Formation and Function of Ascarosides in the Nematodes *C. elegans* and *C. briggsae*

Thesis by
Sarah Michelle Cohen

In Partial Fulfillment of the Requirements for
the Degree of
Doctor of Philosophy in Biology

CALIFORNIA INSTITUTE OF TECHNOLOGY
Pasadena, California

2021
Defended May 17, 2021

Sarah Michelle Cohen
ORCID: 0000-0003-4469-2670

ACKNOWLEDGMENTS

While the following thesis is a product of my research over the past several years at Caltech, it could not have been accomplished without the support and encouragement of many others.

First and foremost, I would like to thank my advisor, Paul Sternberg. I started working for Paul as a summer research assistant while an undergraduate. At that point, I was a chemist who worked with living worms in a cursory fashion, focusing solely on the small molecules they produced. Paul taught me the biology of worms and how that understanding would further my work in small molecules research. When I came to Caltech for my graduate career, Paul helped me in my transition from chemist to biologist, suggesting books to read and classes to take in order to become the best possible scientist I could be. Paul is an amazing mentor – learning how best to push me in my research while always supporting me in every way and even sitting with me in front of a microscope to teach me larval-stage gonad cell development.

I would like to thank my Thesis Committee, Ellen Rothenberg, David Prober, and Jared Leadbetter, for all their valuable and constructive comments over the years. Ellen has consistently been my cheerleader; her suggestions and advice have helped me through this entire process. David's comments and questions are always insightful and have helped guide my research. Jared's enthusiasm has been contagious, and he has always made me comfortable and excited to present my work.

I could not have gotten to where I am today without the support of my undergraduate advisor, Frank Schroeder. I first met Frank while interviewing him for an article in the Cornell Daily Sun.

After becoming fascinated with “worm talk”, I joined Frank’s lab where he taught me how to be a first-rate researcher. Frank is one of the best editors I know, and his comments consistently make me a better writer. And, as I moved into graduate school, Frank continued to support me as a valued collaborator.

I would like to thank all members, past and present, of the Sternberg Lab for always being the best colleagues and friends. Whether we are talking about experiments or having group dinners in Morgan Library, we always have a great time together. I would especially like to thank Margaret Ho, Andrea Choe, James Lee, Alli Akagi, Daniel Leighton, David Angeles, Pei-Yin Shih, Katie Brugman, Mengyi Cao, Heenam Park, Jessica Sun, and Katherine Norton. I would also like to thank Sarah Torres for her constant support and friendship.

I would like to thank my lab-mates and collaborators at the Schroeder Lab at Cornell University. I am thankful for all that you have taught me and for all the fantastic work we have accomplished together. I would especially like to thank Parag Mahanti, Oishika Panda, Pooja Gudibanda, Josh Judkins, Henry Le, Chester Wrobel, Yan Yu, Max Helf, and Bennett Fox. I would also like to thank Josh Baccile, who not only supported me while at the Schroeder lab, but also continued to help me throughout my graduate career with advice, and the use of a rotovap, when he came as a postdoc to Caltech in the Tirrell Lab.

I want to thank my friends both at Caltech and across the country especially Shashank Ghandi, Marissa Tranquilli, Alyssa Scheer, and Yiping Xing for always being there to have fun outside of

work. I would also like to thank my former teacher, Andrew Bezant, for inspiring me to love science even when it's challenging.

I would like to thank my family for their support throughout my life. My parents, Matthew Cohen and Susan Geary, have always given me constant love and encouragement, and I appreciate their reminders that six years is not too long a time to earn a PhD. I would also like to thank Steve Cohen, Nancy Clark, Debbie Gevirtzman, Alex Cohen, Zach Cohen, and especially my uncle, Andy Cohen, for being the first person to show me around a lab and sparking my interest in science by letting me use liquid nitrogen to make ice cream. I would also like to thank my in-laws, Mark Pochapin, Shari Midoneck-Pochapin, and David Pochapin, as well as the rest of the Pochapin family. To all of my family, no matter how much you understand my work (or not) you are always supportive and interested, and I can't thank you enough.

Lastly, I would like to thank my husband, Steven Pochapin, for his constant love, support, and encouragement. Whether he is bringing me food while I'm having a late night in lab, keeping me company while I pour plates on weekends, or helping me with various coding projects, he is always there for me no matter what.

ABSTRACT

As an easily culturable, hermaphroditic, and short-lived species with a fully annotated genome and neural connectome, the nematode *Caenorhabditis elegans* is used as a model organism to study many different biological problems. Examining the communication systems among these worms is important not only to understand how they control and affect one another's behavior, but also gives us clues to the communications systems of closely related parasitic worms.

Nematode worms use small-molecule signaling to send messages about their environments in order to influence behavioral decisions of other animals in their vicinity. A main type of pheromone signaling uses a group of stable small molecules, collectively called ascarosides, that are built modularly from common waste products in cells such as sugars, fatty acids, and amino acid derivatives. Ascarosides are synthesized by *C. elegans* in precise concentrations and combinations to produce finely-tuned messages which control major behaviors such as mating and entry into dauer, an alternative lifestage that allows worms to survive adverse conditions. We are still unraveling exactly how ascarosides are produced and how they affect behaviors in *C. elegans* and other worms species.

