mutations that suppress the slow defecation phenotype of *clk-1(qm30)* animals. Two distinct classes of mutations were identified. The genes defined by these mutations were called *dsc* for **d**efecation **s**uppressor of ***clk-1.*** Although both class I and class II mutations suppress the slow defecation phenotype of *clk-1* mutants at 20°C, only class I mutations can suppress after a temperature shift from 20°C to 25°C (Branicky et al., 2001) (Figure 5). Below, I will discuss in detail the molecular and phenotypic characterization of *dsc-4(qm182)*, a class I suppressor mutation identified in the abovementioned genetic screen.

### 1.7 The *dsc-4* gene

*dsc-4(qm182)* was isolated as a suppressor of the slow defecation phenotype of *clk-1* mutants (Branicky et al., 2001). Recently, it has been shown that the *dsc-4* mutation can dramatically suppress other *clk-1* phenotypes, but not all. As stated earlier, the switch from sperm production to oocyte production is delayed in *clk-1* mutants (compared to the wild-type). This defect can be suppressed by the *dsc-4* mutation (Shibata et al., 2003). This can be seen both by direct examination of the stages of germline development at different time points, as well as by looking at the rate of egg-laying at different time points (Shibata et al., 2003) (Figure 6 and 7).

Shibata et al. (2003) found that *dsc-4* encodes an 892-residue protein, which is similar to the large subunit of the microsomal triglyceride transfer protein (MTP). In humans, MTP is required for apolipoprotein B (apo-B) containing lipoprotein assembly in liver and in intestinal cells (Nakamuta et al., 1996). Mutations in the large subunit of MTP cause abetalipoproteinemia (also known as Bassen-Kornzweig syndrome), a severe deficiency in lipoprotein secretion (Sharp et al., 1993). Unless treated, affected individuals develop gastrointestinal, neurological, ophthalmological, and hematological abnormalities. The *dsc-4(qm182)* allele was found to have two point mutations resulting in amino acid substitutions (Shibata et al., 2003). The mutation sites of *qm182* are located in the apo-B binding domain of MTP and are different from those found in individuals afflicted with abetalipoproteinemia.

### 1.8 Lipoprotein Biology

Lipoproteins are complex macromolecular structures that contain a core of triglycerides and /or cholesterol esters encircled by a monolayer of phospholipids and free cholesterol (Segrest et al., 2001). Lipoproteins differ in their apolipoprotein and lipid content, origin, size and density in an aqueous environment (plasma). Lipoproteins are categorized according to their density in plasma. There are four major classes of serum lipoproteins: chylomicrons, very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL). Apolipoprotein B is a necessary component of LDL, VLDL and chylomicrons. In humans, LDL particles are the main transporters of cholesterol.

LDL can exist in native and oxidized forms. Native LDL is recognized by the LDL receptor (Yamamoto et al., 1984) whereas LOX-1 is responsible for oxidized LDL uptake by endothelial cells (Aoyama et al., 1999; Sawamura et al., 1997). Several lines of evidence demonstrate that LDL is oxidized by ROS (Carr et al., 2000; Yokoyama et al., 2000). Oxidized LDL is believed to be one of the major risk factors for atherosclerosis, which is the primary cause of heart disease and stroke in westernized societies (Lusis, 2000).

### 1.9 The microsomal triglyceride transfer protein

The lipid transfer activity of MTP is required for the assembly and secretion of apoB-containing lipoproteins (Berriot-Varoqueaux et al., 2000), in particular LDL. MTP is a heterodimeric protein that consists of two non-covalently bound polypeptides of 97 (M subunit) and 55 (P subunit) kDa (Gregg and Wetterau, 1994). The P subunit is identical with protein disulfide isomerase (PDI), a ubiquitous endoplasmic reticulum (ER) protein required for native disulphide bond formation in newly synthesized proteins. It has been shown that the isomerase activity of the P subunit is not required for MTP activity and its association with the M subunit (Lamberg et al., 1996). The M subunit is essential for lipid transfer activity and may contain at least three functionally independent domains (the lipid transfer domain, the membrane associating domain and the apoB binding domain) (Bradbury et al., 1999; Mann et al., 1999; Read at el., 2000). The P subunit is required to maintain the lipid transfer activity of the M

subunit and to prevent its aggregation in the ER (Wetterau et al., 1991; Vuori et al., 1992; Ricci et al., 1995).

MTP is found in the ER of apoB secreting cells, particularly those of the liver and intestine. Enzyme kinetic studies with model membranes reveal that MTP transfers lipids by a shuttle mechanism (Atzel et al., 1993). In this mechanism, MTP is proposed to interact momentarily with a membrane, extract lipid molecules, dissociate from the membrane, bind momentarily with another membrane, deliver lipids rapidly to the membrane and become available for another round of lipid transfer (Atzel et al., 1993) (Figure 8). Although MTP is capable of transferring all lipid classes found in apoB-containing lipoproteins, the lipid transfer activity of MTP is optimal with neutrally charged lipids like triglycerides and cholesteryl esters (Atzel et al., 1993). In recent times, MTP has been developed as a target for cholesterol lowering drugs (Wetterau et al., 1998).

### 1.10 Lipoprotein control of germline development

A possible role for *dsc-4* in apoB-containing lipoprotein assembly and secretion inspired additional study to explore the biology of LDL-like particles in the nematode *C. elegans*. As mentioned above, apoB is a necessary component of LDL. It has previously been shown that the genome of *C. elegans* contains five apoB-like genes (*vit-2* to *vit-6*) (Spieth et al., 1991). To determine if altered secretion of apoB-dependent lipoproteins is responsible for the effect of *dsc-4* on germline development, Shibata et al., (2003) disrupted the expression of the *vit* genes by RNAi in a *clk-1(qm30)* background. The authors found that the effect of

the *dsc-4* mutation on germline development could be strongly phenocopied by *vit-5* RNAi (Shibata et al., 2003). Based on that observation, Shibata et al. (2003) speculated that the effect of *dsc-4* on germline development might result from a reduction in secreted LDL-like lipoproteins. To further test their hypothesis, Shibata and colleagues next examined the effect of cholesterol depletion on germline development in *clk-1* mutants. In mammals, cholesterol is a major component of lipoproteins, and reducing its intake or synthesis leads to reduced levels of LDL. As expected, the authors found that the effect of low cholesterol on the development of the *clk-1* germline is indistinguishable from that of the *dsc-4* mutation in the presence of cholesterol.

As stated earlier, *clk-1* encodes a mitochondrial protein required for the biosynthesis of UQ (Ewbank et al. 1997; Miyadera et al. 2001) and *clk-1* mutant mitochondria do not contain detectable levels of UQ, but instead accumulate DMQ (Miyadera et al. 2001; Jonassen et al. 2002). It has been previously shown that the redox properties of DMQ and UQ are quantitatively different (Miyadera et al., 2002). In particular, DMQ retains lower redox potential compared to UQ. This property of DMQ could lead to a reduced production of ROS in *clk-1* mutants (Miyadera et al., 2002). To test whether ROS affects the development of the germline in *clk-1* mutants, Shibata and colleagues performed RNAi against the *C. elegans* superoxide dismutases (SOD) to elevate the level of ROS in a *clk-1(qm30)* background. Shibata et al. (2003) found that *sod-1(RNAi)* is as efficient as the *dsc-4* mutation and *vit-5(RNAi)* in suppressing the slow germline development of *clk-1* mutants, which suggests that this phenotype results from

low levels of ROS. Also, the authors found that the effect of *sod-1(RNAi)* is not additive to that of *dsc-4(qm182)*. Consequently, Shibata et al. (2003) concluded that the oxidation of LDL-like lipoproteins is an important factor in controlling the development of the germline in *C. elegans* (Shibata et al., 2003).

Taken together, the studies described by Shibata and colleagues promote a model in which native LDL-like lipoproteins and oxidized LDL-like lipoproteins control *C. elegans* germline development (Figure 9). In this model, decreasing the oxidation of LDL-like lipoproteins retards the rate of germline development (as in *clk-1* mutants), whereas decreasing the production and/or secretion of native LDL-like lipoproteins (as in *clk-1; dsc-4* mutants) can restore a normal rate of germline development (Shibata et al., 2003). Thus, oxidized LDL-like lipoproteins promote, and native LDL-like lipoproteins slow down the development of the germline in *C. elegans* (Shibata et al., 2003).

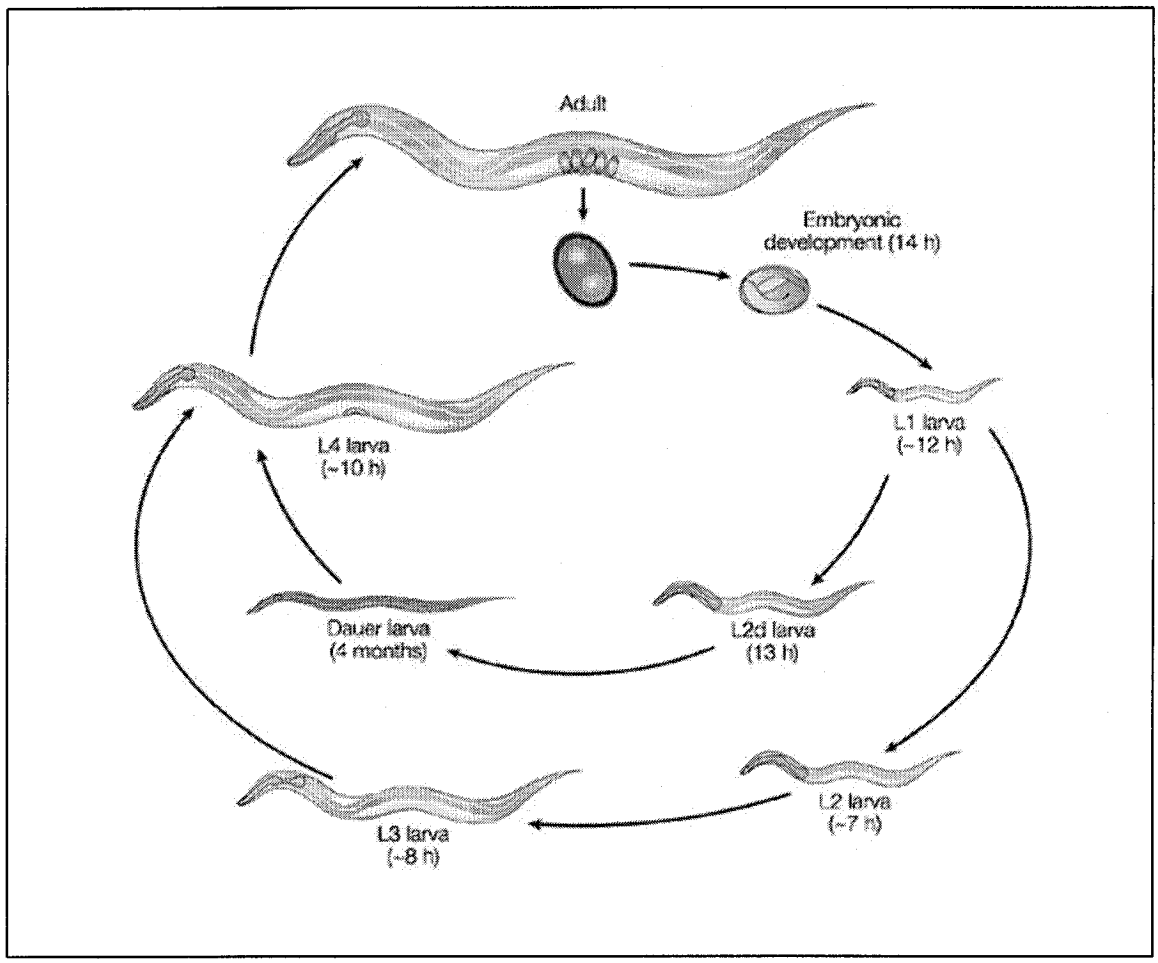


**Figure 1: The lifecycle of *C. elegans*.** *C. elegans* eggs are fertilized inside the adult hermaphrodite and laid a few hours later. Eggs hatch and animals advance through four larval stages. The four larval stages are separated by molts. *C. elegans* can adopt an alternative larval stage, called the dauer larval stage, in preference to becoming an L2 larva when placed under conditions of environmental stress, low food supply, or crowding (Cassada and Russell, 1975; Riddle, 1988). Dauer larvae are thin and non-feeding.

(Adapted from http://thalamus.wustl.edu/nonetlab/ResearchF/LifeCycle.jpg.)

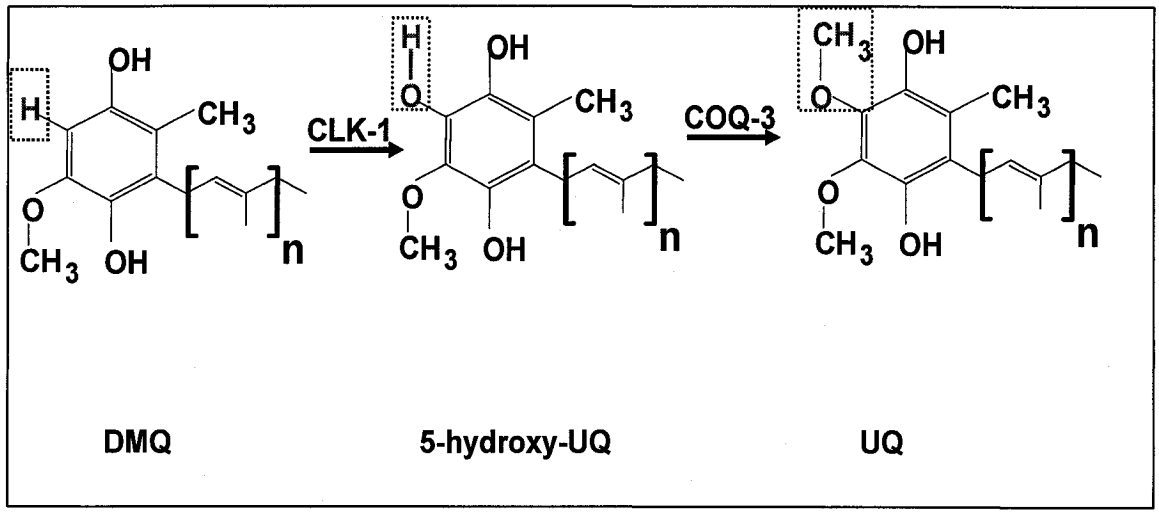


**Figure 2: *clk-1* encodes a mitochondrial protein required for the biosynthesis of ubiquinone (UQ).** CLK-1 converts demethoxyubiquinone (DMQ) into 5-hydroxyubiquinone (5-hydroxy-UQ) and COQ-3 converts 5-hydroxy-UQ into ubiquinone (UQ). *clk-1* mutants accumulate DMQ. n denotes the number of isoprene units (which is species-dependent). In *C. elegans*, n=9.

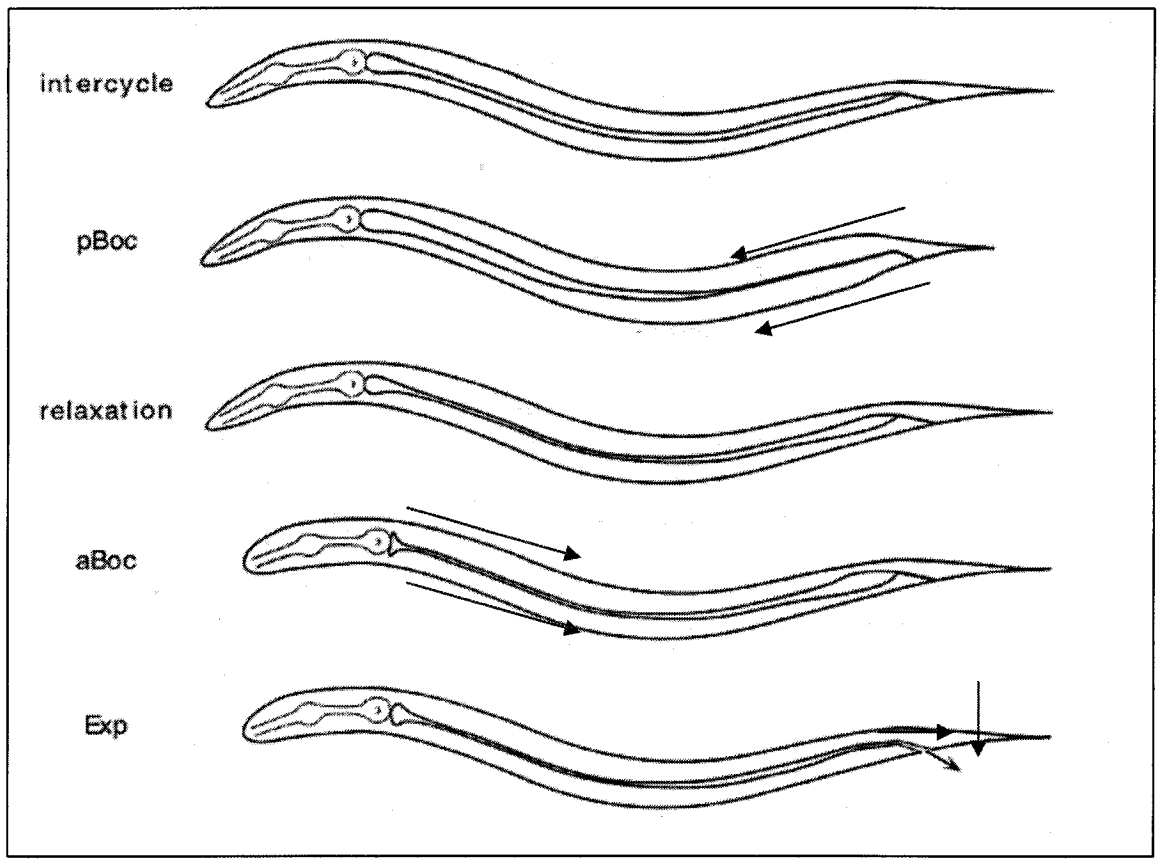


**Figure 3: Movements of the defecation motor program.** In *C. elegans* defecation occurs approximately every 55 seconds. Defecation is carried out by three distinct motor steps: the posterior body muscle contraction (pBoc), the anterior body muscle contraction (aBoc), and the expulsion muscle contraction (Exp).