To further understanding of the formation of ascarosides in *C. elegans*, I studied the O-acyltransferase gene class to see if they helped catalyze the 4' modifications of ascarosides, as predicted based on their chemistry. Surprisingly, *oac* genes were found to be uninvolved in the biosynthesis of ascarosides; but they do affect ascaroside production and secretion. To understand the underlying mechanisms of formation and function of ascarosides across worm species, I also studied ascarosides in the closely-related species *Caenorhabditis briggsae*. First, I

developed an efficient CRISPR/Cas9 method for use in *C. briggsae*. From there, I was able to make the *C. briggsae* mutants *Cbr-glo-1* and *Cbr-daf-22*, genes that we showed were one-to-one orthologs of their *C. elegans* counterparts. I then showed that ascr#2 was the main component of daumone in *C. briggsae*. Additionally, I found that there is an anti-dauer signal, hypothesized to be another type of small signaling molecule – a glucoside. These findings further our understanding of the formation and function of ascaroside signaling molecules in nematode worms.

Thesis Supervisor: Paul W Sternberg
Committee Chair: Ellen V Rothenberg
Committee Member: David Prober
Committee Member: Jared R Leadbetter

PUBLISHED CONTENT AND CONTRIBUTIONS

Cohen SM, Sternberg PW. 2019. Genome editing of *Caenorhabditis briggsae* using CRISPR/Cas9 co-conversion marker *dpy-10*. In: microPublication Biology. doi:10.17912/micropub.biology.000171.

Sarah Cohen designed the method, performed the experiments, and wrote the paper.

Le HH, Wrobel CJ, Cohen SM, Yu J, Park H, Helf MJ, Curtis BJ, Kruempel JC, Rodrigues PR, Hu PJ, *et al*. 2020. Modular metabolite assembly in *Caenorhabditis elegans* depends on carboxylesterases and formation of lysosome related organelles. In: eLife. 9:e61886. doi:10.7554/eLife.61886.

Sarah Cohen participated in designing the project and performed research on the *C. briggsae* strains.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

Figures

Tables

Chapter 1

AN INTRODUCTION TO THE FORMATION AND FUNCTION OF ASCAROSIDE SIGNALING MOLECULES IN NEMATODES

1.1 Introduction

The nematode *Caenorhabditis elegans* has been developed and used as a model organism in many fields due to its fully annotated genome, complete connectome, short lifespan, fixed cell lineages during development, and many other qualities that make it an ideal candidate to aid in the understanding of the underlying biology of eukaryotes (The *C. elegans* Sequencing Consortium 1998; Cook *et al*. 2019; Brenner 1974, Sulston *et al*. 1983, Sulston and Horvitz 1977). However, equally interesting are the nematode- and species-specific aspects of this organism's biology.

Lacking sight or speech as a means to perceive and communicate about their environment, nematodes such as *C. elegans* rely on chemical and mechanical cues in order to make behavioral decisions (Chute and Srinivasan 2014; Goodman 2006). The small molecules used by worms for communication are collectively called ascarosides (Ludewig and Schroder 2012). The wide-ranging complexity and diversity of these molecules, and their myriad functionality in regulating complex behaviors, makes them a fascinating avenue of inquiry that has yet to be fully explored.

1.2 An Early History of Ascarosides

That nematodes use pheromones to communicate with one another is unsurprising given the ubiquity of pheromones and other similar signaling molecules in bacteria, insects, and higher eukaryotes (Miller and Bassler 2001; Regnier and Law 1968; Wyatt 2009). Worm-conditioned

media contains both volatile and soluble pheromones, the latter of which will be the topic of this work (Golden and Riddle 1984a; Chasov *et al*. 2007).

Pheromone signaling was first identified in the nonparasitic roundworm *C. elegans* during investigations into the major lifespan and behavioral decision of whether or not to enter dauer (Golden and Riddle 1982). The dauer diapause lifestage, analogous to the infective juvenile (IJ) stage in parasitic nematodes, is an alternate developmental pathway that is lethargic, non-feeding, and generally resistant to adverse conditions and which occurs instead of the third larval stage (Cassada and Russell 1975). *C. elegans* preferentially decide to enter into dauer when they encounter unfavorable growing conditions such as high population density, high temperature, or lack of food prior to the L1 molt and through the L2d stage (Cassada and Russell 1975; Golden and Riddle 1984b; Schaedel *et al*. 2012). Crude pheromone was distilled by concentrating the supernatant of liquid worm cultures that had been deprived of food and was shown to be the signal through which these adverse conditions were communicated to one another by the worms (Golden and Riddle 1984b; Golden and Riddle 1984c).

Crude pheromone was known to be a mixture of related molecules that were stable, non-volatile, and chemically similar to hydroxylated fatty acids (Golden and Riddle 1984a). This crude pheromone was purified and identified as an ascaroside now known as ascr#1 (Jeong *et al*. 2005).