(Adapted from http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=ce2.figgrp.841.)

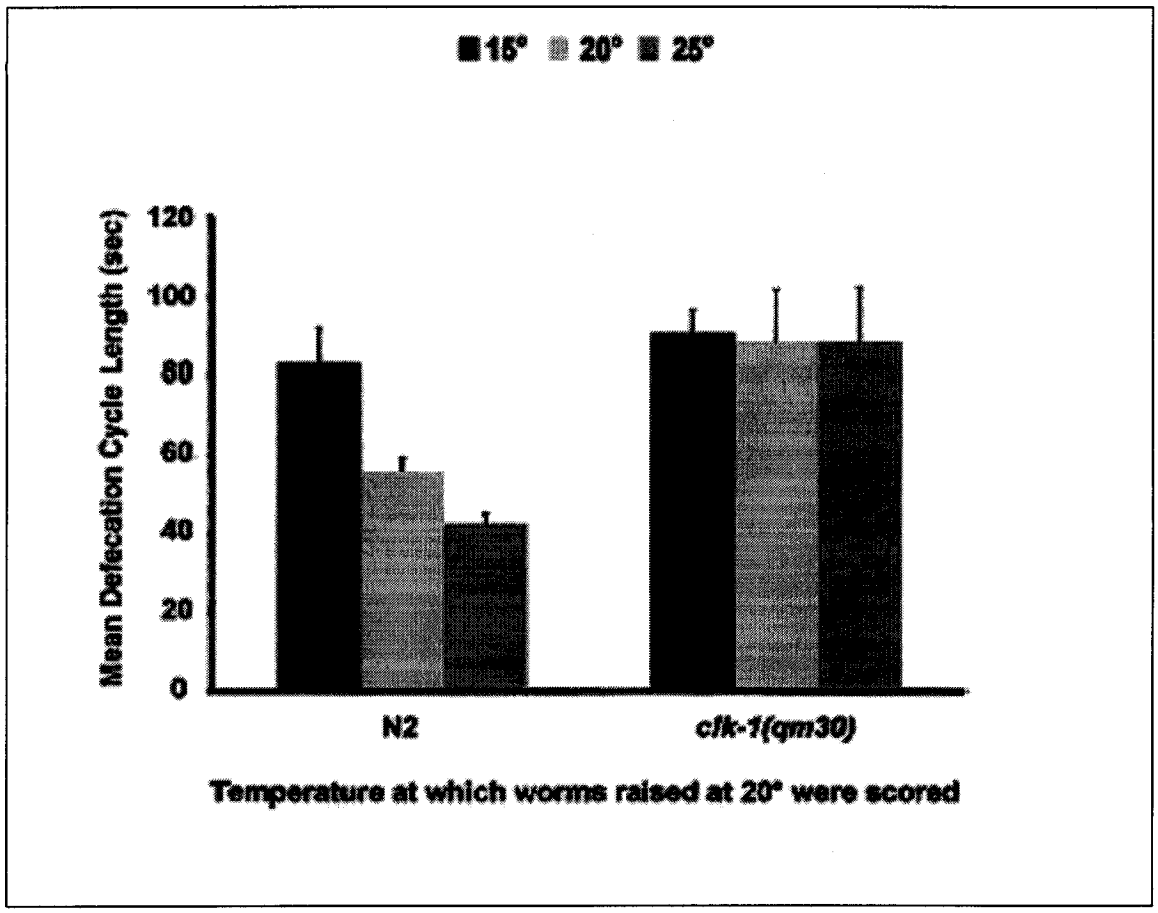


**Figure 4: Temperature shift experiments.** In contrast to wild-type (N2) animals, when *clk-1* mutants are grown at 20°C and shifted to a new temperature, the defecation cycle length is unaffected by that new temperature. (Adapted from Branicky et al., 2001.)

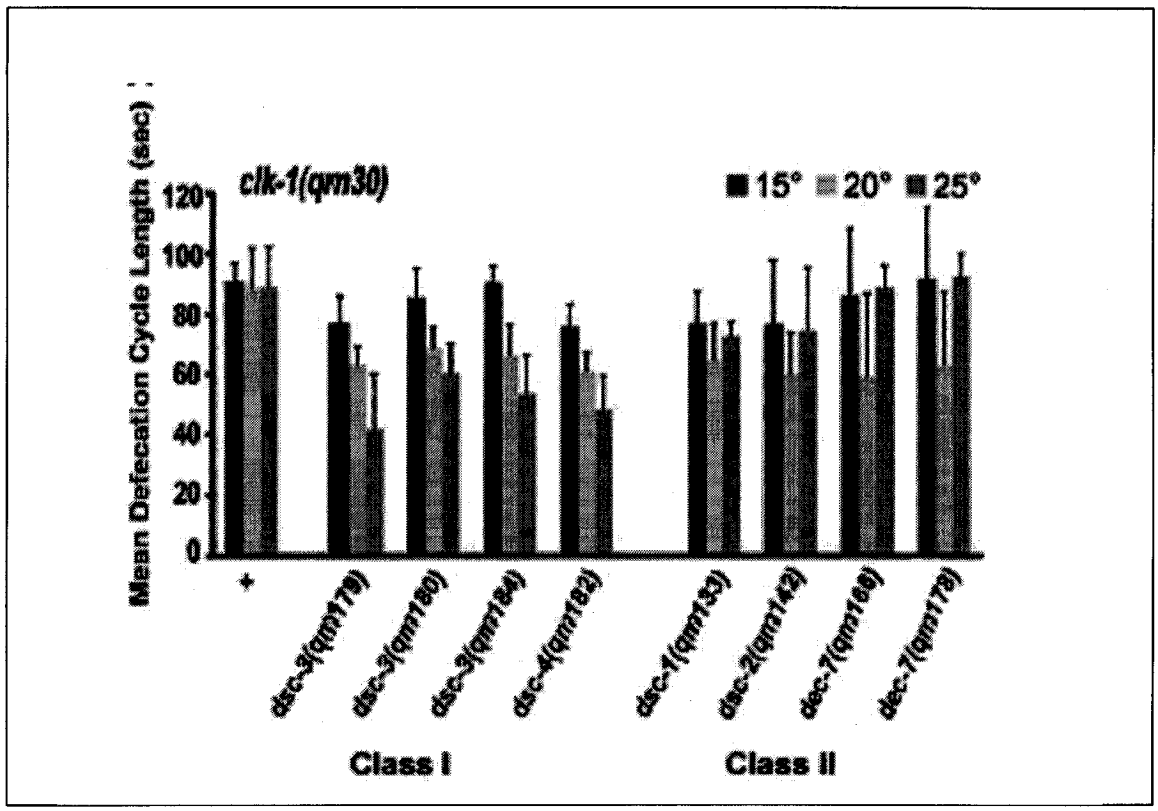


**Figure 5: The effect of the *dsc* mutations on the background of *clk-1(qm30)*.** Class I mutants strongly suppress the slow defecation cycle of *clk-1* mutants at 20°C as well as after a temperature shift to 25°C. In contrast to class I mutants, the class II mutants suppress the slow defecation cycle of *clk-1* mutants weakly or not at all following a temperature shift to 25°C. (Adapted from Branicky et al., 2001.)

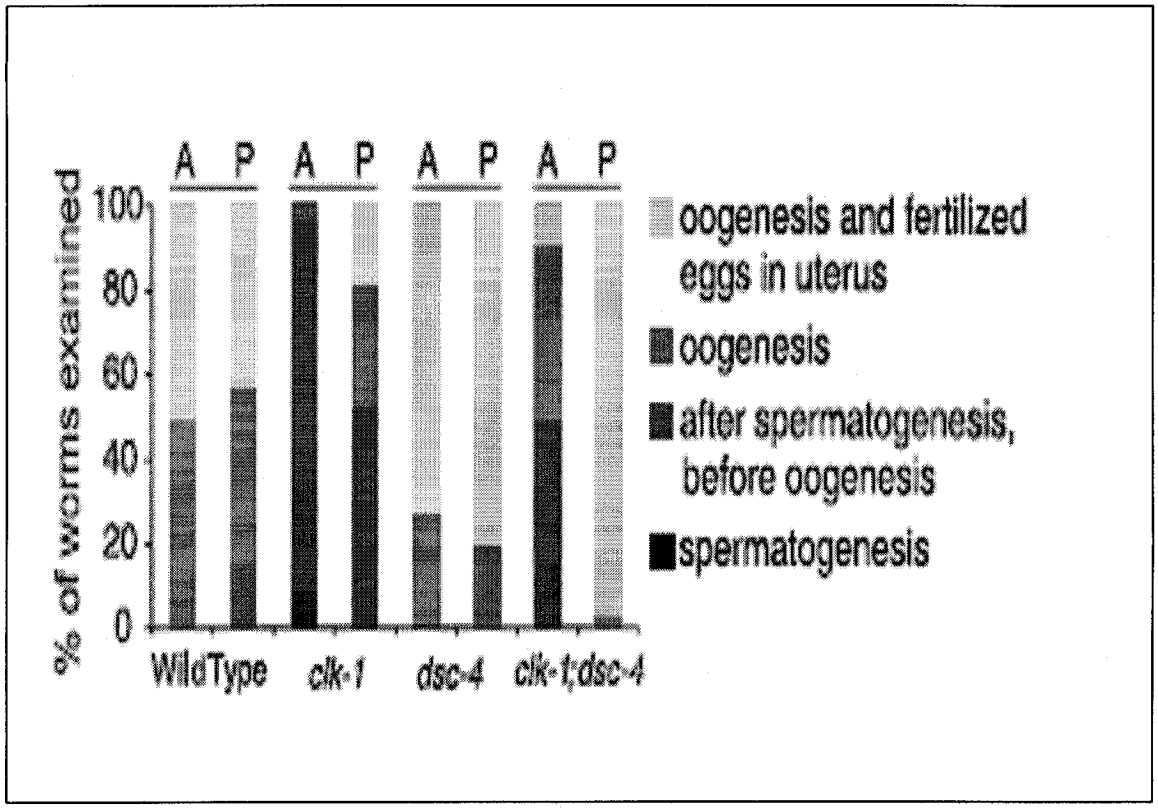


**Figure 6: Germline development at 6 hours after the adult molt.** All wild-type animals are in the process of oogenesis and approximately half of them have fertilized eggs. In comparison, the onset of oogenesis is dramatically delayed in *clk-1* mutants. The *dsc-4* mutation can suppress the slow germline development of *clk-1* mutants. (Adapted from Shibata et al., 2003.)

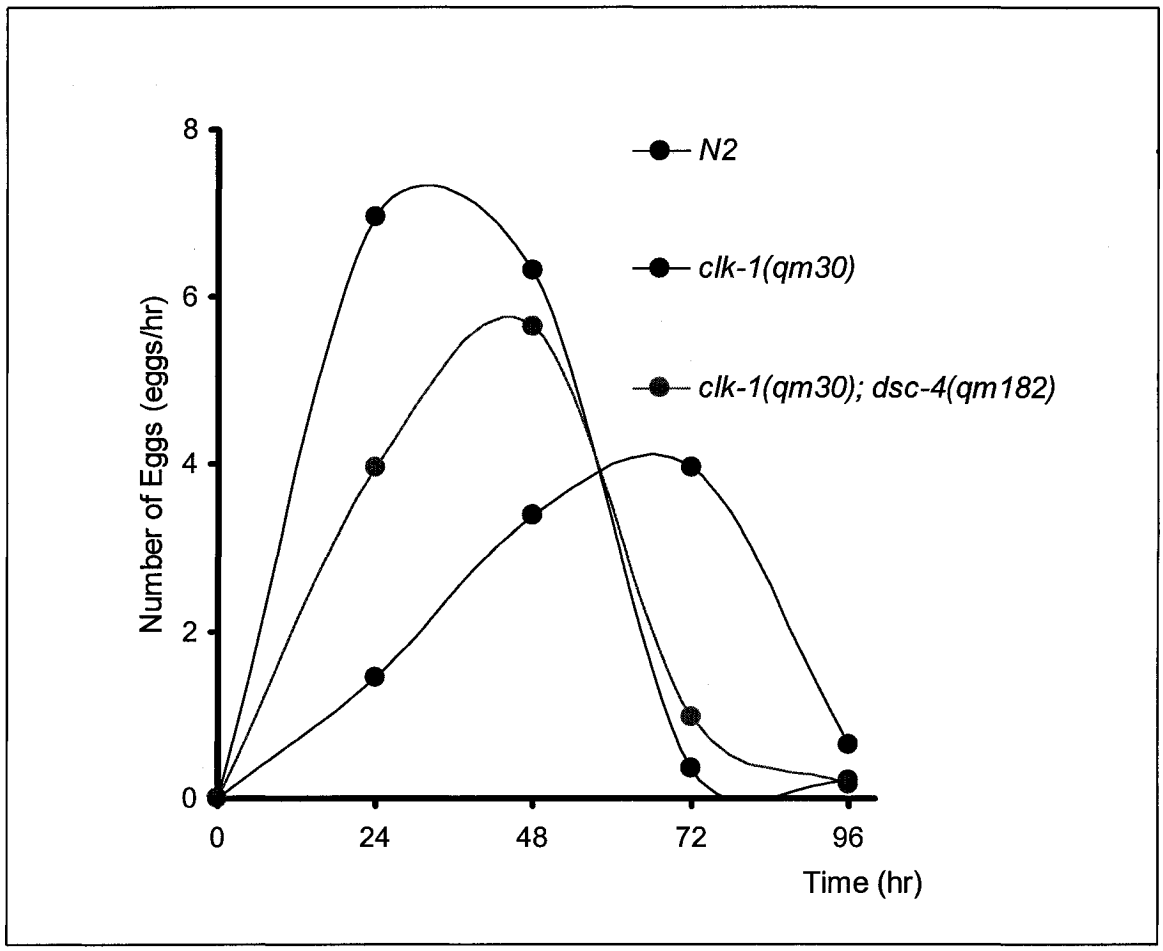


**Figure 7: Time course analysis of egg-laying rate.** Wild-type animals reach their peak of egg laying between 24 and 48 hours, whereas *clk-1* mutants reach their peak of egg laying by 72 hours. This delay can be suppressed by the *dsc-4* mutation, since *clk-1; dsc-4* double mutants reach their peak of egg laying by 48 hours. (Y. Shibata unpublished observations.)

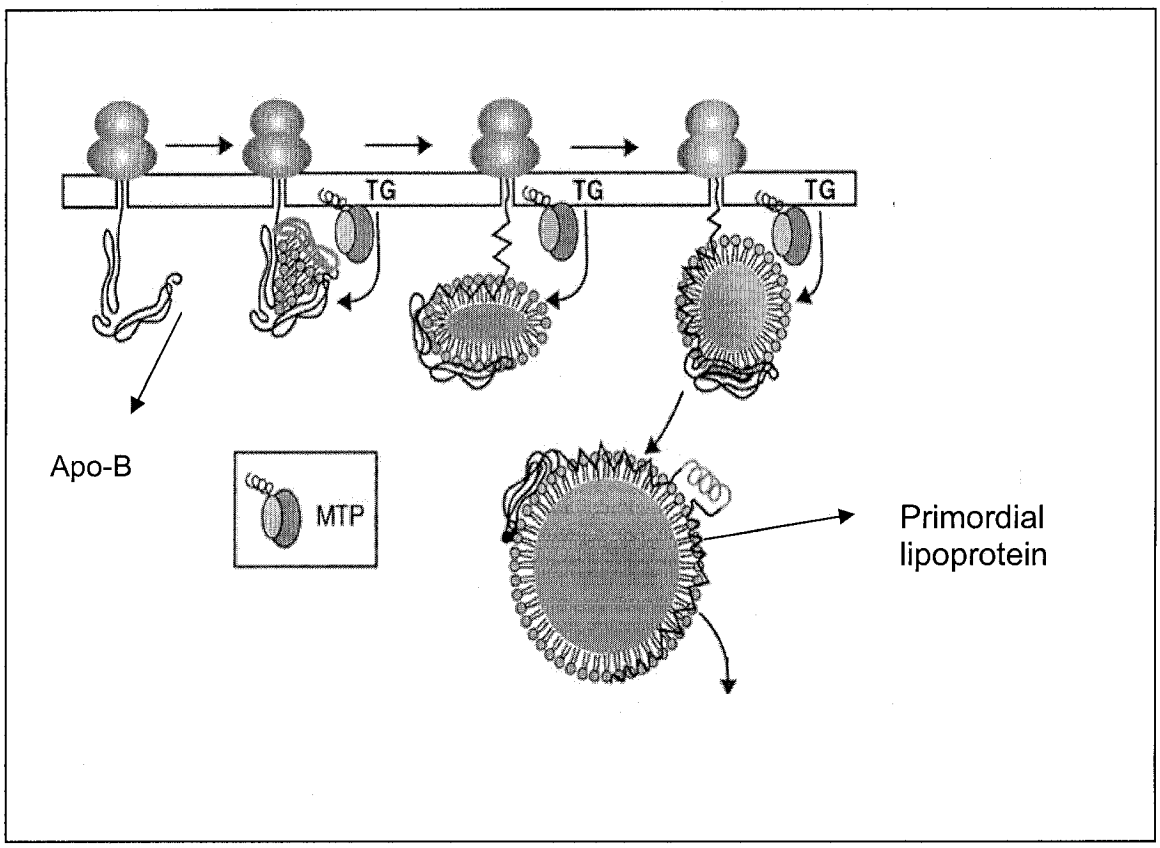


**Figure 8: Formation of primordial lipoproteins requires MTP.** Free MTP can bind nascent apoB. After that, MTP can extract lipid molecules from the membrane and transfer them to apoB. Many rounds of this process will result in immense lipidation of apoB. Subsequent release of MTP results in the formation of a secretion competent primordial lipoprotein. (Adapted from Shelness and Sellers, 2001.)

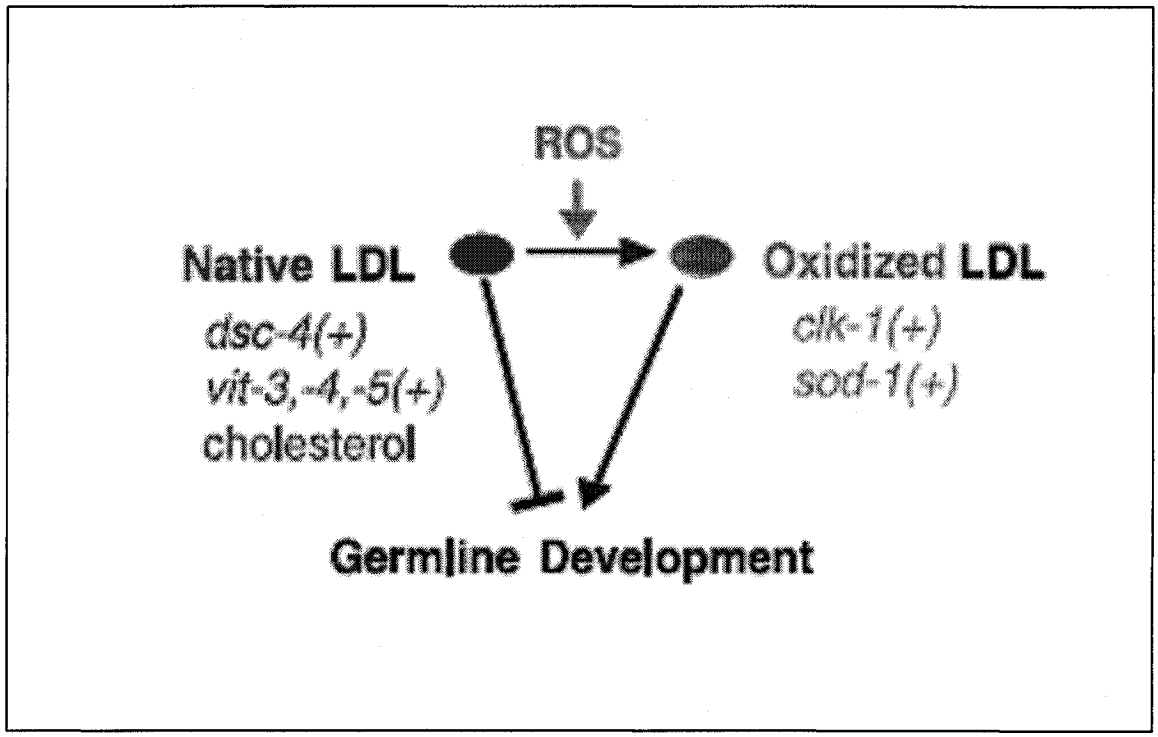


**Figure 9: Lipoprotein control of germline development.** Germline development is stimulated by oxidized LDL-like lipoproteins and inhibited by native LDL-like lipoproteins in the worm. (Adapted from Shibata et al., 2003.)

# Chapter 2.0 – Genetic screen for defecation suppressors of *clk-1*

## 2.1 Introduction

As explained in Chapter 1, *clk-1* mutants have a lengthened defecation cycle that is not affected by changes in temperature (Figure 4). To reiterate, Branicky et al. (2001) carried out a genetic screen for mutations that suppress the slow defecation phenotype of *clk-1* mutants at 20°C. In order to do so, the progeny (F2) of the animals mutagenized with 25 mM EMS were scored for one defecation cycle each at 20°C. Animals that had a defecation cycle length of <65 seconds were kept for further phenotypic and genetic analysis. In this manner, Branicky and colleagues screened a total of 5421 F2 animals and identified 10 suppressor mutations. It has previously been found that these suppressor mutants fall into two distinct classes (Branicky et al., 2001). The class I mutants strongly suppress the slow defecation cycle of *clk-1* mutants at 20°C as well as after a temperature shift to 25°C. In contrast to class I mutants, the class II mutants can suppress only weakly or not at all after shifts to 25°C (Figure 5). The goal of our screen was to isolate and characterize new class I suppressors. In order to do so, we repeated the screen carried out by Branicky et al. (2001), but screened for mutants at 25°C instead of 20°C.

## 2.2 Materials and Methods

### 2.2.1 Nematode strains and cultivation methods

Animals were cultured on nematode growth medium (NGM) agar plates, and were fed the *E. coli* strain OP50 (Brenner 1974). The N2 (Bristol) strain was used as wild type. All animals were cultured at 20°C unless otherwise stated. Genetic strains analyzed in the study include: **MQ1247**: *dsc-6(qm192)* II, **MQ99**: *clk-1(qm30)* III, **MQ1248**: *dsc-7(qm193)* V, **MQ1299**: *dsc-6(qm192)* II; *clk-1(qm30)* III and **MQ1232**: *clk-1(qm30)* I; *dsc-7(qm193)* V.

### 2.2.2 Genetic screen for defecation suppressors of *clk-1*

*clk-1(qm30)* animals were mutagenized with 50mM ethyl methanesulfonate (EMS), as described (Sulston and Hodgkin 1988). Groups of five mutagenized hermaphrodites (P0) were picked to 60-mm petri dishes and left to self-fertilize. Groups of twenty-five F1 animals were plated on 90-mm petri dishes as young adults and were left to lay eggs for approximately 24 hours. F2 animals at the young adult stage were shifted to 25°C for a minimum of two hours and then scored for one defecation cycle each at that temperature. Animals that had a defecation cycle of < 68 seconds were picked to 60-mm plates, singled, and left to self-fertilize. The progeny (F3) of the singled worms were shifted to 25°C as young adults for a minimum of two hours and then scored for one defecation cycle each at that temperature. Only those strains that had a significant proportion of fast defecating worms in the F3 generation were kept for further genetic and phenotypic analysis.

### 2.2.3 Rate of defecation

Animals were grown at 20°C and shifted to 15°C or 25°C as young adults. Animals were then scored for five consecutive defecation cycles each at 15°C or 25°C, 2-6 hours after being shifted to that temperature. The defecation cycle length was defined as the period between the pBoc step of one defecation cycle and the pBoc step of the next defecation cycle (Figure 3). To prevent animals from being over-heated by the microscope lamp during the scoring session, the plates were positioned on heat sinks (petri dishes filled with water) and were kept on the microscope for a maximum of fifteen minutes. All animals were scored at 15°C or 25°C unless otherwise mentioned.

### 2.2.3 Statistical analysis

We performed one-tailed t-tests, taking into account the unequal variance of samples.

## 2.3 Results

### 2.3.1 Isolation of suppressor mutations

To identify additional class I *dsc* suppressors, we carried out a genetic screen to isolate mutations that could suppress the slow defecation phenotype of *clk-1*(*qm30*) mutant animals subsequent to a two hour temperature shift to 25°C. In our screen, the progeny (F2) of the animals mutagenized with 50 mM EMS were scored for one defecation cycle each at 25°C. Animals that had a defecation cycle of < 68 seconds were kept for further phenotypic analysis. In this manner, I

screened a total of 2490 F2 animals, a number equivalent to ~1223 haploid genomes (Ellis and Horvitz 1991), and identified two suppressor mutations. The critical data are summarized in Table 1. The two mutants isolated were outcrossed with *clk-1(qm30)* males to determine the heritability. We found that *dsc-6(qm192)* is a recessive mutation (data not shown) and that *dsc-7(qm193)* is a semi-dominant mutation (Figure10).

### 2.3.2 Screen results

A total of 2490 F2 animals were examined and 40 mutants that had a defecation cycle length of <68 seconds were isolated in our genetic screen for defecation suppressors of *clk-1*. We analyzed the progeny (F3) of the 40 candidates identified and found that only five could be kept for further phenotypic and genetic study. In general, candidates were discarded for one of three reasons: candidate animals were sterile, a significant proportion of slow defecating F3 worms were recovered at 25°C, or F3 animals were unable to develop beyond the L3 stage (Table 2). The five candidates kept were outcrossed three times with *clk-1(qm30)* males to clean up the strain. For three of these candidates, fast and slow defecating worms were recovered at 25°C following the first or second outcross (Table 2). Since the heritability of the fast defecation phenotype was unclear, we discontinued our study of these strains. The frequency of suppressor isolation in our genetic screen was 1/1245.

### 2.3.3 The effect of the new suppressors on defecation

It has previously been found that *dsc-3(qm179)* and *dsc-4(qm182)*, which were originally isolated by their ability to suppress the slow defecation of *clk-1* mutants at 20°C, also suppressed defecation after a temperature shift to 25°C (Branicky et al. 2001). We carried out a genetic screen (that included a temperature shift to 25°C) to find similar suppressor mutations and isolated two new suppressors. As a first step, we examined the effect of the suppressors on the slow defecation of *clk-1* mutants at 20°C and 25°C. We found that *dsc-6(qm192)* and *dsc-7(qm193)* strongly suppress the slow defecation phenotype of *clk-1(qm30)* animals when grown at 20°C and shifted to 25°C (which is how they were isolated), as well as when grown and scored at 20°C (Figure 11).

Unlike *dsc-3(qm179)* and *dsc-4(qm182)*, which suppress the slow defecation of *clk-1(qm30)* animals more strongly at 25°C than at 20°C (Figure 5), *dsc-6(qm192)* and *dsc-7(qm193)* suppress the slow defecation of *clk-1* mutants equally at both temperatures (Figure 11). One possibility is that, although the new suppressor mutants suppress the lengthened defecation cycle of *clk-1(qm30)* mutants after a temperature shift to 25°C, they are unable to adjust their rate of defecation in response to a change in temperature (similar to *clk-1(qm30)* animals). To test the ability of the new suppressor strains to adjust their defecation cycle length after a temperature shift, animals were scored for defecation after a shift to 15°C. We found that when the suppressor mutants are shifted from 20°C to 15°C, the mean defecation cycle length is increased (Figure 11). This suggests that the new suppressor mutants are able to adjust their rate

of defecation in response to a change in temperature. Notably, the *dsc-6* mutation cannot suppress the lengthened defecation cycle of *clk-1* mutants after a temperature shift to 15°C, whereas the *dsc-7* mutation can. It has previously been found that both the *dsc-3* and the *dsc-4* mutation suppress the increased cycle length of *clk-1* animals moderately at 15°C (Figure 5) (Branicky et al 2001).

### 2.3.4 Isolation of the new suppressor mutations on a wild type background

*dsc-6(qm192)* and *dsc-7(qm193)* were re-isolated on a wild-type background to determine how the mutations affect defecation in the presence of *clk-1(+)* activity. Animals were grown at 20°C and scored for 3-5 consecutive defecation cycles each at 20°C and 25°C. Similar to *dsc-3(qm179)* and *dsc-4(qm182)* mutants (Branicky et al., 2001), we found that *dsc-6(qm192)* and *dsc-7(qm193)* mutants defecate faster than wild type at 20°C as well as after a temperature shift to 25°C (Figure 12). Interestingly, in the wild-type background, *dsc-6(qm192)* and *dsc-7(qm193)* decrease the length of the defecation cycle more at 25°C than at 20°C.

## 2.4 Discussion

I examined a total of 2490 mutagenized F2 animals and identified 2 new suppressors. For comparison, Branicky et al. (2001) screened 5421 F2 animals and identified 10 suppressor mutations, which correspond to 7 different complementation groups. Note that, the frequency of suppressor isolation in their screen was about 1/540. In view of that, we expected to isolate at least 5 new

suppressors; yet, we isolated only two. We reasoned that our low recovery of suppressors might be due to the design of our genetic screen. As a reminder, Branicky and colleagues screened for suppressors at 20°C and isolated two classes of suppressors: class I and class II. The goal of our study was to isolate class I suppressors only, so we screened for mutants at 25°C instead of 20°C. Consequently, we might have overlooked a number of class II suppressors in our genetic screen.

Interestingly, 42.5% of the candidates identified in our screen were sterile (Table 2.). Most of these animals had a defecation rate of about 35 seconds at 25°C (data not shown), which is faster than the wild-type at that temperature (Figure 4). One possible explanation for this finding is that these animals were mutant in germline proliferation (oogenesis mutants or mutants with altered sperm) as well.

Intriguingly, the class I suppressors identified in our screen are similar to, but not the same as *dsc-3(qm179)* and *dsc-4(qm182)*. It has previously been found, that *dsc-3(qm179)* and *dsc-4(qm182)* suppress the slow defecation of *clk-1(qm30)* animals more strongly at 25°C than at 20°C (Branicky et al., 2001). In fact, the *dsc-3* mutation can shorten the defecation cycle length of *clk-1* mutants to less than the wild-type length at 25°C (Branicky et al., 2001). In contrast, *dsc-6(qm192)* and *dsc-7(qm193)* suppress the slow defecation of *clk-1* mutants equally at both temperatures (Figure 11). This might be due to the design of our genetic screen, in which we score initially at 25°C and test later at 20°C (which is the reverse of what was done in the previous screen) (Branicky et al., 2001). At

the present time it is unclear why this would result in different types of temperature sensitivity in the isolated mutants. In Chapter 3, we describe in detail a complete phenotypic analysis of the new suppressor strains.

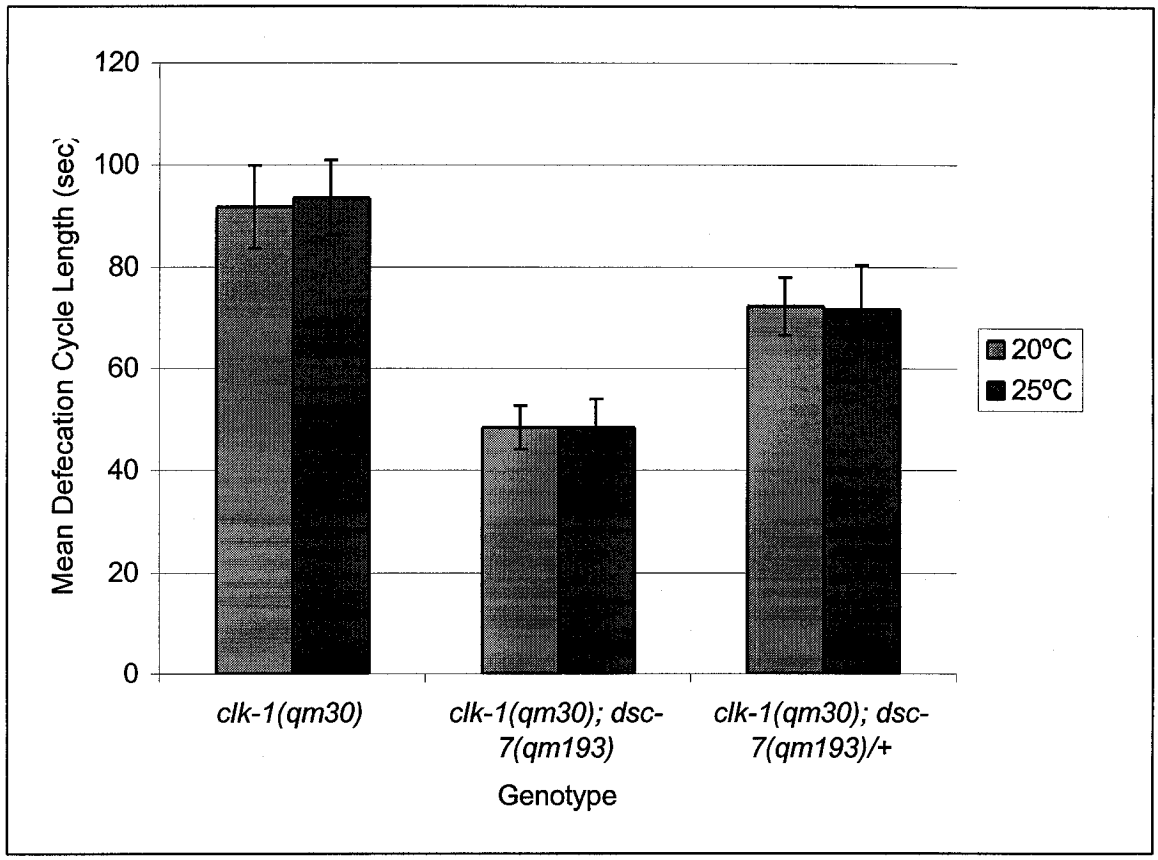


**Figure 10: The *dsc-7(qm193)* mutation has a semi-dominant effect in a *clk-1(qm30)* background.** Animals were grown at 20°C and scored at 20°C and 25°C. The bars represent the means of animals that had each been scored for three to five consecutive defecation cycles. The error bars correspond to the standard deviation of the means. For each genotype n = 15. The numerical values are as follows: for *clk-1(qm30)* at 20°C and 25°C respectively, 91.8 ± 8.2 and 93.5 ± 7.3; for *clk-1(qm30); dsc-7(qm193)* at 20°C and 25°C respectively, 48.5 ± 4.3 and 48.4 ± 5.5; for *clk-1(qm30); dsc-7(qm193)/+* at 20°C and 25°C respectively, 72.3 ± 5.7 and 71.7 ± 8.6.