Ascarosides, small molecules that generally contain an ascarylose sugar attached to a fatty acid side chain, were first discovered in the egg membranes of several parasitic roundworm species

including *Parascaris equorum* (equine roundworm), *Ascaris lumbricoides* (human roundworm), and *Ascaris suum* (pig roundworm) (Fouquey *et al*. 1958, Jezyk and Fairbairn 1967; Bartley *et al*. 1996).

1.3 A Wide Variety of Ascarosides Discovered in *C. elegans*

The ascaroside family of small molecules was expanded in *C. elegans* as new methods allowed for the discovery of several other "simple" ascarosides that contain only an ascarylose sugar and a fatty acid side chain where the penultimate (ω-1) carbon of a fatty acid is attached to the first (1') position of the carbohydrate (Figure 1.1). (Butcher *et al*. 2007; Butcher *et al*. 2008; Pungaliya *et al*. 2009, Srinivasan *et al*. 2012). The simple ascarosides differ from one another in the length of the side chain and its saturation, most often with only one double bond (Edison 2009). Ascarosides can further be modified in a modular fashion through the addition of terminal and head groups; terminal groups are various moieties attached to the terminal (α) end of the fatty-acid side chain while head groups are made of those same types of components but attached to the second (2') or fourth (4') carbon of the ascarylose sugar (Srinivasan *et al*. 2008).

There are several hundred known ascarosides, and they show an array of modifications (Figure 1.2) (Ludewig and Schroeder 2012). Ascarosides that have a terminal group are exemplified by ascr#8, which contains a 4-aminobenzoic acid moiety possibly derived from bacteria (Pungaliya *et al*. 2009). Classes of ascarosides that contain head groups include indole carboxy ascarosides (icas), 4-hydroxybenzoyl ascarosides (hbas), tigloyl ascarosides (mbas), succinylated octopamine ascarosides (osas), tyramine ascarosides (tsas), and glucosyl uric acid ascarosides (uglas), among others (Butcher *et al*. 2009a; Srinivasan *et al*. 2012; von Reuss *et al*. 2012; Artyukhin *et al*. 2013;

Artyukhin *et al*. 2018). A further ascaroside class includes the omega-linked ascarosides (oscr), and their indole counterparts (icos), which have an (ω)-linked fatty-acid side chain rather than the (ω-1)-link common in simple ascarosides (von Reuss *et al*. 2012).

In addition to glycosides of the dideoxysugar ascarylose, there are other glucosides excreted in the *C. elegans* metabolome such as indole glucosides (iglus), anthranilic acid glucosides (angls), and tiglic acid glucosides (tyglus) (Stupp *et al*. 2013; Le *et al*. 2020).

A library of all known ascaroside molecules, including their chemical and structural information, can be found at the *C. elegans* Small Molecule Identifier Database (SMID-DB) (Artyukhin *et al*. 2018).

1.4 Formation of Ascarosides in *C. elegans*

Ascarosides are built modularly from cellular metabolic waste products (Pungaliya *et al*. 2009; Butcher *et al*. 2009a). This occurs in the peroxisomes and liposomal-like gut granules of intestinal cells in worms, where the various moieties necessary for ascaroside formation can also be found (Butcher *et al*. 2009b; Panda *et al*. 2018). Cellular products that have been implicated in ascaroside formation include endogenous amino acids and their derivatives, which can account for the formation of most 4’ modifications including the icas, hbas, and mbas ascaroside families (Srinivasan *et al*. 2012, von Reuss *et al*. 2012).

Although some pieces are as yet missing, we have a rough picture of the formation of ascarosides in *C. elegans* (Figure 1.3). We do not know how the fatty acid side-chain is attached

to the carbohydrate, but the ascarylose sugar is known to be made endogenously by the worms (Srinivasan *et al*. 2012). Previous studies also show that once the glycolipid side-chain is attached, these very-long chain ascarosides are first processed using peroxisomal β-oxidation to create medium and short chain simple ascarosides (Butcher *et al*. 2009b; Joo *et al*. 2009; Joo *et al*. 2010; von Reuss *et al*. 2012).

Mutation of the gene *daf-22* creates worms that are unable to form dauers using their own endogenous pheromone – this was the first gene known to be defective in pheromone production (Golden and Riddle 1985). It was later found that the *daf-22* gene product catalyzes the last step of the peroxisomal beta-oxidation pathway in conjunction with ACOX-1, MAOC-1, and DHS-28, to shorten the fatty-acid side chain of ascarosides to between 3-9 carbons (Butcher *et al*. 2009b; Joo *et al*. 2009; Joo *et al*. 2010; von Reuss *et al*. 2012). There are five *acox-1* genes – specific ones are used for the parallel formation pathways of (ω)-linked ascarosides and (ω-1)-linked ascarosides (Zhang *et al*. 2015; Zhang *et al*. 2018).

While peroxisomal β-oxidation shortens the fatty acid side chains, it does not explain the proliferation of ascarosides through head and terminal group modification. Head group modification was found to occur in the lysosomal related organelles (LROs), birefringent acidic gut granules in the *C. elegans* intestine (Coburn *et al*. 2013; Panda *et al*. 2018). Mutants of the genes *glo-1*, *glo-3*, and *apb-3* lack or have defective acidic gut granules and are unable to produce 2’ or 4’ modified ascarosides while the absence of other types of gut granules does not affect ascaroside formation (Hermann *et al*. 2005; Rabbitts *et al*. 2008; Panda *et al*. 2018). The formation of some glucosides is also lessened or abolished when gut granules are absent,

indicating that gut granules also play a role in their formation (Le *et al*. 2020). These lysosomal-like organelles are an ideal place for head group modifications as they act as storage for cellular waste products, from which the ascaroside head groups are derived (Coburn and Gems 2013).

One class of enzymes – encoded by the *cest* gene family – has been implicated in the direct attachment of 4' modifiers to simple ascarosides (Faghih *et al*. 2020; Le *et al*. 2020). Of the 34 genes in the *cest* gene family, *cest-3* and *cest-9.2* cannot produce any icas or mbas; *cest-4* cannot produce some iglu; *cest-1.1* is unable to produce uglas#1 and its derivatives; *cest-8* is unable to produce osas; and *cest-2.2* is unable to produce ascr#8 or its derivatives (Faghih *et al*. 2020; Le *et al*. 2020). The gene *acs-7*, an acyl-CoA synthetase that localizes to the LROs, is also found to be required for the biosynthesis of icas#9 and osas#9 (Panda *et al*. 2018; Zhou *et al*. 2018). After being processed in the gut granules, some modified ascarosides are returned to the peroxisome for further peroxisomal β-oxidation (Zhou *et al*. 2018). These known modification steps can be broadly applied or extremely specific, depending on the enzyme involved, allowing for a high degree of regulation in the biosynthesis of ascaroside molecules.

1.5 Functions of Ascarosides in *C. elegans*

Although there are over 200 identified ascaroside and glucoside metabolites, we only know the function of a handful (Table 1.1) (Ludewig and Schroeder 2012). Generally, these small molecules affect major complex behaviors depending on concentration and combination (Srinivasan *et al*. 2008). Often, ascarosides modified with head groups act antagonistically to their non-modified "simple" counterparts (Srinivasan *et al*. 2008). Ascarosides in high and low concentrations also often direct antagonistic behaviors (Srinivasan *et al*. 2008).

Ascaroside production is dependent on life-stage with larval or juvenile worms producing different ascaroside profiles than their adult counterparts; ascaroside profiles are also sex-dependent (Choe *et al*. 2012a; Izrayelit *et al*. 2012; Chasov *et al*. 2007). Dauer larvae, however, are not known to produce exogenous ascarosides (Kaplan *et al*. 2011). Ascarosides are selectively released by nematodes into their environments – these stable small molecules can then interact with G protein-coupled receptors in recipient worms' chemosensory neurons around the mouth (von Reuss *et al*. 2012; Zwaal *et al*. 1997, for example). Worms then integrate these various signals to affect a behavioral response (Bargmann and Horvitz 1991). The study of various worm behaviors, especially dauer formation, mating, and attraction, has given us insight into how ascarosides function.

Through studies of dauer formation, dauer pheromone was found to be primarily composed of ascr#2, ascr#3, ascr#5, ascr#8, and icas#9 (Butcher *et al*. 2007; Butcher *et al*. 2008; Butcher *et al*. 2009a, Srinivasan *et al*. 2008). In particular, ascr#2 was determined to be a signal of population density while ascr#3 is an indication of food availability – in high concentrations (nanomolar to micromolar) these metabolites signal high population density and low food availability, respectively (von Reuss *et al*. 2012; Kaplan *et al*. 2011). Furthermore, ascr#2 and ascr#3, normally thought of as dauer-inducing pheromones in the early larval stages, induce stress-resistance and longevity in L4 and adult worms (Ludewig *et al*. 2013).

In low concentrations (femtomolar to picomolar), however, ascr#2 and ascr#3 indicate low population density and high food availability (Srinivasan *et al*. 2012; von Reuss *et al*. 2012).

Under these conditions, instead of dauer-inducing pheromones, they become male attractants, as do ascr#4 and ascr#8 (Pungaliya *et al*. 2009; Srinivasan *et al*. 2008; Srinivasan *et al*. 2012). ascr#3 is the most abundant ascaroside in the hermaphrodite metabolome and is thought to be an indicator for males seeking to mate that a worm is a hermaphrodite (Aprison and Ruvinsky 2017).

In contrast, ascr#10 is the most abundant ascaroside in males; high levels of ascr#10 are an indicator of their sex to hermaphrodites (Aprison and Ruvinsky 2017). Hermaphrodites are attracted to extremely low levels (attomolar range) of ascr#10; males also release other hermaphrodite attractants including low concentrations of icas#3 and icas#9, an indole version of a known male-attractant and a known dauer ascaroside, respectively (Izrayelit *et al*. 2012; Srinivasan *et al*. 2012). In their relation to mating and attraction in worms, ascr#3 and ascr#10 have also been implicated in faster aging in hermaphrodites (Aprison and Ruvinsky 2016).