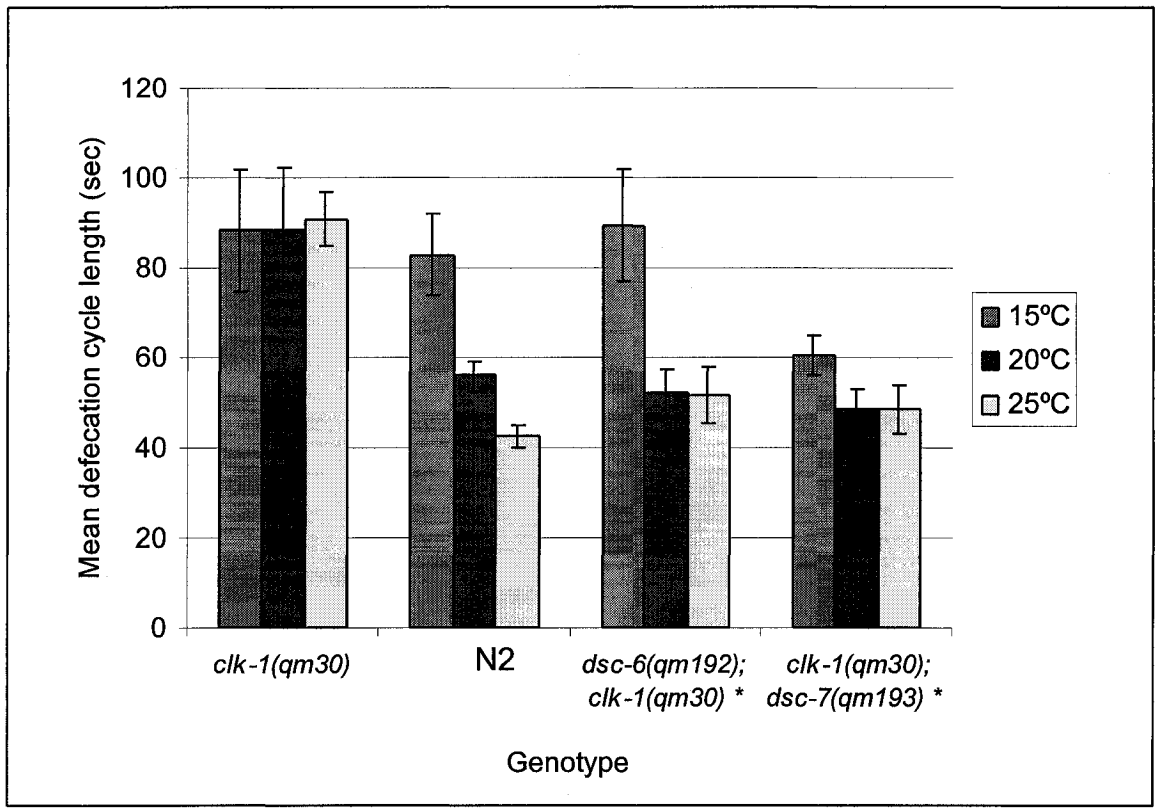


**Figure 11: The effect of temperature on the defecation cycle length of the new suppressor strains.** Animals were grown at 20°C and scored at 15°C, 20°C and 25°C. The bars represent the means of animals that had each been scored for five consecutive defecation cycles at 20°C and 25°C and for three consecutive defecation cycles at 15°C. The sample sizes are as follows: at 20°C, n = 25; at 25°C, n = 25; at 15 °C, n=15. The numerical values are as follows: for *clk-1(qm30)* at 15°C, 20°C and 25°C respectively, 90.7 ± 6.0, 88.3 ± 13.5 and 88.4 ± 13.8; for N2 at 15°C, 20°C and 25°C respectively, 82.8 ± 9.2, 56.1 ± 2.9 and 43.5 ± 4.3; for *dsc-6(qm192); clk-1(qm30)* at 15°C, 20°C and 25°C respectively, 89.3 ± 12.4, 52 ± 6.5 and 51.7 ± 3.11; for *clk-1(qm30); dsc-7(qm193)* at 15°C, 20°C and 25°C respectively, 60.25 ± 4.4, 48.5 ± 4.4 and 48.4 ± 5.5.

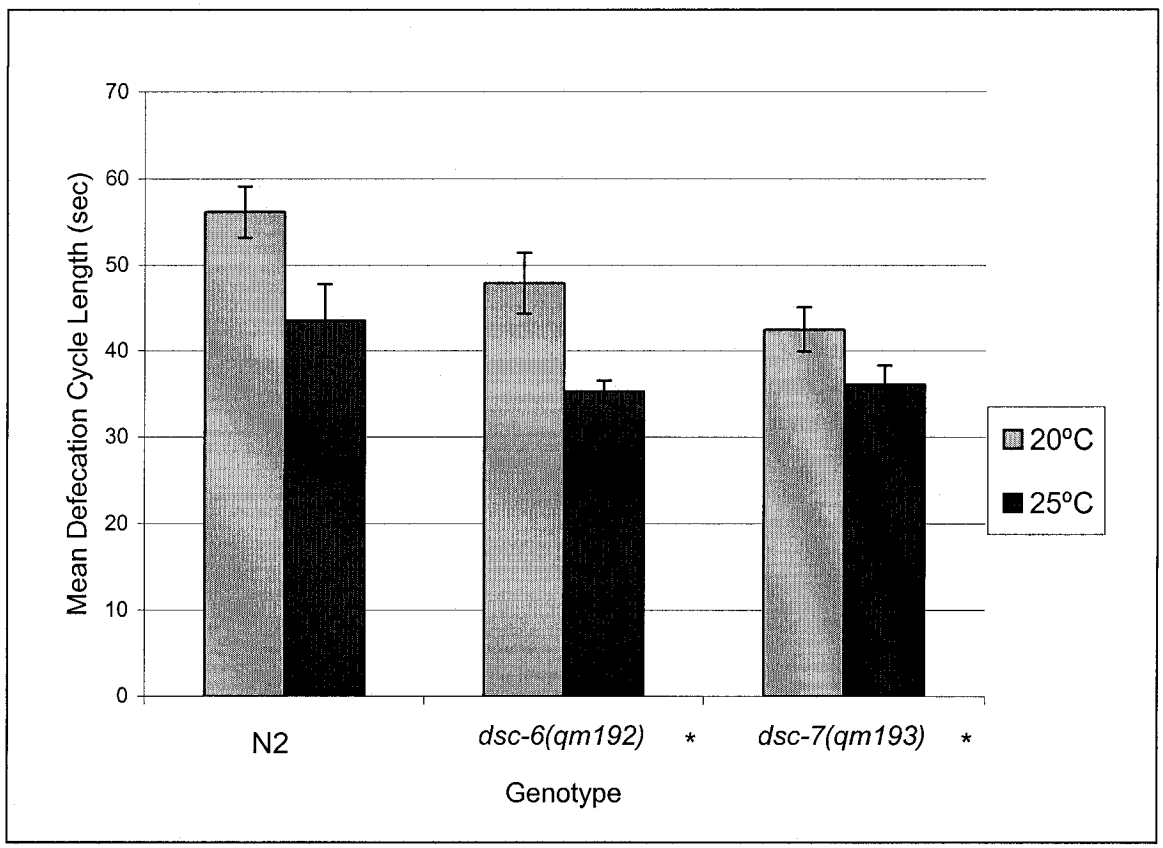


**Figure 12: The new suppressor mutations on a wild-type background.** Strains were grown at 20°C and scored at either 20°C or 25°C. The bars represent the means of animals that had each been scored for 5 consecutive defecation cycles at 20°C and 25°C. The error bars correspond to the standard deviation of the means. n = 25 at both temperatures. The numerical values are as follows: for N2 at 20°C and 25°C respectively, 56.1 ± 2.9 and 43.5 ± 4.3; for *dsc-6(qm192)* at 20°C and 25°C respectively, 47.9 ± 3.6 and 35.2 ± 1.3; for *dsc-7(qm193)* at 20°C and 25°C respectively, 42.5 ± 2.6 and 36.1 ± 2.1.

* Suppressor mutations had a significant effect on the defecation cycle length of *clk-1(+)* animals at 20°C and 25°C ($P < 0.05$).

| | |
|---|---|
| Rounds of mutagenesis | 20 |
| Number of F2 animals screened | 2490 |
| Number of candidates isolated | 40 |
| Number of mutants isolated | 2 |

**Table 1: Summary of screen results.**

| | |
|---|---|
| Candidate animals were sterile | 42.5% |
| A significant proportion of slow defecating F3 worms were recovered at 25°C | 40% |
| F3 animals were unable to develop beyond the L2/L3 stage | 5% |
| Fast and slow defecating worms were recovered at 25°C following the first or second outcross | 7.5% |

**Table 2: Reasons for eliminating candidate worms from our study.**

# Chapter 3 – Phenotypic analysis of suppressor mutants

## 3.1 Introduction

As stated in Chapter 1, *dsc-4(qm182)* was isolated as a suppressor of the slow defecation phenotype of *clk-1* mutants (Branicky et al., 2001). Close examination of the *dsc-4* mutation revealed that it can suppress other *clk-1* phenotypes but not all (Shibata et al., 2003). In view of that, we examined the effect of *dsc-6(qm192)* and *dsc-7(qm193)* on other physiological processes slowed down and deregulated in *clk-1* mutants, such as growth, germline development and ageing. The phenotype of the other previously identified class I *dsc* mutation, *dsc-3(qm179)*, has not been examined in detail. Consequently, we analyzed *dsc-3* mutants as well. Part of our phenotypic analysis was to determine if all class I suppressors are alike.

## 3.2 Materials and Methods

### 3.2.1 Nematode strains and cultivation methods

Animals were cultured on nematode growth medium (NGM) agar plates, and were fed the *E. coli* strain OP50 (Brenner 1974). The N2 (Bristol) strain was used as wild type. All animals were cultured at 20 °C unless otherwise stated. Genetic strains analyzed in the study include: **MQ1247**: *dsc-6(qm192)* II, **MQ99**: *clk-1(qm30)* III, **MQ914**: *dsc-3(qm179)* IV, **MQ920**: *dsc-4(qm182)* IV, **MQ1248**: *dsc-7(qm193)* V, **MQ1229**: *dsc-6(qm192)* II; *clk-1(qm30)* III, **MQ1225**: *clk-*

*1(qm30)* III; *dsc-3(qm179)* IV, **MQ891**: *clk-1(qm30)* III; *dsc-4(qm182)* IV, **MQ1232**: *clk-1(qm30)* I; *dsc-7(qm193)* V.

### 3.2.2 Rate of defecation

Described in Chapter 2. All animals were scored at 25°C unless otherwise mentioned.

### 3.2.3 Statistical analysis

Described in Chapter 2.

### 3.2.4 Time course analysis of egg laying rate

L4 animals were picked to 60-mm plates and examined three hours later. Fifteen animals that had molted to adults during this time interval were selected for the experiment and were considered to be 1.5 hours old (at the end of the interval). Animals were singled 24, 48, 72 and 96 hours later and allowed to lay eggs for three hours. The average number of eggs laid per hour per worm following each time interval was calculated.

### 3.2.5 Rate of embryonic development

Two-cell embryos were dissected out of gravid hermaphrodites and picked to a clean plate. Embryos were examined at hourly intervals, from 14-16 hours after dissection, until all embryos had hatched.

### 3.2.6 Rate of post-embryonic development

Eggs were picked to a clean plate and examined three hours later. 50 animals that had hatched during this time interval were chosen for the experiment. Worms were examined at hourly intervals and the percentage of animals that had reached adulthood by each time point was noted.

### 3.2.7 Developmental stage of the germline

L4 animals were picked to 60-mm plates and examined three hours later. Ten animals that had molted to adults during this time interval were selected for the experiment and were considered to be 1.5 hours old (at the end of the interval). The posterior and anterior germline of each animal was examined using DIC microscopy subsequent to the three-hour time interval.

### 3.2.8 Brood size

Twelve L4 animals were singled and transferred to new plates daily (over a period of 3 days). The total number of progeny produced by each worm was determined and the mean brood size for each genotype was calculated.

### 3.2.9 Lifespan analysis

Thirty gravid adults were picked to a fresh plate and allowed to lay eggs for 6 hours. The eggs were left to hatch overnight at 20°C. Five groups of ten larvae that had hatched during the night were picked to 60-mm petri dishes and examined daily until death. Animals were considered dead when they no longer

reacted to a gentle touch on the tail or head. Note that, the animals were transferred to new plates each day during the period of egg laying, to keep them separate from their brood.

### 3.2.10 Rate of defecation on NGM+C

NGM plates were prepared with extra cholesterol (NGM+C: 10μg/ml, 25 μg/ml and 50 μg/ml). Worms were cultured on the NGM+C plates for one generation before the rate of defecation was examined. As a control, the defecation rate for each genotype was also examined on standard NGM plates (0.5 μg/ml).

### 3.2.11 Percent larval arrest on NGM-C

NGM plates were prepared with a reduced amount of cholesterol (NGM-C: 0.50 μg/ml). Worms were cultured on the NGM-C plates for two generations. 20 F1 animals were picked to a clean NGM-C plate and allowed to lay eggs for 6 hours. Animals were examined 2-5 days later, and the percent larval arrest was calculated. As a control, the percent larval arrest was also calculated on standard NGM plates (0.5 μg/ml).

## 3.3 Results

### 3.3.1 *dsc-6(qm192)* and *dsc-7(qm193)* partially suppress the slow embryonic development of *clk-1* mutants

To score the effect of the new suppressors on the rate of embryonic development of *clk-1* mutants, two-cell embryos were dissected out of gravid hermaphrodites, picked to a clean plate and observed at hourly intervals, until hatching. We found that the new suppressor mutants have a rate of embryonic development much faster than *clk-1(qm30)* worms (Figure 13). For example, *clk-1(qm30)* embryos start hatching before 13 hours and finish hatching by 29 hours whereas, *dsc-6(qm192)*; *clk-1(qm30)* embryos start hatching before 12 hours and finish hatching 19 hours after dissection. For comparison, Y. Shibata (unpublished observations) previously found that *clk-1(qm30); dsc-4(qm182)* embryos start hatching before 15 hours and finish hatching by 24 hours. In contrast to *dsc-6(qm192)* and *dsc-7(qm193)*, we found that the *dsc-3* mutation does not suppress the lengthened embryonic development of *clk-1* mutants (Figure 13).

### 3.3.2 The lengthened post-embryonic development of *clk-1* mutants is partially suppressed by *dsc-7(qm193)*

To study the effect of the new suppressors and *dsc-3(qm179)* on the rate of post-embryonic development of *clk-1* mutants, L1 staged animals were observed at hourly intervals and the percentage of animals that had reached

adulthood by each time point was scored. Yukimasa Shibata (unpublished observations) previously found that the *dsc-4* mutation does not suppress the slow post-embryonic development of *clk-1(qm30)* animals. In contrast to the *clk-1(qm30); dsc-4(qm182)* strain, we found that the *dsc-7* suppressor strain has a rate of post-embryonic development that is faster than the *clk-1(qm30)* strain (Figure 14). To illustrate, 100% of *clk-1(qm30); dsc-7(qm193)* animals reach adulthood by 69 hours, whereas 100% of *clk-1(qm30)* animals reach adulthood by 86 hours. Interestingly, *dsc-6(qm192); clk-1(qm30)* animals have a slower rate of post-embryonic development than *clk-1* mutants while *clk-1(qm30); dsc-3(qm179)* animals have a similar rate (Figure 14).

### 3.3.3 The effect of *dsc-6(qm192)* and *dsc-7(qm193)* on the egg-laying rate of *clk-1* mutants

As stated in Chapter 1, it has previously been found that *clk-1(qm30)* affects the rate of germline development (Shibata et al., 2003). In particular, the switch from sperm production to oocyte production is delayed in *clk-1* mutants. Given that neither oocytes nor eggs can be produced before the end of spermatogenesis, we can examine the delayed oogenesis of *clk-1* mutants by counting the number of eggs laid per hour after the worms have molted into young adults. Wild type animals reach their peak of egg laying at approximately 36 hours, whereas *clk-1(qm30)* animals reach their peak rate at 48 hours. The *dsc-6* mutation suppresses this delay as the peak egg-laying rate of *dsc-6; clk-1* double mutants is reached by 36 hours (Figure 15). Seeing as the delayed egg

laying of *clk-1* mutants is due to their delayed production of oocytes, this result suggests that the *dsc-6* mutation suppresses the delayed oogenesis of *clk-1* mutants. Unlike the *dsc-6* mutation, the *dsc-7* mutation does not suppress the delayed egg laying of *clk-1* mutants (Figure 15). Yet, at 24 hours *clk-1; dsc-7* double mutants lay a considerably greater number of eggs than *clk-1(qm30)* animals. One possible explanation for this finding is that *dsc-7(qm193)* weakly suppresses the delayed germline development of *clk-1* mutants.

### 3.3.4 *dsc-6(qm192)* and *dsc-7(qm193)* suppress the slow germline development of *clk-1* mutants

Next we examined the development of the posterior and anterior germline of N2 and *clk-1* mutants using DIC microscopy. At 1.5 hours after the adult molt, we found that over 30% of wild-type worms examined had oocytes at the proximal end of the anterior and posterior germline. In comparison, we found that the onset of oogenesis is considerably delayed in *clk-1* mutants, in which 100% of the anterior gonads and 91% of the posterior gonads examined (in 1.5 hour old adults) were still undergoing spermatogenesis. To more directly study the effect of the new suppressor mutations and *dsc-3(qm179)* on the development of the *clk-1* germline, we used DIC microscopy to examine the germline of the *clk-1(qm30); dsc* double mutants (Figure 16). Consistent with the earlier production of eggs (Figure 15), we found that at 1.5 hours after the adult molt, 95% of *clk-1; dsc-6* double mutants examined had oocytes at the proximal end of the anterior germline. As expected, we found that the *dsc-7* mutation also suppresses the

slow germline phenotype of *clk-1* mutants. To illustrate, 58% of the anterior gonads and 50% of the posterior gonads examined in 1.5 hour old adults were undergoing oogenesis. Furthermore, we found that the *dsc-3* mutation does not suppress the slow germline development of *clk-1* mutants. For example, 1.5 hours after the adult molt, just 12% of *clk-1; dsc-3* double mutants examined had oocytes at the proximal end of the posterior gonad, compared to 9% of *clk-1* mutants (Figure 16).

### 3.3.5 Brood size

We found that the new suppressor mutations do not have a significant effect on the low brood size phenotype of *clk-1* mutants ($P>0.05$). The numerical values are as follows: for N2, 302 ± 38 animals; for *clk-1(qm30)*, 187 ± 42 animals; for *dsc-6(qm192); clk-1(qm30)*, 204 ± 37 animals; for *clk-1(qm30); dsc-7(qm193)*, 208 ± 25 animals.

### 3.3.6 *dsc-6(qm192)* suppresses the long life span of *clk-1* mutants

We also examined the effect of the new suppressors on the increased lifespan of *clk-1* mutants. In our study, we found that *dsc-7(qm193)* cannot suppress the lifespan phenotype of *clk-1(qm30)* animals, similar to *dsc-3(qm179)* and *dsc-4(qm182)* (Y. Shibata, unpublished observations) (Figure 17). In contrast to the other class I suppressors, we found that the *dsc-6* mutation can restore the extended life span of *clk-1(qm30)* worms to wild type, as *dsc-6; clk-1* double mutants have a mean life span of 17.1 ± 3.8 days (Figures 17). For comparison,

*clk-1* mutants have a mean life span of 22.1 ± 7.6 days (Figure 17). Additionally, we found that *dsc-6(qm192)* does not have an effect on the life span of N2 (19.5 ± 14.3 days) (Figure 18). This is evidence that the action of *dsc-6* is specific to *clk-1* mutants. So far, *dsc-6(qm192)* is the only suppressor identified capable of reducing the extended lifespan of *clk-1* mutants. Thus, determining the molecular identity of *dsc-6* might provide further insight into the molecular mechanism(s) responsible for the longevity of *clk-1(qm30)* worms.