There is also an antagonistic ascaroside-mediated connection between reproductive and dauer developmental pathways. icas#1, icas#3, and hbas#3 attract all worms, both hermaphrodites and males, at high concentrations while their non-modified ascaroside counterparts (ascr#1 and ascr#3) are dauer signals (Srinivasan *et al*. 2012, von Reuss *et al*. 2012). These indole ascaroside attractants are also implicated in suppressing foraging behaviors, as well as promoting attraction and aggregation behaviors (Srinivasan *et al*. 2012; Green *et al*. 2016). Another modification of ascr#3, mbas#3 is a dispersal signal which occurs even in the presence of food (Zhang *et al*. 2017).

The succinylated octopamine ascarosides (osas) are mainly produced by L1 larvae with osas#9 and osas#10 produced by starved and well-fed worms, respectively (Artyukhin *et al*. 2013). Osas#9 is also part of an avoidance pheromone for all larval worms, the rest of which has yet to be deduced (Artyukhin *et al*. 2013).

Some ascarosides that are highly enriched in the worms but not excreted, such as the O-glycoside ester ascarosides (glas), are thought to be used for storage of ascaroside-based molecules in advance of modification and excretion based on environmental factors (von Reuss *et al*. 2012). Ascaroside-based pheromone has also been shown to increase the rate of egg-laying in adult worms, also known as pheromone-dependent reproductive plasticity (PDRP), and the acceleration of larval development, showing that ascarosides are not only produced but also reacted to in a life-stage dependent manner (Wharam *et al*. 2017).

A function of indole glucosides (iglu) may be to catabolize toxic moieties such as phenazines and indole given off by certain species of bacteria like *Pseudomonas aeruginosa* or *Escherichia coli*, which exist in the environment alongside *C. elegans* and are, in the case of *E. coli*, used as the primary food source in laboratory settings (Stupp *et al*. 2013). There may be other behavioral functionalities of glucosides that have yet to be explored.

1.6 Ascarosides in the *Caenorhabditis* Genus

Ascaroside signaling is not unique to *C. elegans* and is found in many other nematode species (Choe *et al*. 2012a). These signals are often unique to each species either through different ratios of the same ascarosides, or by using different ascaroside classes altogether (Choe *et al*. 2012a;

Dong *et al*. 2016). Within the *Caenorhabditis* genus, ascarosides have only been studied within the elegans subgroup, but they can still give us clues as to how ascaroside signaling has evolved and differs among species (Figure 1.4).

Most closely related to *C. elegans*, *C. inopinata* produces many of the same major ascarosides including ascr#1 and ascr#3 (Kanzaki *et al*. 2018, Bergame *et al*. 2019). *C. inopinata* males are also known to be more sensitive to the synthetic version of ascr#3 than are *C. elegans* males (Kanzaki *et al*. 2018).

The *C. briggsae* metabolome is dominated by derivatives of ascr#2 and ascr#6.1 (Dong *et al*. 2016). Icas#2 and icas#6.2, indole ascarosides unique to *C. briggsae*, were found to be sex-pheromones used in conjunction to attract males (Dong *et al*. 2016). In *C. briggsae*, the importance of gut granules in 4' modified ascaroside formation was also underlined by studies of *Cbr-glo-1* which lacks both gut granules and 4' modified ascarosides (Le *et al*. 2020).

C. nigoni produces some of the same ascarosides as *C. elegans* including ascr#3, but they also produce metabolites that use the sugar caenorhabdose (caen ascaroside class) rather than ascarylose (Bergame *et al*. 2019). *C. remanei* also produces a novel class of ascarosides, known as fatty acid ascarosides (fasc), which appear to encourage male attraction (Dolke *et al*. 2019). The fasc ascarosides were not found in any other *Caenorhabditis* species other than *C. latens* (Dolke *et al*. 2019).

Other observations of ascarosides in caenorhabditids are that indole ascaroside (icas) signaling is highly prevalent, and blends of icas molecules are species-specific in the elegans group within the *Caenorhabditis* genus but not used widely outside that group (Dong *et al*. 2016). Generally, indole ascarosides also function as sex pheromones but are attractive to different or both sexes depending on the species (Dong *et al*. 2016).

1.7 Ascarosides in Other Nematodes

From wide-ranging studies on ascarosides across nematode clades, we know that the fatty acid chain length can be species-specific with some species, such as those in the *Heterohabditis* genus, producing many medium-chain ascarosides instead of the short-chain ascarosides more familiar in studies of the *Caenorhabditis* genus (Choe *et al*. 2012a). Although ascaroside profiles are thought to be species-specific, ascarosides can also be conserved across species which share behaviors or ecological niches – ascr#9, for example, is conserved among all insect-parasitic nematodes (Choe *et al*. 2012a). This indicates that there may be benefits to using certain ascarosides under specific situations, especially when it relates to an interaction with another group of organisms.

Pristionchus pacificus uses paratose rather than ascarylose for the base sugar in some of its communication molecules (Bose *et al*. 2012; Bergame *et al*. 2019). This modification, and several other small modifications of known *C. elegans* ascarosides, creates a new library of *Pristionchus* ascarosides and glucosides including the ubas, npar, paso, bhas, nuclas, puglas, pugl, and bkas groups (Artyukhin *et al*. 2018, Curtis *et al*. 2020).

The importance of the *daf-22* pathway in ascaroside formation is underlined by the necessity of *Ppa-daf-22.1* and *Ppa-daf-22.2* in the formation of ascarosides in *P. pacificus*; however, the fact that the *daf-22* homologs, as well as the other homologs in the beta-oxidation pathway are expanded upon in *P. pacificus* indicates that there are likely unique pathways for the formation of signaling molecules using different sugars (Markov *et al*. 2016; Artyukhin *et al*. 2018).

The gene *Ppa-uar-1*, a *cest* homolog, was essential in learning more about the connection of head groups in ascaroside formation, as it cannot create the very specific ascarosides ubas#1 and ubas#2 (Falke *et al*. 2018). Other ascarosides discovered through studies of *P. pacificus* include npar#1, ubas#1, ubas#2, and pasc#9, which have been confirmed as a part of the *P. pacificus* dauer pheromone, and dasc#1, found to induce alternate mouth development to encourage predacious rather than bacterivourous feeding (Bose *et al*. 2012; Artyukhin *et al*. 2018; Yim *et al*. 2015; Bento *et al*. 2010; Ragsdale *et al*. 2013).

Outside the original findings of ascarosides in the egg membranes of *Ascaris* worms and the substantial findings in *P. pacificus*, only scattered information can be found on ascarosides in other worms. The sex pheromones for both male and female *Panagrellus redivivus* have been identified as ascr#1 (female-produced male-attractant) and dhas#18 (male-produced female-attractant) (Choe *et al*. 2012b). The parasite *Heterohabditis bacteriophora* uses ascarosides to control infective juvenile (IJ) development by preventing recovery to the J4 stage; this makes sense as the IJ stage is closely related to the ascaroside-induced dauer stage in *C. elegans* and other non-pathogenic nematode species (Noguez *et al*. 2012).

Finally, in the pinewood nematode *Bursaphelenchus xylophilus*, ascarosides are used not only by the worms to drive specifics of its host beetle's development but are also produced by the host beetle itself – this may indicate that ascarosides have been developed in parasitic species to co-opt the host's own biology to form better conditions for the parasite (Zhao *et al.* 2016).

1.8 Conclusion

Understanding the formation of ascaroside small molecules and their biological function in *C. elegans* and other nematodes is important for understanding the behavioral complexity of these animals. It is especially important to expand upon the ascaroside discoveries in *C. elegans* to include parasitic nematodes as they cost hundreds of billions of US dollars in agricultural losses each year and affect over 50% of the world's human population causing hundreds of thousands of deaths (Phani *et al.* 2021; Stepek *et al.* 2006). Previous work using pheromones as a management agent for field pests shows that ascarosides may be beneficial as anti-helminthic agents which are non-harmful to the host organism, and further exploration of nematode-specific small molecule signaling will help with this endeavor (Meyer *et al.* 1997).

Although we have come far in our understanding of ascaroside-based worm communication since the first isolation of ascarosides in *Ascaris* worms and daumone in *C. elegans*, there is still much we do not know. While we have a clear understanding of the peroxisomal β-oxidation pathway that shortens the fatty acid side chain, we still do not know how the side chain is attached to the ascarylose sugar. And although we are making headway in understanding the 2' and 4' modifications made by carboxylesterases in the gut granules, this work is as-yet incomplete.

We also continue to discover small molecules at a faster rate than we determine their functionality. The sheer expanse of small molecules expressed in the metabolomes of *C. elegans* and other worms hints at their great capacity to determine even more behaviors than we have already uncovered. Additionally, the continued expansion of metabolite research into other nematode species furthers our understanding of the complexities of these signals and their conservation or specificity across species.

1.9 Thesis Summary

In this work, we seek more information on the formation and function of ascarosides in the nematodes *C. elegans* and *C. briggsae*. In Chapter 2, we create mutant strains for all genes in the *oac* gene family. Through this, we are able to determine that the *oac* gene family is, surprisingly, not associated with ascaroside biosynthesis, as predicted due to O-acyltransferase enzymatic activity. However, *oac* mutants do affect the production and secretion of ascarosides. We also show that *bus-1* affects longevity by extending lifespan by 50%.

In Chapter 3, we discuss the creation of a new CRISPR/Cas9 method for the nematode *C. briggsae*. This enables us to create precise mutations previously inaccessible to researchers. In Appendix A, we examine the *Cbr-glo-1* mutant, found to be phenotypically equivalent to the *Cel-glo-1* mutant strain in its inability to produce 4’ modified ascarosides.