### 3.3.7 The effect of high cholesterol on the defecation rate of class I suppressors

Seeing as *dsc-4* encodes a protein similar to the large subunit of MTP (Shibata et al. 2003), and cholesterol is a major component of lipoproteins we reasoned that adding more cholesterol to the NGM might increase the defecation cycle length of *clk-1; dsc-4* double mutants. L4 animals were grown on NGM plates prepared with extra cholesterol and F1 animals were scored at 25°C for defecation. As predicted, we found that the cholesterol treatment altered the defecation rate of *clk-1(qm30); dsc-4(qm182)* animals (Figure 19). To illustrate, *clk-1; dsc-4* double mutants have a mean defecation cycle length of 71.3 ± 13 seconds when grown and scored on NGM plates containing 15µg/ml of cholesterol. For comparison, the mean defecation rate of *clk-1(qm30); dsc-4(qm182)* animals grown and scored on standard NGM (5 µg/ml of cholesterol) is 48.6 ± 5 seconds (Figure 19).

As a next step, we examined the effect of high cholesterol on the defecation rate of the new suppressor strains. As we had hoped, we found that the cholesterol treatment increased the defecation cycle length of *dsc-6(qm192); clk-1(qm30)* and *clk-1(qm30); dsc-7(qm193)* animals, similar to *clk-1(qm30); dsc-4(qm182)* animals (Figure 19). For example, *dsc-6; clk-1* double mutants have a mean defecation cycle length of 64.0 ± 7.6 seconds when grown and scored on NGM plates containing 15µg/ml of cholesterol (Figure 19). To compare, the mean defecation rate of *dsc-6(qm192); clk-1(qm30)* animals grown and scored on standard NGM is 51.5 ± 3 seconds (Figure 19). Based on our observations, we reasoned that *dsc-6* and *dsc-7* might encode proteins required for cellular lipoprotein trafficking in the worm, similar to *dsc-4*.

In contrast to the other *clk-1(qm30); dsc* double mutants, we found that adding extra cholesterol to the NGM did not alter the defecation rate of *clk-1(qm30); dsc-3(qm179)* animals (Figure 19). This observation is consistent with the molecular function of *dsc-3*. It has previously been found that *dsc-3* encodes a protein required for the uptake of cholesterol (R. Branicky, unpublished observations). Consequently, increasing the amount of exogenous cholesterol will not improve or improve only very little the inability of *clk-1; dsc-3* double mutants to absorb adequate amounts of cholesterol from their surroundings.

As a control, we examined the effect of high cholesterol on the defecation cycle length of *clk-1* mutants. We found that adding extra cholesterol to the NGM altered the defecation rate of *clk-1(qm30)* animals, similar to *clk-1(qm30); dsc-4(qm182)*, *dsc-6(qm192); clk-1(qm30)* and *clk-1(qm30); dsc-7(qm193)* animals

(Figure 19). In an attempt to clarify our results, we next examined the effect of high cholesterol on the defecation cycle length of *dsc-3(qm179)*, *dsc-4(qm182)*, *dsc-6(qm192)* and *dsc-7(qm193)* animals (Figure 20). Surprisingly, we found that the cholesterol treatment altered the defecation rate of *dsc-3* mutants only. To illustrate, *dsc-3(qm179)* animals have a mean defecation cycle length of 41.2 ± 3 seconds when grown and scored on NGM plates containing 15µg/ml of cholesterol (Figure 20). For comparison, the mean defecation rate of *dsc-3* mutants grown and scored on standard NGM is 34.7 ± 1 seconds (Figure 20). Additionally, we found that the cholesterol treatment altered the defecation rate of N2. At this time, there is no clear explanation for these results. Variation in the scoring and in the scoring temperature (due to a difference in time and location) may possibly account for the unexpected data obtained in our study.

### 3.3.8 The effect of low cholesterol on *clk-1(qm30); dsc* double mutants

We next examined the effect of reduced levels of cholesterol on all *clk-1(qm30); dsc* double mutant strains and all *dsc* wild type strains. While all strains grow more slowly on plates containing 0.5ug/ml of cholesterol when compared to their growth on standard NGM (5 ug/ml), only the *dsc-3* strains were hyper sensitive to a reduced amount of cholesterol (Figure 21). Again, this finding is consistent with the molecular function of *dsc-3*. To reiterate, *dsc-3* encodes a protein required for the uptake of cholesterol (R. Branicky, unpublished observations). Consequently, reducing the amount of exogenous cholesterol will further impair the ability of *dsc-3* mutants to absorb cholesterol from their

surroundings. It has previously been shown that worms require sterol (usually supplied as cholesterol) and sterol deprivation can result in arrested growth as larvae. In view of that, hypersensitivity was measured by determining the percent of larval arrest (Figure 21). As a control, the percent larval arrest was calculated for each genotype on standard NGM (0.5 µg/ml) plates.

### 3.4 Discussion

It has previously been found that the *dsc-4* mutation strongly suppresses the long defecation cycle of *clk-1* mutants at 20°C as well as after a temperature shift to 25°C (Branicky et al 2001). Closer examination of the *dsc-4* mutation revealed that it can suppress other aspects of the *clk-1* phenotype (such as the slow embryonic development and slow germline development of *clk-1(qm30)* mutants) but not all (i.e. the *dsc-4* mutation does not suppress the slow post-embryonic development or increased lifespan of *clk-1(qm30)* animals) (Shibata et al., 2003). As discussed in Chapter 2, we carried out a genetic screen to isolate similar defecation suppressors of *clk-1* and identified two new suppressors: *dsc-6(qm192)* and *dsc-7(qm193)*. We analyzed the phenotype of the new suppressor mutants in a number of different ways to see if *dsc-6(qm192)* and *dsc-7(qm193)* could also suppress other aspects of the *clk-1* phenotype. The results are summarized in Table 3.

In our phenotypic study of the new suppressor strains, we found that *dsc-6(qm192)* and *dsc-7(qm193)* strongly suppress the slow embryonic and slow germline development of *clk-1* mutants (Figure 13 and 16), similar to *dsc-*

*4(qm182)*. Additionally, we found that the *dsc-6* mutation can suppress the increased lifespan of *clk-1* mutants (Figure 17) and that the *dsc-7* mutation can moderately suppress the lengthened postembryonic development of *clk-1* mutants (Figure 14). Note that, the *dsc-4* mutation cannot suppress either of these *clk-1* phenotypes (Y. Shibata, unpublished observations) (Table 3). In contrast to the other class I suppressors, our phenotypic analysis of the *clk-1; dsc-3* double mutant strain revealed that the *dsc-3* mutation can only suppress the defecation phenotypes of *clk-1* mutants (Table 3).

It has previously been found that *dsc-3* encodes a protein required for the uptake of cholesterol (R. Branicky, unpublished observations). Consistent with a role in cholesterol uptake, we found that *clk-1; dsc-3* double mutants are hypersensitive to a reduced amount of cholesterol in the NGM (Figure 21). As stated in Chapter 1, Shibata et al. (2003) recently cloned *dsc-4* and found that it encodes the large subunit of MTP. Consistent with a role in lipoprotein assembly and secretion, we found that adding extra cholesterol to the NGM can rescue the fast defecation phenotype of *clk-1; dsc-4* double mutants (Figure 19). Intriguingly, adding extra cholesterol to the NGM can also rescue the fast defecation phenotype of *dsc-6; clk-1* and *clk-1; dsc-7* double mutants (Figure 19). For that reason, we believe that *dsc-6* and *dsc-7* might also encode activities required for lipid transport in the worm.

Additionally, we have examined the rate of defecation and germline development of *dsc-6(qm192); clk-1(qm30); dsc-4(qm182)* animals. We found that *dsc-6; clk-1; dsc-4* triple mutants exhibit a mean defecation cycle length

similar to *clk-1; dsc-4* and *dsc-6; clk-1* double mutants at 20°C as well as after a temperature shift to 25°C (Figure 22). As for germline development, we found that the effects of *dsc-4(qm182)* and *dsc-6(qm192)* on the *clk-1(qm30)* background are not additive (data not shown). Moreover, It was previously found that the effects of *dsc-3(qm179)* and *dsc-4(qm182)* on the defecation rate of *clk-1* mutants are not additive (Y. Shibata, unpublished observations). These findings provide further evidence that the class I *dsc* suppressors are acting in the same or a similar cellular pathway. Note that, we had difficulty mapping the *dsc-7* mutation. Consequently, we could not construct a *clk-1, dsc-4; dsc-7* triple mutant strain to phenotypically examine.

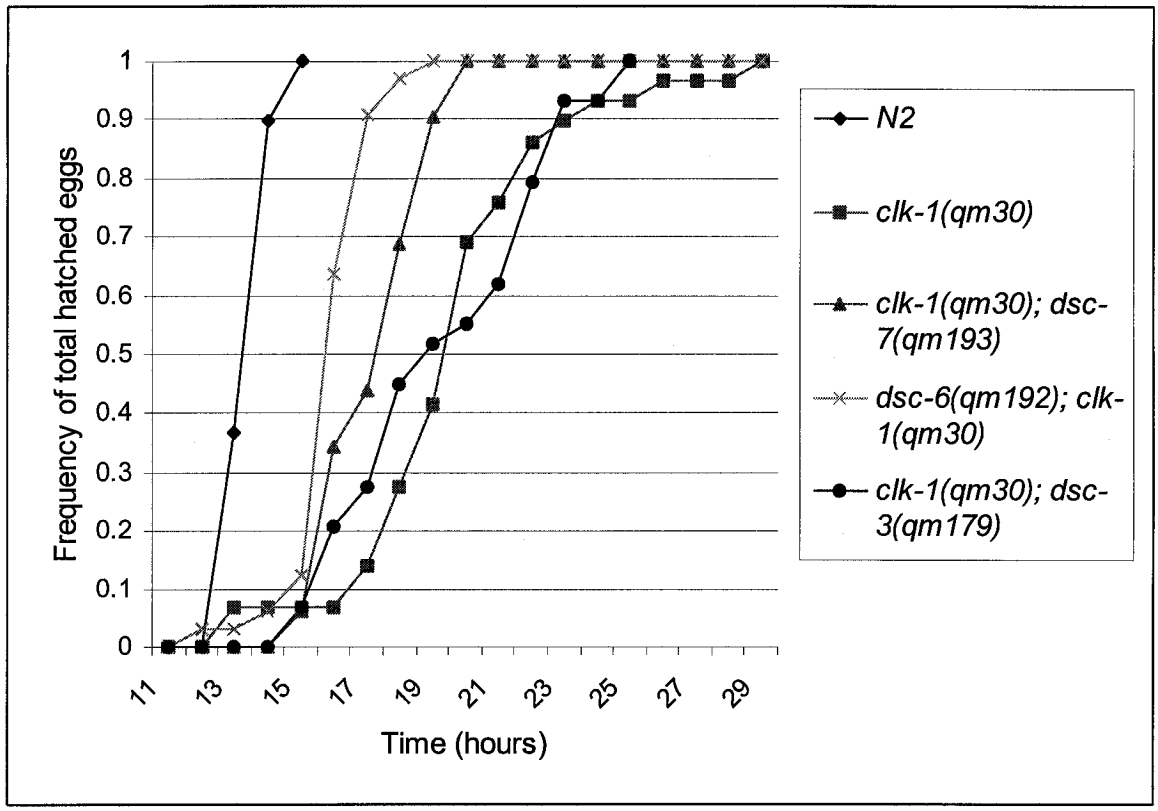


**Figure 13: *dsc-6(qm192)* and *dsc-7(qm193)* suppress the lengthened embryonic development of *clk-1(qm30)* mutants.** Two-celled embryos were dissected out of gravid hermaphrodites and picked to a clean plate. Embryos were examined at hourly intervals, from 12-14 hours after dissection, until all embryos had hatched. The sample sizes are as follows: for N2, n = 49; for *clk-1(qm30)*, n = 29; for *clk-1(qm30); dsc-7(qm193)*, n = 32; for *dsc-6(qm192); clk-1(qm30)*, n = 33; for *clk-1(qm30); dsc-3(qm179)*, n = 35. The mean and standard error for each genotype is as follows: for N2, 13.73 ± 0.638; for *clk-1(qm30)*, 19.93 ± 3.229; for *clk-1(qm30); dsc-7(qm193)*, 17.56 ± 1.501; for *dsc-6(qm192); clk-1(qm30)*, 16.24 ± 1.12; for *clk-1(qm30); dsc-3(qm179)*, 19.7 ± 3.0.

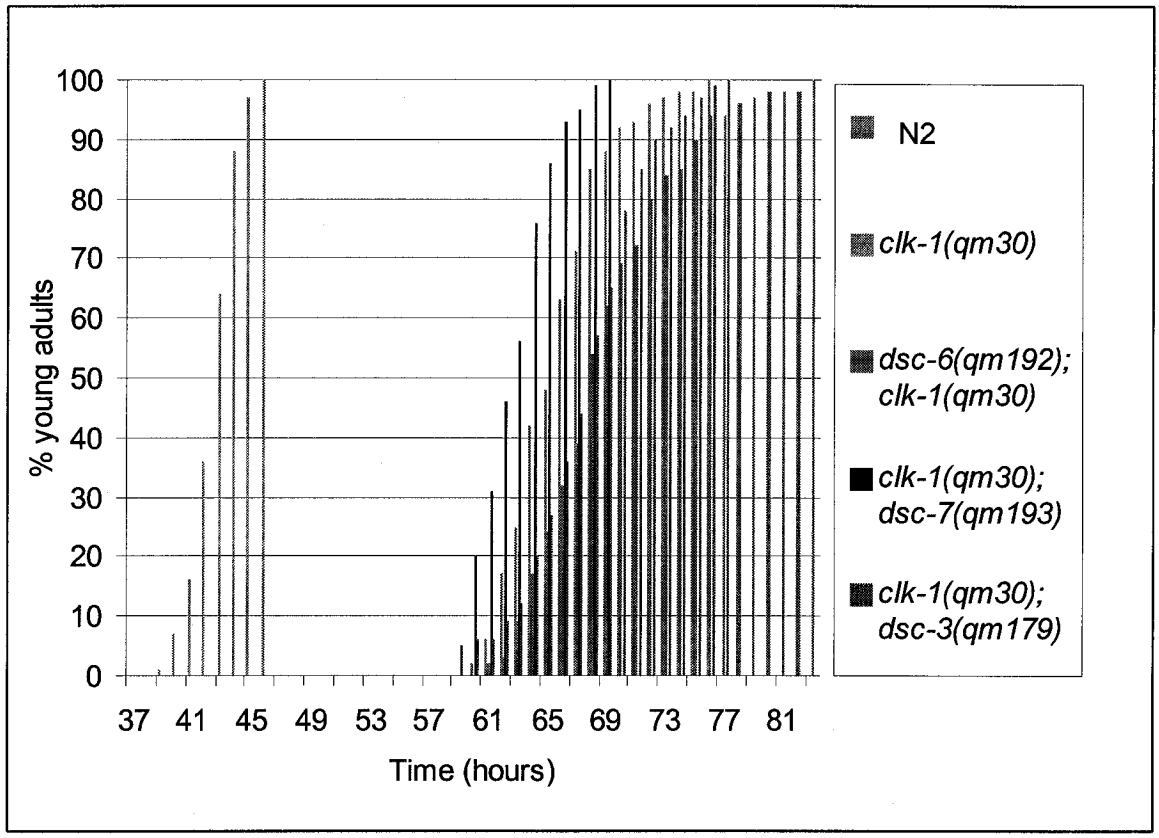


**Figure 14: The *dsc-7* mutation partially suppresses the slow post-embryonic development of *clk-1* mutants.** Staged L1s were examined at hourly intervals and the percentage of animals that had reached adulthood by each time point was noted. The sample sizes are as follows: for N2, n = 107; for *clk-1(qm30)*, n = 89; for *clk-1(qm30); dsc-3(qm179)*, n = 97; for *dsc-6(qm192); clk-1(qm30)*, n = 120; for *clk-1(qm30); dsc-7(qm193)*, n = 100.

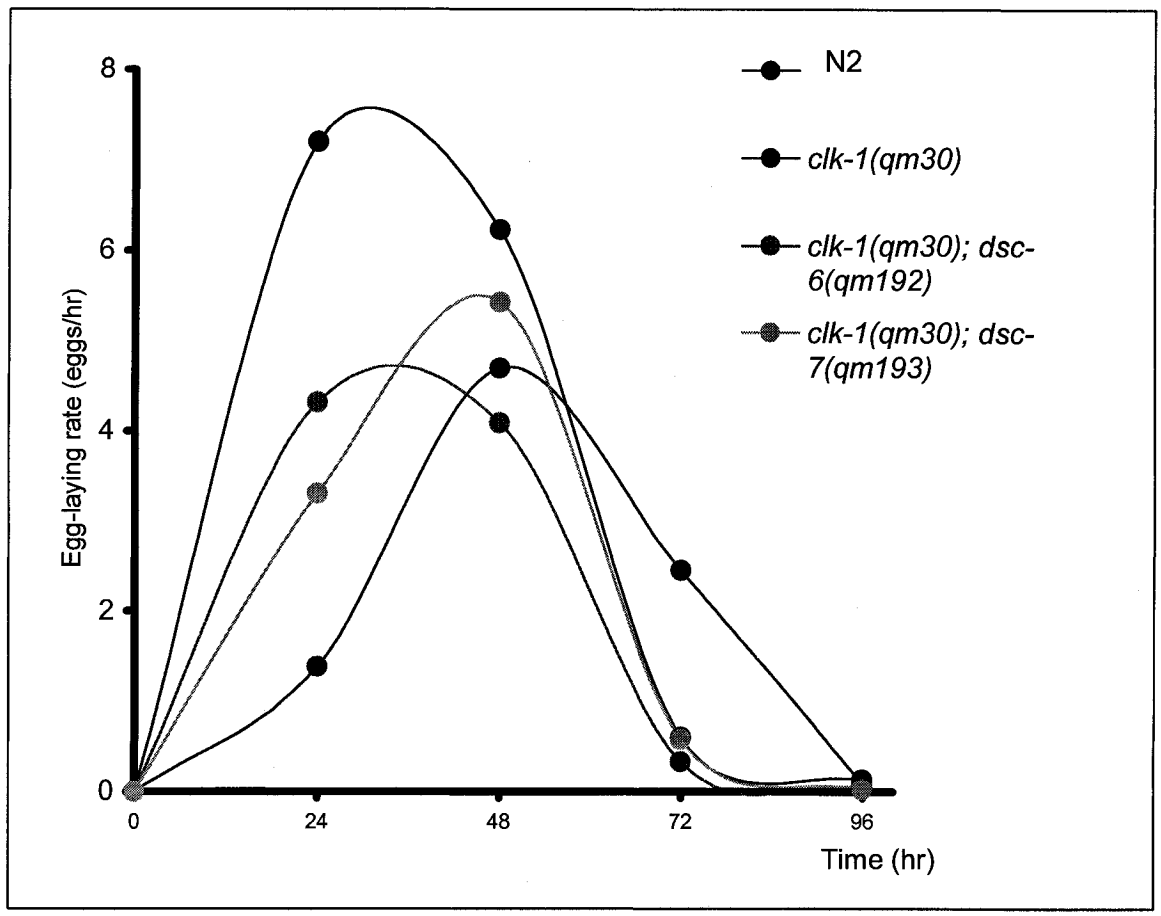


**Figure 15: The effect of *dsc-6(qm192)* and *dsc-7(qm193)* on the egg-laying rate of *clk-1* mutants.** Wild type animals reach their peak of egg laying at approximately 36 hours, whereas *clk-1(qm30)* animals reach their peak at 48 hours. *dsc-6(qm192)* suppresses this delay by slightly more than 12 hours. In contrast, *dsc-7(qm193)* cannot suppress the delayed egg laying of *clk-1* mutants. For each genotype n=15.

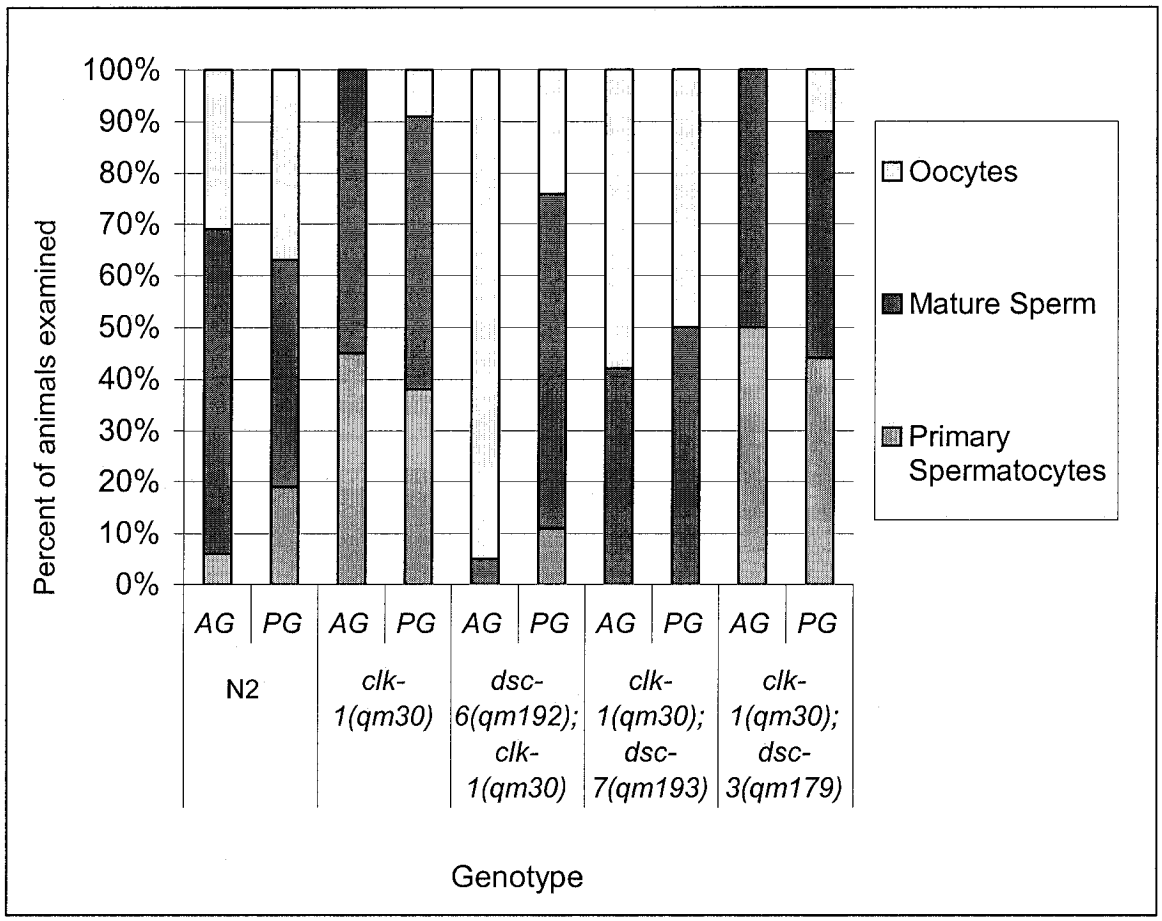


**Figure 16: Germline development 1.5 hours after the adult molt.** The percent of animals with oocytes, sperm or spermatocytes at the proximal end of the anterior and posterior germline is shown. For each genotype n≥30. 1.5 hours after the adult molt, more than 30% of wild-type animals are in the process of oogenesis. In contrast, most of the *clk-1* mutants are still undergoing spermatogenesis. The *dsc-6* and *dsc-7* mutations suppress the slow germline development of *clk-1* mutants. In contrast, the *dsc-3* mutation cannot suppress the slow germline development of *clk-1* mutants.

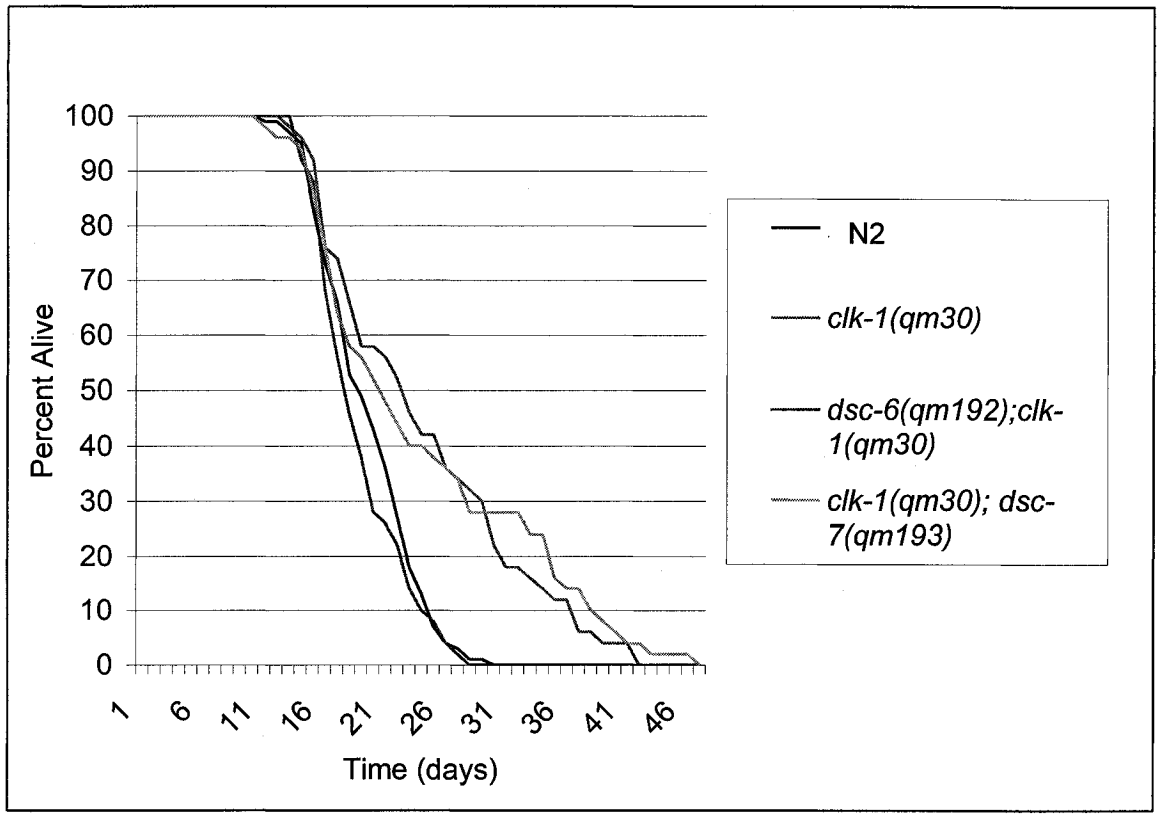


**Figure 17: Death curves for the new suppressor strains.** *dsc-6; clk-1* double mutants live substantially shorter than *clk-1* mutants. The mean life span and standard error for each genotype is as follows: N2, 19.5 ± 4.3; for *clk-1(qm30)*, 22.1 ± 7.6; for *clk-1(qm30); dsc-7(qm193)*, 23.5 ± 9.9; for *dsc-6(qm192); clk-1(qm30)*, 17.1 ± 3.8. The sample sizes are as follows: for N2, n = 200; for *clk-1(qm30)*, n = 100; for *clk-1(qm30); dsc-7(qm193)* n = 100; for *dsc-6(qm192); clk-1(qm30)*, n = 100.

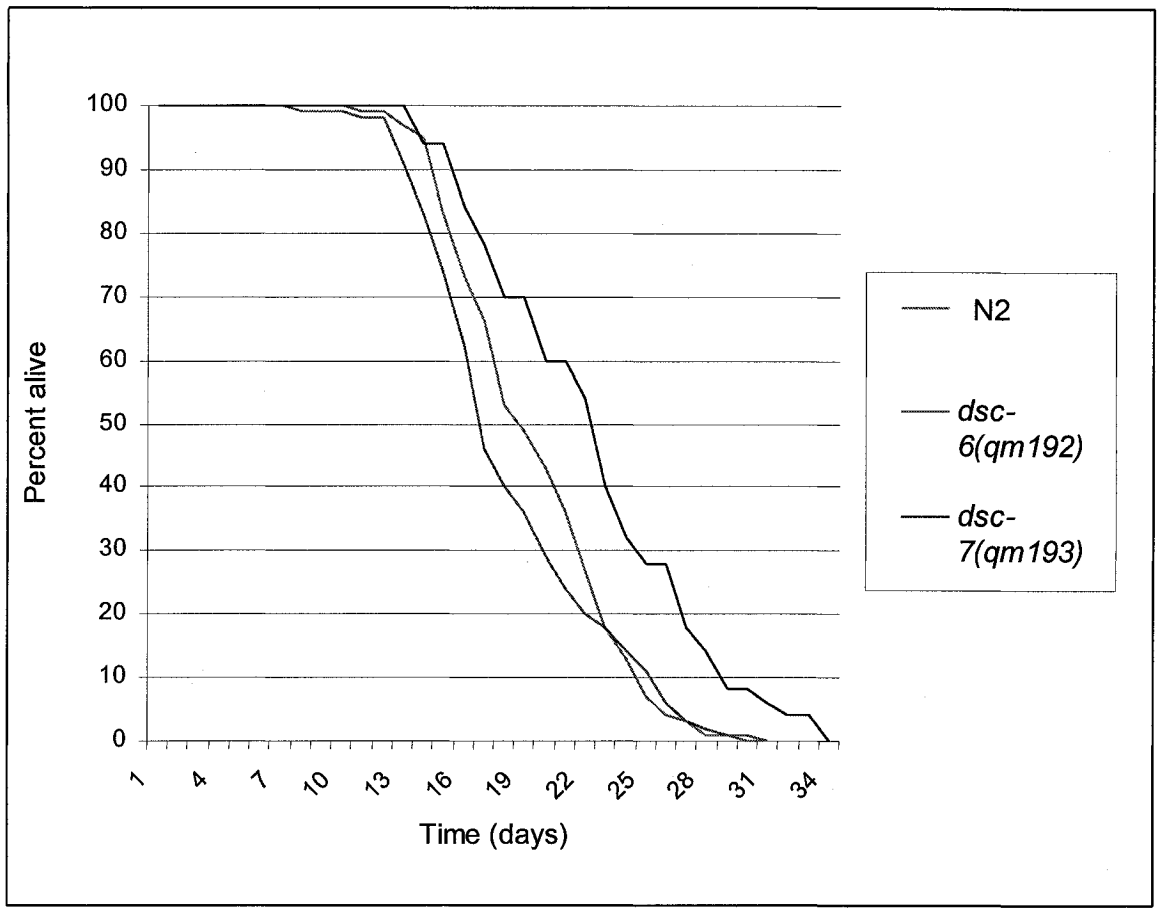


**Figure 18: Death curves for N2 and the *dsc* wild-type strains.** *dsc-6* mutants do not live shorter than N2. The mean life span and standard error for each genotype is as follows: for N2, 19.5 ± 4.3; for *dsc-6(qm192)*, 18.1 ± 4.5; for *dsc-7(qm193)*, 19.7 ± 5.9.The sample sizes are as follows: for N2, n = 200; for *dsc-6(qm192)*, n = 200; for *dsc-7(qm193)* n =100.

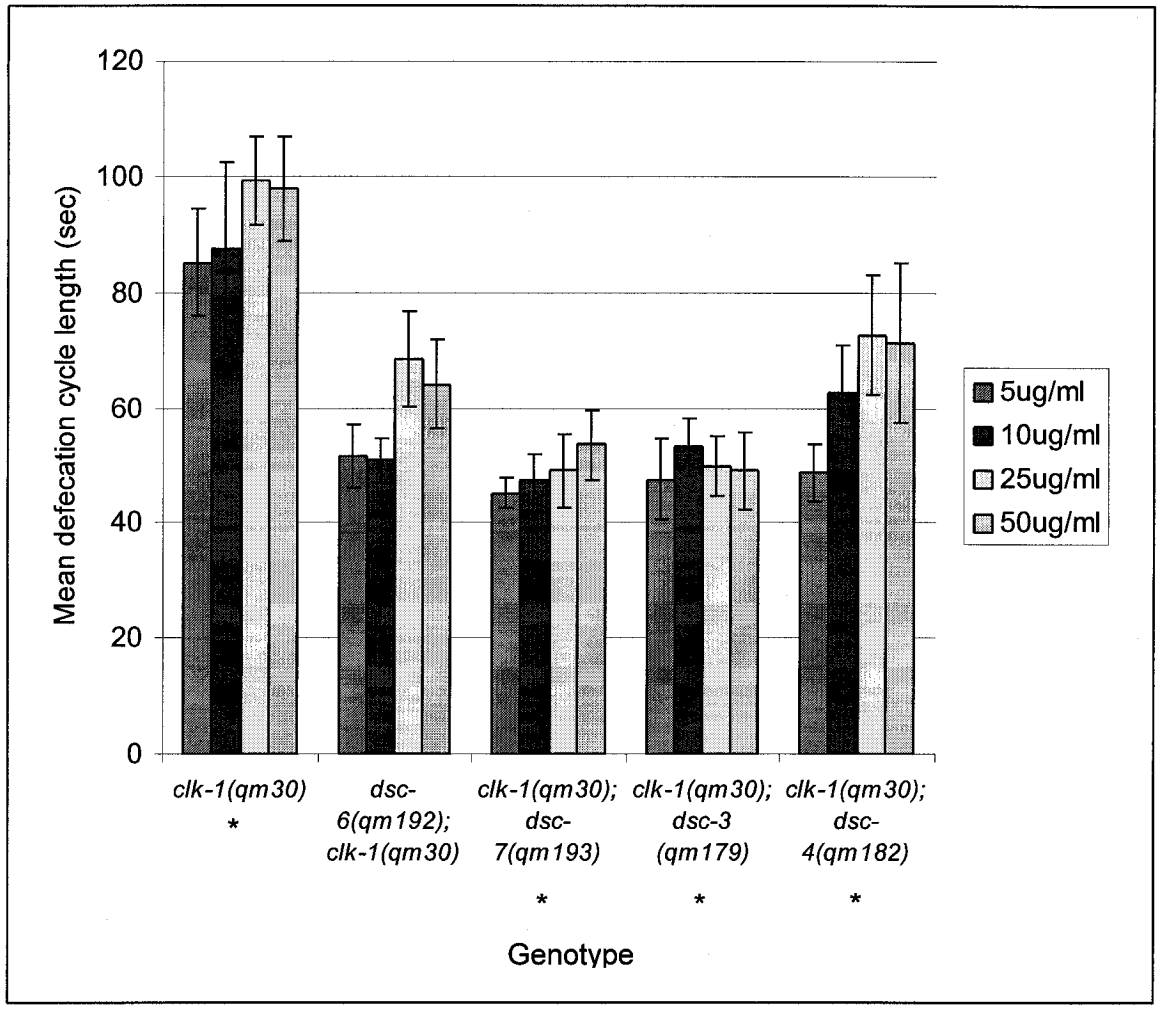


**Figure 19: The effect of high cholesterol on the defecation rate of *clk-1(qm30); dsc* double mutants.** Adding extra cholesterol to the NGM rescues the fast defecation phenotype of *clk-1; dsc-4*, *dsc-6; clk-1* and *clk-1; dsc-7* double mutants. For each genotype n = 15. Each animal was scored for three consecutive defecation cycles at 25°C following a two hour temperature shift to that temperature. We tested whether 50 µg/ml of cholesterol had a significant effect on defecation by comparing the rate of defecation on 50 µg/m plates with the rate of defecation on 5µg/ml plates. * indicates a significant effect ($P < 0.05$).