In Chapter 4, we find that the *Cbr-daf-22* mutant is also phenotypically equivalent to its *C. elegans* counterpart, *Cel-daf-22*, in that it cannot produce any short-chain ascarosides. It can, however, produce another group of small molecules, glucosides. This information, coupled with

our knowledge of *Cbr-glo-1*, allows us to perform studies on crude dauer pheromone in both *C. elegans* and *C. briggsae* where we find that, like *C. elegans*, a main component of the *C. briggsae* dauer pheromone is ascr#2. Additionally, we discover the existence of an anti-dauer compound, or group of compounds, produced by both *Cbr-daf-22* and *Cel-daf-22* mutants, which we hypothesize to be glucosides.

1.10 Figures and Tables

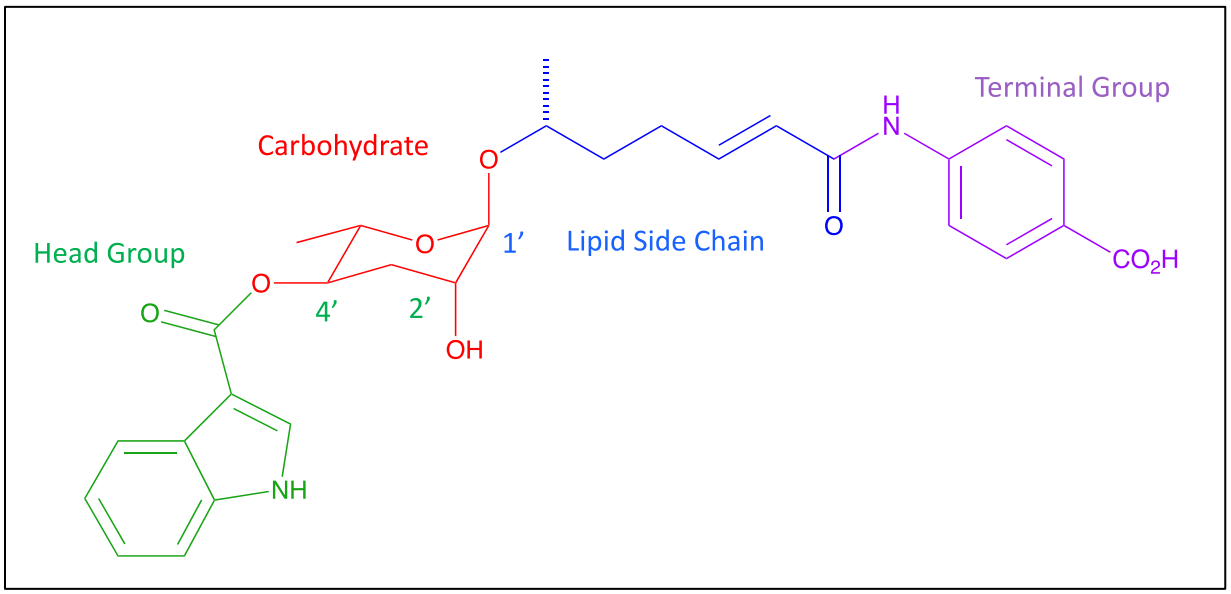


Figure 1.1 Ascarosides are formed modularly. Ascarosides start with an ascarylose sugar base. On this base, a 1' modification, usually a fatty acid side-chain, is added. This side chain can be modified by length or through saturation. Further modifications can be made by adding terminal groups to the α-end of the fatty acid, or by adding head groups to the 4' or 2' positions of the carbohydrate.