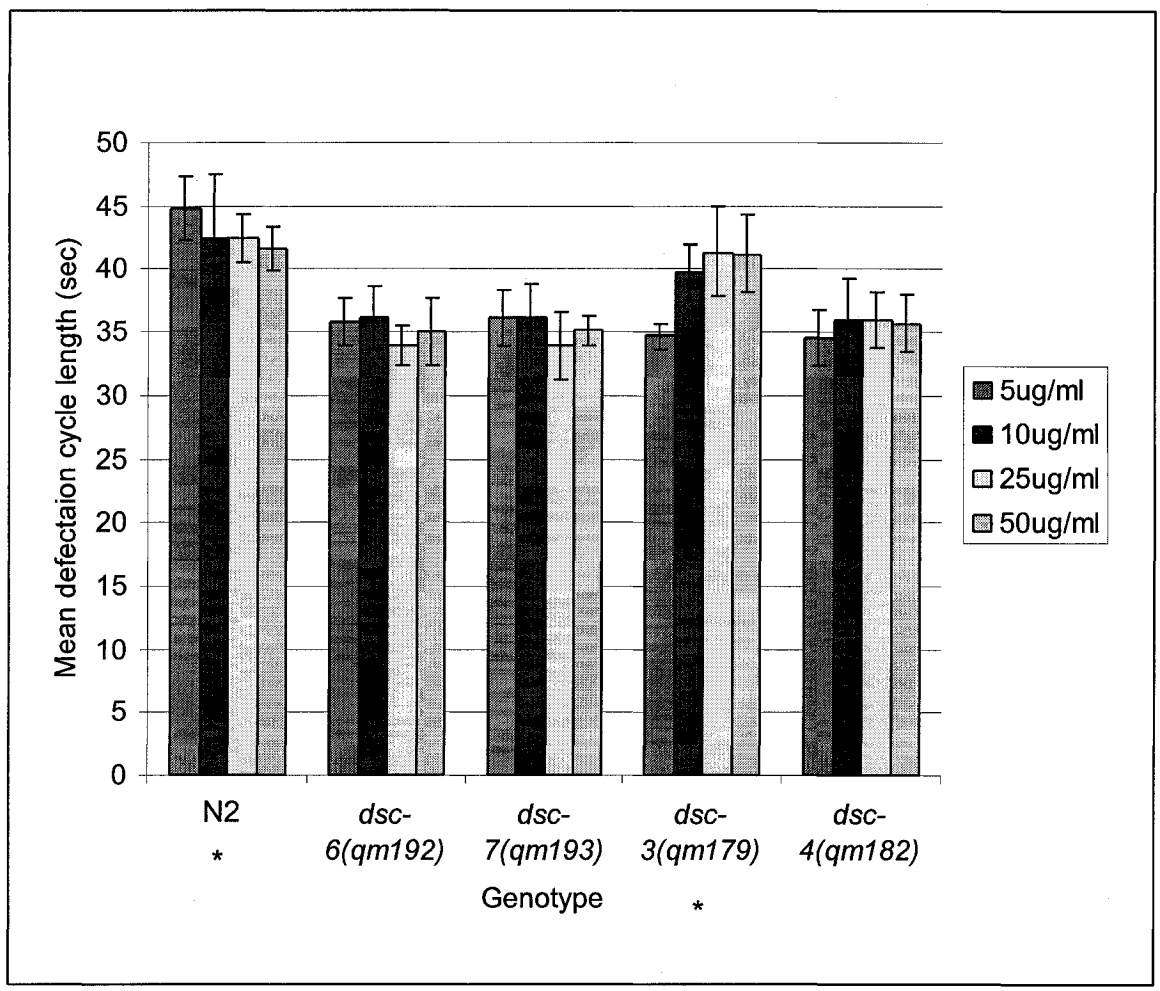


**Figure 20: The effect of high cholesterol treatment on the defecation rate of *dsc* mutants.** Adding extra cholesterol to the NGM rescues the fast defecation phenotype of *dsc-3* mutants only. For each genotype n = 15. Each animal was scored for three consecutive defecation cycles at 25°C following a two hour temperature shift to that temperature. We tested whether 50 µg/ml of cholesterol had a significant effect on defecation by comparing the rate of defecation on 50 µg/m plates with the rate of defecation on 5µg/ml plates. * indicates a significant effect ($P < 0.05$).

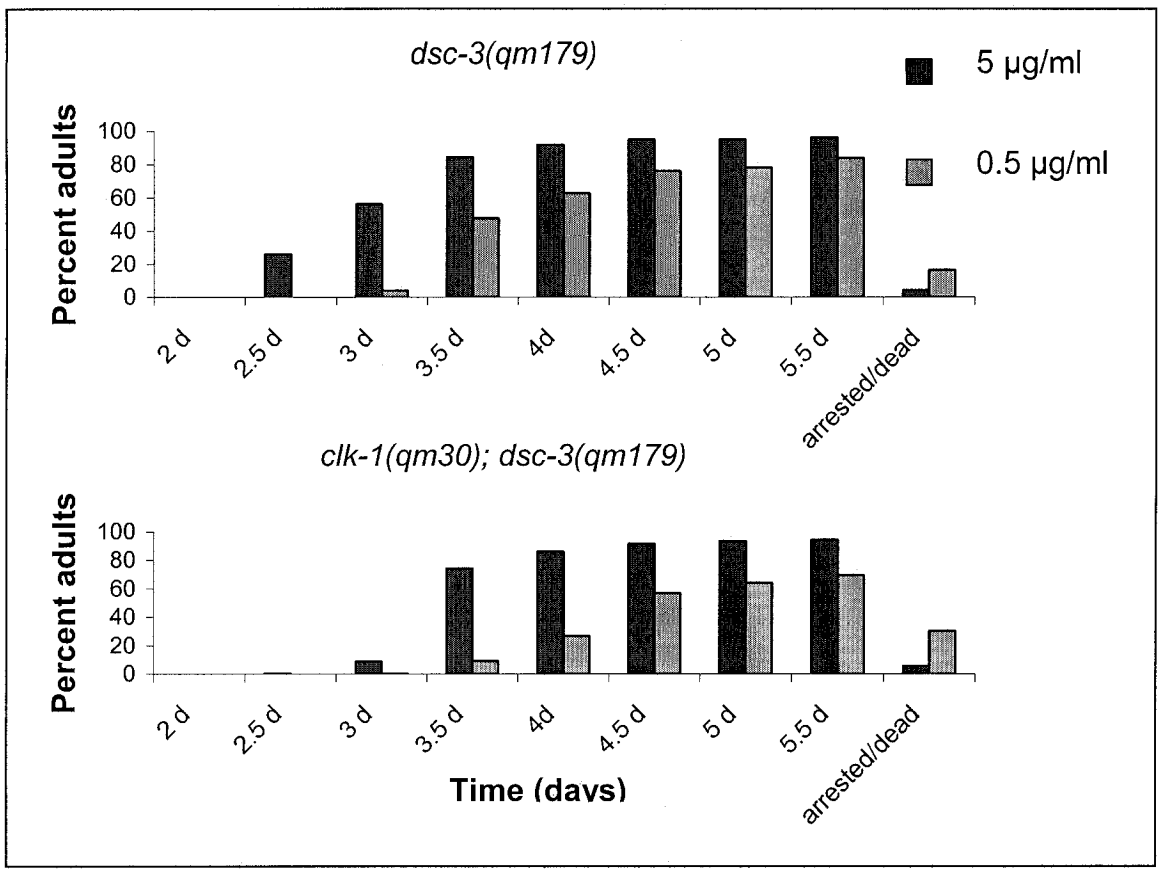


**Figure 21: *dsc-3* mutants are hypersensitive to low cholesterol.** Animals were cultured on NGM-C (0.5 µg/ml cholesterol) and NGM (5 µg/ml cholesterol) plates. The percent larval arrest was calculated for each genotype. Numerical values are as follows: for *dsc-3(qm179)* on NGM and NGM-C respectively, 4% and 16.3% of animals were dead or arrested; for *clk-1(qm30); dsc-3(qm179)* on NGM and NGM-C respectively, 5.7% and 30.5% of animals were dead or arrested. The sample sizes are as follows: for *dsc-3(qm179)* on NGM and NGM-C respectively, n = 398 and n = 332; for *clk-1(qm30); dsc-3(qm179)* on NGM and NGM-C respectively, n = 451 and n = 364. This work was done in collaboration with Robyn Branicky.

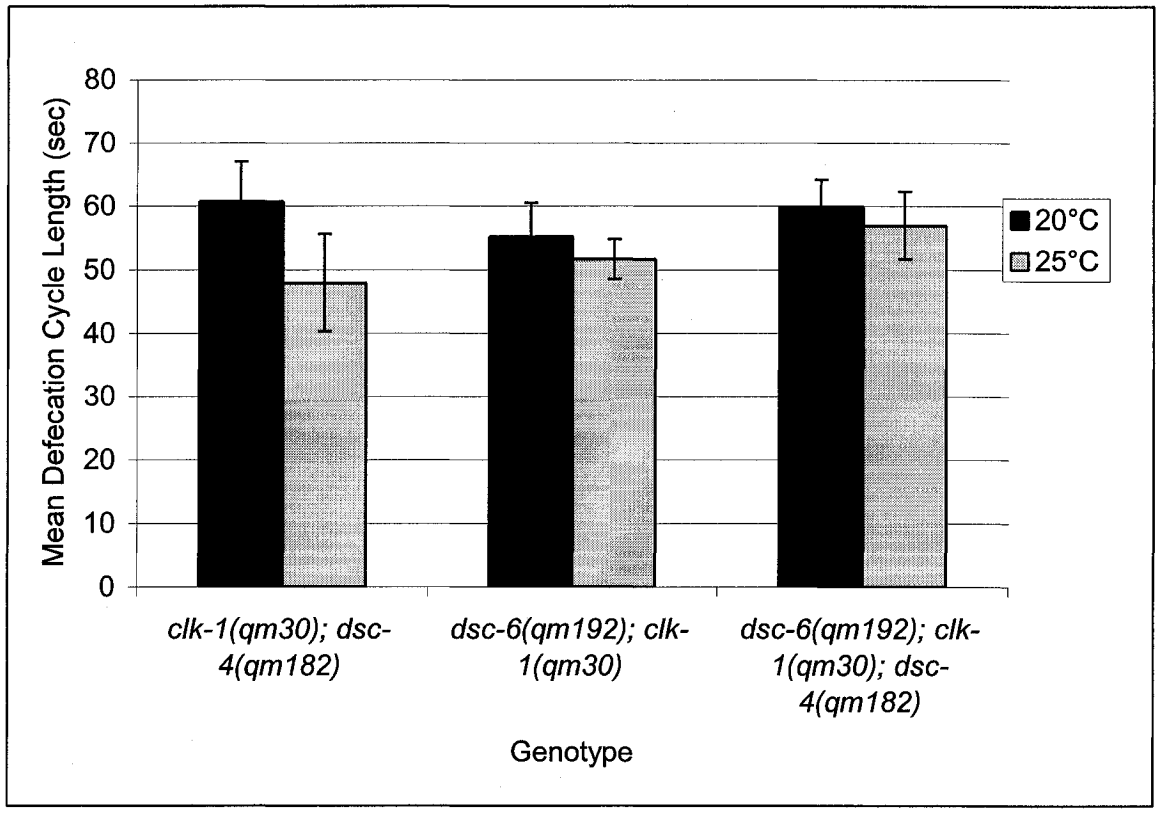


**Figure 22: *dsc-6; clk-1; dsc-4* triple mutants exhibit a mean defecation cycle length similar to *clk-1; dsc-4* and *dsc-6; clk-1* double mutants.** Strains were grown at 20°C and scored at either 20°C or 25°C. The bars represent the means of animals that had each been scored for 5 consecutive defecation cycles at 20°C and 25°C. The error bars correspond to the standard deviation of the means. n = 25 at both temperatures for *clk-1(qm30); dsc-4(qm182)* and *dsc-6(qm192); clk-1(qm30)*. n = 10 at both temperatures for *dsc-6(qm192); clk-1(qm30); dsc-4(qm182)*. The numerical values are as follows: for *clk-1(qm30); dsc-4(qm182)* at 20°C and 25°C respectively, 60.7 ± 6.3 and 48 ± 7.7; for *dsc-6(qm192); clk-1(qm30)* at 20°C and 25°C respectively, 55.2 ± 5.2 and 51.6 ± 3.1; for *dsc-6(qm192); clk-1(qm30); dsc-4(qm182)* at 20°C and 25°C respectively, 59.9 ± 4.2 and 57 ± 5.3.

| Phenotype / Genotype | Defecation | Embryonic development | Postembryonic development | Germline development | Lifespan |
|---|---|---|---|---|---|
| *clk-1(qm30); dsc-3(qm179)* | Suppressed | Not suppressed | Not suppressed | Not suppressed | Not suppressed |
| *clk-1(qm30); dsc-4(qm182)* | Suppressed | Suppressed | Not suppressed | Suppressed | Not suppressed |
| *dsc-6(qm192); clk-1(qm30)* | Suppressed | Suppressed | Not suppressed | Suppressed | Suppressed |
| *clk-1(qm30); dsc-7(qm193)* | Suppressed | Suppressed | Suppressed | Suppressed | Not suppressed |

**Table 3: Summary of *clk-1(qm30)* phenotypes suppressed by class I mutations.**

# Chapter 4.0 – Toward the molecular identification of *dsc-6* and *dsc-7*

## 4.1 Introduction

Two mutations – *dsc-6(qm192)* and *dsc-7(qm193)* were isolated in a genetic screen for defecation suppressors of *clk-1*. Below, I will discuss in detail the mapping techniques we carried out to determine the genomic position of *dsc-6* and *dsc-7*. Briefly, the new suppressor mutations were linked to a chromosome using two-factor data generated from one or more crosses. Once linkage to a chromosome was established, we performed several three-factor mapping experiments to position *qm192* and *qm193* to a chromosomal subregion. In our study, we were able to accurately map the *dsc-6* mutation only.

## 4.2 Materials and Methods

### 4.2.1 Nematode strains and cultivation

Animals were cultured on nematode growth medium (NGM) agar plates, and were fed the *E. coli* strain OP50 (Brenner 1974). The N2 (Bristol) strain was used as wild type. All animals were cultured at 20 °C unless otherwise stated. Genetic strains used in this work are as follows: **MQ1247**: *dsc-6(qm192)* II, **CB2001**: *dpy-10(e128) unc-53(e404)* II, **CB3970**: *unc-4(e120) bli-1(e769)* II, **MQ99**: *clk-1(qm30)* III, **MQ1248**: *dsc-7(qm193)* V, **MQ112**: *unc-34(e315) dpy-11(e224)* V, **CB2030**: *unc-62(e663) dpy-11(e224)* V, **CB1942**: *dpy-11(e224) unc-42(e270)* V, **DR181**: *unc-60(m35) dpy-11(e224)* V, **CB2065**: *dpy-11(e224) unc-*

*76(e911)* V, **MQ1229**: *dsc-6(qm192)* II; *clk-1(qm30)* III and **MQ1232**: *clk-1(qm30)* I; *dsc-7(qm193)* V.

### 4.2.2 Defecation rate

Described in Chapter 2. All animals were scored at 25°C unless otherwise mentioned.

### 4.2.3 Complementation tests

In general, males homozygous for one *dsc* mutation were mated to hermaphrodites homozygous for a different *dsc* mutation. Trans-heterozygous F1 animals were scored for five consecutive defecation cycles each at 25°C, 2-6 hours after being shifted to that temperature.

### 4.2.4 Two-factor mapping

Linkage to each chromosome was tested individually using strains homozygous for a visible recessive marker mutation (*m*) in a *clk-1(qm30)* background. *clk-1(qm30); dsc* double mutant males were mated to each marker strain. Animals in the F2 generation were shifted to 25°C for a minimum of two hours and then scored for 2-3 consecutive defecation cycles at that temperature. Fast defecating worms (defecation cycle length of < 60 seconds) in the F2 generation were picked to 60-mm plates, singled, and left to self-fertilize. The progeny (F3) of the singled F2 worms were examined for animals homozygous for *m*. If the suppressor mutation and *m* were linked, no fast defecating worms in

the F2 generation produced *m* homozygotes. If the suppressor mutation and *m* were unlinked, 2/3 of the fast defecating worms isolated in the F2 generation produced animals homozygous for *m*.

### 4.2.5 Three-factor mapping

The new suppressor mutations were mapped more accurately using three point mapping experiments. In order to do so, *dsc* mutant males were mated to strains that contained two linked visible recessive marker mutations on a wild-type background. Ten F1 animals were isolated and left to self fertilize. Plates were examined 4-5 days later for F2 recombinants. All F2 recombinants were singled and left to self fertilize. Six F3 recombinant animals were isolated from each F2 recombinant. Only F4 plates homozygous for the recombinant chromosome were kept for the experiment. To avoid marker effects, homozygous recombinant progeny were mated to *dsc* mutant males. Defecation was scored in the F1 cross progeny. Given that *dsc-6* and *dsc-7* mutants defecate faster than wild-type worms at 20°C as well as after a temperature shift to 25°C (Figure 12), we performed all three-point mapping experiments on a wild-type background. This accelerated each step of our three-point mapping strategy by about one day.

### 4.2.6 Cosmid preparation

Cosmids transformed in *E.coli* (strain TJ1) were obtained from the *C. elegans* genomic map project at the Sanger Center in Cambridge, England.

Cosmid DNAs for microinjection were extracted from *E.coli* by a standard miniprep protocol. Large RNAs were removed by a LiCl treatment.

### 4.2.7 PCR of *gbh-1*

The *gbh-1* gene was amplified from cosmid DNA by a nest PCR protocol (See Table 4).

### 4.2.8 Transgenic worms

Hermaphrodites were microinjected with DNA as described to create transgenic lines (Mello and Fire, 1995). In general, the injection mixture included pRF4 DNA (a coinjection marker) and cosmid DNA. pRF4 is a plasmid that carries the *rol-6* marker. The mutant gene encoded by *rol-6(su1006)* causes animals to roll and move in circles (Mello and Fire, 1995). This behavior is easily detected using a dissecting microscope. Transgenic lines were established by picking F1 progeny that posses the co-injection marker phenotype.

## 4.3 Results

### 4.3.1 Initial genetic mapping of the mutants isolated in the suppressor screen

As stated in Chapter 1, Branicky and colleagues identified two class I *dsc* genes (*dsc-3* and *dsc-4)* in their genetic screen for defecation suppressors of *clk-1.* Complementation tests were performed to determine if the new suppressor

mutations correspond to these or different complementation groups. We found that *qm192* and *qm193* are not alleles of *dsc-3* or *dsc-4*. As a next step, a complementation test was preformed between the isolated mutants. We found that *qm192* and *qm193* compliment each other. Thus, the new suppressor mutations belong to two distinct complementation groups. After that, linkage to each chromosome was tested separately using strains homozygous for a visible recessive marker mutation in a *clk-1(qm30)* background. We found that *dsc-6(qm192)* is located on chromosome II and *dsc-7(qm193)* is located on chromosome V.

### 4.3.2 Mapping results for *dsc-7(qm193)*

Three-point mapping experiments with *unc-34(e315) dpy-11(e224)* suggested that *dsc-7* was to the right of *unc-34* (-20.39 cM) and to the left of *dpy-11* (0.00 cM): in a cross between *dsc-7(qm193)* and *unc-34(e315) dpy-11(e224)*, 24/25 Unc non-Dpy animals picked up *dsc-7(qm193)*, while 2/45 Dyp non-Unc animals picked up *dsc-7(qm193)*.

Contrary to our initial mapping of *dsc-7(qm193)*, three-point mapping experiments with *unc-62(e663) dpy-11(e224)* suggested that *dsc-7* could be located to the right of *dpy-11*: in a cross between *dsc-7(qm193)* and *unc-62(e663) dpy-11(e224)*, 15/15 Unc non-Dpy animals picked up *dsc-7(qm193)*, while 0/40 Dyp non-Unc animals did not pick up *dsc-7(qm193)*. For that reason, we carried out a number of additional three-point and two-point mapping experiments to better position the *dsc-7* mutation. Despite our efforts, the genetic

location of the *dsc-7* gene still remains unclear. The critical data are summarized in Table 5.

### 4.3.3 Mapping results for *dsc-6(qm192)*

Three point mapping experiments with *dpy-10(e128) unc-53(e404)* suggested that *dsc-6* was to the left of or very close to *unc-53* (3.07 cM): in a cross between *dsc-6(qm192)* and *dpy-10(e128) unc-53(e404)*, 11/11 Dpy non-Unc recombinants picked up *dsc-6(qm192)*, while only 2/16 Unc non-Dpy recombinants picked up *dsc-6(qm192)*.

To better position *dsc-6*, we further mapped *dsc-6* with respect to *unc-4(e120)* and *bli-1(e769)*. Three point mapping experiments with *unc-4(e120) bli-1(e769)* suggested that *dsc-6* was to the left of or very close to *bli-1* (3.03 cM): in a cross between *dsc-6(qm192)* and *unc-4(e120) bli-1(e769)*, 14/16 Unc non-Blister recombinants picked up *dsc-6(qm192)*, while 2/2 Blister non-Unc recombinants did not pick up *dsc-6(qm192)*. To molecularly identify the *dsc-6* gene, 15 cosmids that roughly correspond to this genetic region were assayed for rescuing activity.

### 4.3.4 Candidate gene

As stated in Chapter 1, Shibata et al. (2003) recently cloned *dsc-4* and found that it encodes the large subunit of MTP. To reiterate, MTP is an ER protein required for the assembly and secretion apoB-containing lipoproteins (Berriot-Varoqueaux et al. 2000). In view of that, we were able to identify a suitable

candidate gene located in close proximity to the predicted genetic location of *dsc-6*. Like *dsc-4*, the candidate gene encodes for a protein, gamma-butyrobetaine hydroxylase -1 (GBH-1), that plays a key role in lipid metabolism. More specifically, GBH-1 is the last enzyme in the biosynthetic pathway of carnitine. Carnitine is a small, water-soluble amino acid derivative that enables activated fatty acids to enter the mitochondria, where they are broken down via beta-oxidation (Friedman and Fraenkel, 1955). The complete oxidation of a fatty acid molecule generates many ATP molecules.

As a first step, we amplified *gbh-1* from cosmid DNA by PCR for injection (Table 4). In our mix, the concentration of *gbh-1* was 50 ng/µl and the concentration of the co-injection marker (pRF4) was 150 ng/µl. Initially, this mix was injected into *dsc-6(qm192)* mutants and 6 stable lines were obtained. Several transformant worms from each line were shifted to 25°C for a two-hour period and then scored for defecation at that temperature. As a reminder, *dsc-6(qm192)* animals defecate faster then wild type animals at 20°C as well as after a temperature shift to 25°C (Figure 12). We found that *gbh-1* rescued (partially) the fast defecation phenotype of *dsc-6(qm192)* mutants (data not shown). However, upon further investigation we found that *gbh-1* cannot rescue *dsc-6(qm192); clk-1(qm30)* double mutants (data not shown). Note that, similar results were obtained following the injection of D2089, the cosmid on which the *gbh-1* gene is located. We reasoned that over expression of *gbh-1* might slow down the defecation rate of *clk-1(+)* animals leading to a false positive result.

Consequently, all additional injections were carried out in a *dsc-6(qm192); clk-1(qm30)* background. A summary of our injection data can be found in Table 7.

### 4.3.5 Injection of cosmid groups

As stated above, *dsc-6* was mapped to the left of or very close to *bli-1(e769)*. 15 cosmids which roughly correspond to this genetic region were obtained from the Sanger Center in Cambridge, England. They were divided into 4 pools (pool 1; ZK971, ZK970, M02G9 and C07E3, pool 2; C07E3, T09F3, E04D5 and ZK673, pool 3; ZK673, ZK666, F59B10, R166 and C06A1, pool 4; C06A1, C14A4, M28, D2089 and C09G5) and each pool was injected into *dsc-6(qm192); clk-1(qm30)* mutants. For each pool, the total concentration of cosmids was 50 ng/µl and the concentration of the co-injection marker (pRF4) was 150 ng/µl. We found that none of the pools rescued the fast defecation of *dsc-6; clk-1* double mutants. The critical data are summarized in Table 7.

## 4.4 Discussion

We carried out a genetic screen to isolate class I defecation suppressors of *clk-1*, and identified two new suppressors that correspond to two different complementation groups. In our study, we found that *dsc-6(qm192)* is located on chromosome II and *dsc-7(qm193)* is located on chromosome V. To map the new suppressor mutations more precisely, we carried out a number of three-point mapping experiments. We were able to map *dsc-6* to a small genetic interval located on the right arm of chromosome II. 15 cosmids that roughly correspond to

this genetic region were assayed for transformation rescue. They were divided into 4 pools (Table 6) and each pool was injected into dsc-*6(qm192); clk-1(qm30)* animals. We found that, none of the pools rescued the fast defecation of *dsc-6; clk-1* double mutants (Table 7). Accordingly, we reasoned that it might be necessary to better position *dsc-6* on chromosome II using more two- and three-point mapping experiments.

Despite our intense efforts to map *dsc-7*, its position on chromosome V remains very unclear. As stated earlier, we have carried out a number of two- and three-point mapping experiments and found that *dsc-7* can be mapped to the right and to the left arm of chromosome V (Table 5). We reasoned that are conflicting results might be due to a chromosomal rearrangement of the *dsc-7(qm193)* chromosome. Thus, we discontinued our mapping of the *dsc-7* gene. Recall that *dsc-7(qm193)* can suppress a number of *clk-1* phenotypes (Table 3). Consequently, determining the molecular identity of *dsc-7* might disclose important clues about the origin of the complex pleiotropic phenotype of *clk-1* mutants. In view of that, we hope to isolate and map more *dsc-7* suppressor mutations by performing the same genetic screen over again. We expect to reveal the molecular identity of *dsc-7* in this manner.

| Forward Primer | Reverse Primer | PCR Program | PCR Reagents |
|---|---|---|---|
| First Nest | | 94°C – 1 min<br>94°C – 30 sec<br>58°C – 30 sec<br>72°C – 5 min 30 sec } X25<br>72°C – 10 min<br>4°C | 50 ng DNA<br>10X*taq* buffer<br>200 µmoles dNTPs<br>Elongase<br>Distilled water |
| GTC AGC TAG AAG ACA TCG AGA GC | GAT CTG TGT CAC ATT CTC ATC G | | |
| Second Nest | | 94°C – 1 min<br>94°C – 30 sec<br>58°C – 30 sec<br>72°C – 5 min } X30<br>72°C – 10 min<br>4°C | 50 ng DNA<br>10X*taq* buffer<br>200 µmoles dNTPs<br>Elongase<br>Distilled water |
| CAT TGA AGT CTC CCT GCT CC | GGT AAT AAT CCC CGA TGC TTC G | | |

**Table 4: Summary of the PCR reagents and programs used for the amplification of *gbh-1* from cosmid DNA.**

| Mutation | Genetic mapping data [a] |
|---|---|
| *dsc-6(qm192)* II | [*unc-53 dpy-10 / dsc-6*]<br>*unc-53* (2/27) *dsc-6* (15/27) *dpy-10*<br><br>[*unc-4 bli-1 / dsc-6*]<br>*unc-4* (16/18) *dsc-6* (2/28) *bli1*<br><br>Complements *dsc-3(qm179)*, *dsc-4(qm182)* and *dsc-7(qm193)* |
| *dsc-7(qm193)* V | [*dyp-11 unc-42 / dsc-7*]<br>*dpy-11* (1/23) *dsc-7* (22/23) *unc-42*<br><br>[*unc-34 dpy-11 / dsc-7*]<br>*unc-34* (26/70) *dsc-7* (44/70) *dpy-11*<br><br>[*unc-62 dpy-11 / dsc-7*}<br>*unc-62* (0/55) *dpy-11* (55/55) *dsc-7*<br><br>[*unc-60 dpy-11 / dsc-7*]<br>*unc-60* (0/50) *dpy-11* (50/50) *dsc-7*<br><br>[*dpy-11 unc-76 / dsc-7*]<br>*dsc-7* (9/9) *dpy-11* (0/0) *unc-76*<br><br>Complements *dsc-3(qm179)*, *dsc-4(qm182)* and *dsc-6(qm192)* |

**Table 5: Summary of three-factor mapping data.**

[a] The genotypes given in brackets are those of the F1 animals whose descendants were scored to obtain three- factor mapping data.

| Pool Number | Cosmids |
|---|---|
| Pool 1 | ZK971, ZK970, M02G9 and C07E3 |
| Pool 2 | C07E3, T09F3, E04D5 and ZK673 |
| Pool 3 | ZK673, ZK666, F59B10, R166 and C06A1 |
| Pool 4 | C06A1, C14A4, M28, D2089 and C09G5 |

**Table 6: Summary of cosmid pools used for microinjection.**

| Injection mix | Genotype injected | Number of lines | Result |
|---|---|---|---|
| *gbh-1* | *dsc-6(qm192)* | 6 | 3 lines show partial rescue |
| *gbh-1* | *dsc-6(qm192); clk-1(qm30)* | 3 | No rescue |
| D2089 | *dsc-6(qm192)* | 9 | 4 lines show partial rescue |
| D2089 | *dsc-6(qm192); clk-1(qm30)* | 4 | No rescue |
| Pool 1 | *dsc-6(qm192); clk-1(qm30)* | 8 | No rescue |
| Pool 2 | *dsc-6(qm192); clk-1(qm30)* | 5 | No rescue |
| Pool 3 | *dsc-6(qm192); clk-1(qm30)* | 8 | No rescue |
| Pool 3 | *dsc-6(qm192)* | 3 | No rescue |
| Pool 4 | *dsc-6(qm192); clk-1(qm30)* | 3 | No rescue |
| Pool 4 | *dsc-6(qm192)* | 5 | 3 lines show partial rescue |

**Table 7: Summary of injection mixes and results.**

## Chapter 5.0 – General discussion

The phenotype of *clk-1* mutants is extremely pleiotropic, with nearly all aspects of development, behavior and reproduction being slowed down on average. To understand the origin of this complex pleiotropic phenotype, several genetic screens have been carried out to identify feature-specific suppressor mutations. Generally speaking, suppressors have been used widely to analyze genetic pathways and to identify functionally related genes since Sturtevant first described them in 1920 (Sturtevant, 1920). Each class of suppressor provides different types of information, and not all are useful for defining new proteins that are functionally related to the original protein. For example, intragenic suppressors (suppressors that restore the original DNA sequence) are not at all useful for defining genetic pathways. In contrast, a screen for feature-specific suppressors might allow for the discovery of suppressors that can act either upstream or downstream of the original gene or suppressors that act in a second related pathway. Six variations of suppression mechanisms have been described previously (Prelich, 1999).

*dsc-4 (qm182)*, a suppressor of the slow defecation phenotype of *clk-1* mutants, was isolated as a *clk-1* feature-specific suppressor (Branicky et al., 2001). While the *dsc-4* mutation does not suppress all aspects of the *clk-1* phenotype, it does suppress other phenotypes including the slow embryonic and slow germline development of *clk-1* mutants (Table 3) (Shibata et al., 2003). *dsc-4* encodes the worm homologue of the large subunit of MTP, which is required for the secretion of LDL (Shibata et al., 2003). A study carried out by Shibata and

colleagues provides evidence that the Dsc-4 phenotype is caused by a reduction in native LDL-like lipoproteins. In addition, it has recently been found that DMQ is less likely to produce ROS than UQ (Miyadera et al., 2002), and that lipid oxidation is reduced in *clk-1* mutants (Braeckman et al., 2002). Together, these findings suggest that the presence of DMQ instead of UQ in *clk-1* mutants results in altered oxidation of LDL-like lipoproteins in the worm. This suggests a model in which at least part of the complexity of the *clk-1* phenotype is due to oxidative modification of LDL by ROS. Suppressor mutations that suppress other *clk-1(qm30)* phenotypes could identify other cellular components whose level of oxidation represents a signal that affects the development of particular features of *clk-1* mutants.

The goal of our study was to isolate and characterize other *dsc-4* like genes. In order to do so, we carried out a genetic screen for defecation suppressors of *clk-1* and identified two suppressor mutations. On the basis of the mapping and complementation tests we performed, it is likely that these mutations define two new complementation groups. In our study, we were able to map *dsc-6* only, yet; we were unable to determine its molecular identity. Also, we analyzed the phenotype of the new suppressor strains and have found that, like *dsc-4(qm182)*, *dsc-6(qm192)* and *dsc-7(qm193)* can suppress a number of *clk-1* phenotypes, but not all (Table 3). We find this result fascinating and it brings to light a number of interesting questions. (1) Would it be possible to identify *dsc-4, dsc-6* and *dsc-7* in a genetic screen for germline suppressors of *clk-1*? In fact, this type of genetic screen is already in progress, and even though mutants in

these genes strongly suppress the slow germline development of *clk-1* mutants, they have not been reisolated. Given that the same chemical mutagen (EMS) was used for the abovementioned screen and the screen for defecation suppressors of *clk-1* it is remarkable that the *dsc-4*, *dsc-6* and *dsc-7* genes were not identified again. The sensitivity of either screen for the identification of a suppressor mutant might account for this result. (2) Why are some *clk-1* phenotypes suppressed and not others? It might be that, certain aspects of *clk-1* development are more sensitive than others to the physiological change that is triggered by a mutation in the suppressor gene. Consequently, some phenotypes are suppressed and others are not. (3) If all class I suppressors are altering lipid metabolism in the worm, how come they do not all suppress the same subset of *clk-1* mutant phenotypes? There are many aspects of lipid metabolism and each *dsc* suppressor could be affecting lipid biology in the worm differently. For example, the weakest *dsc* suppressor gene, *dsc-3*, encodes a protein required for the uptake of exogenous cholesterol (R. Branicky, unpublished observations). On the other hand, *dsc-4,* a much stronger suppressor gene, encodes a protein required for apo-B containing lipoprotein assembly (Shibata et al., 2003). So, it is possible that mutations in *dsc-3* and *dsc-4* alter the overall physiology of the worm differently. If this is the case, it is not surprising that *dsc-3(qm179)* and *dsc-4(qm182)* suppress a different subset of *clk-1(qm30)* phenotypes.

Intriguingly, we found that the *dsc-6* mutation can suppress the increased life span of *clk-1* mutants. To date, the *dsc-6* mutation is the only *clk-1* suppressor identified able to do this. We hope that the molecular identity of *dsc-6*

will shed some light on the molecular mechanism(s) responsible for the extended lifespan of *clk-1* mutants. To illustrate, the identification of *dsc-6* might allow us to explain better the role of ROS with respect to the increased longevity of *clk-1* mutants, in which UQ is replaced by DMQ. Several lines of evidence indicate that the effect of *clk-1(qm30)* on the lifespan of the worm could be mediated through an alteration of the metabolism of ROS. To illustrate, it has recently been found that DMQ might be less prone to ROS production than UQ (Miyadera et al., 2002). It will be interesting to see if the level of ROS is restored to normal in *dsc-6; clk-1* double mutants. If this is the case, we can speculate that the wild-type lifespan of *dsc-6(qm192); clk-1(qm30)* animals might be due to an increase in the level of ROS. Since it is difficult to measure the amount of ROS in an organism, we might have to rely on the molecular identity of *dsc-6* to come to this conclusion. For example, *dsc-6* might encode an activity that controls the amount of fatty acid beta-oxidation that takes place in the mitochondria and/or peroxisomes. Consequently, we might speculate that the *dsc-6* mutation results in an increased amount of fatty acid beta-oxidation resulting in an increased level of ROS in *dsc-6; clk-1* double mutants.

Based on additional phenotypic analysis, including determining the effect of increased dietary cholesterol on the defecation rate of the suppressor strains, we believe that, the molecular identification of *dsc-6* and *dsc-7* will reveal additional components involved in lipid metabolism in the worm (Figure 20). Thus, the work presented here might allow for the opportunity to better understand the relationship between LDL-like lipoproteins and the pleiotropic

phenotype of *clk-1* mutants. Also, in the future it will be interesting to see if other types of suppressor mutations will disclose other important clues about the complexity of the Clk-1 phenotype.

## Chapter 6.0 – References

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