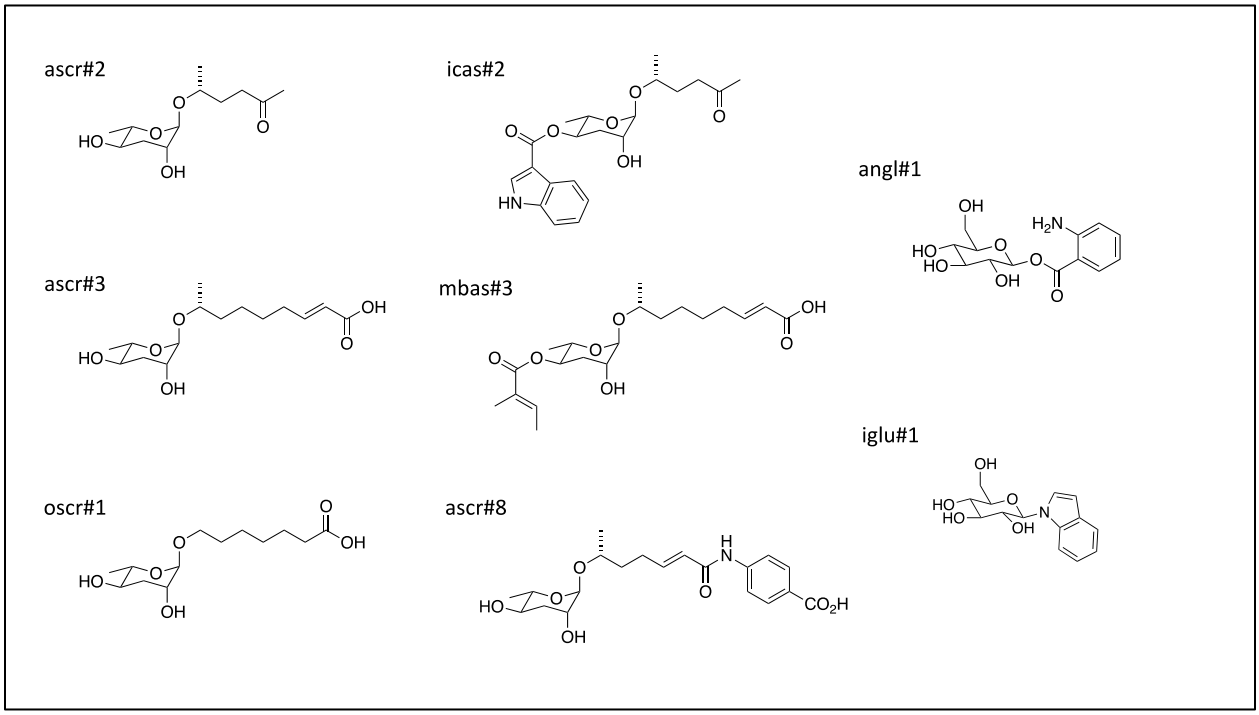


Figure 1.2 The wide diversity of ascaroside signaling molecules. ascr#2 exemplifies a simple saturated ascaroside while ascr#3 shows a simple unsaturated ascaroside. oscr#1 shows a (ω)-linked fatty-acid side chain rather than the (ω-1)-link found in ascr#2 and ascr#3. icas#2 and mbas#3 exemplify 4' head group modifications while ascr#8 shows a terminal group modification. Small molecules that use a different sugar than ascarylose include angl#1 and iglu#1.

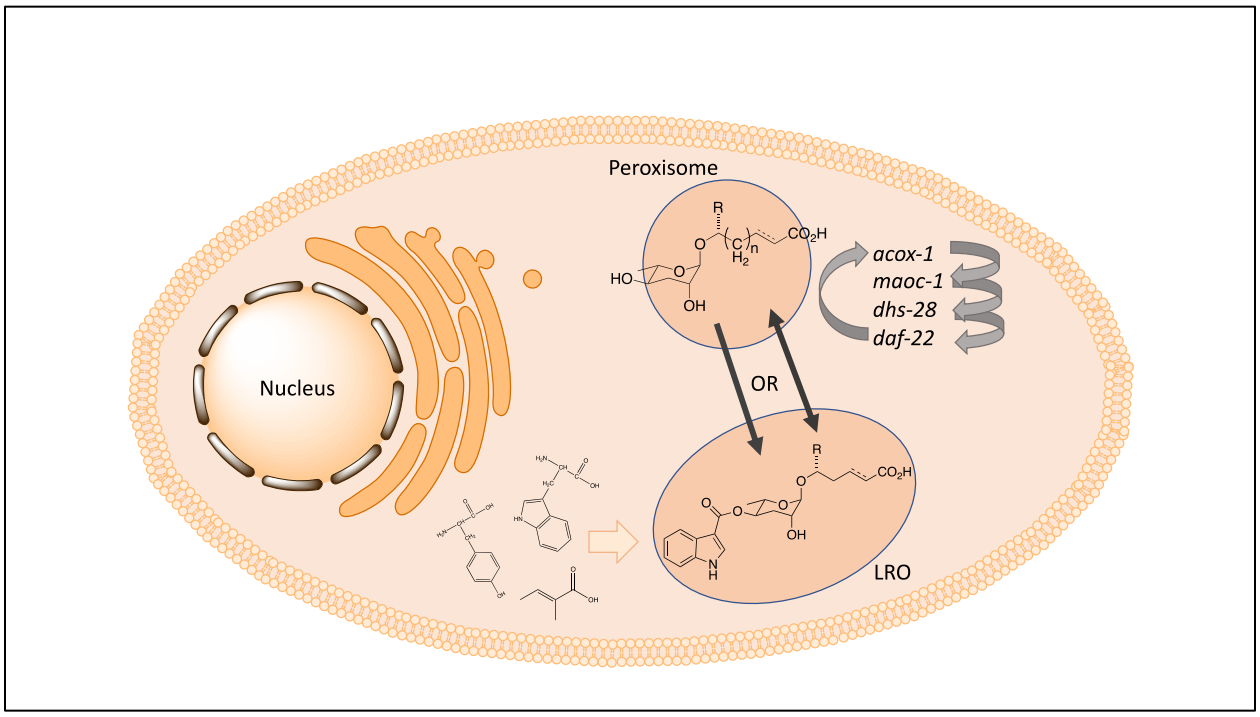


Figure 1.3 A model for ascaroside biosynthesis. Although we do not currently know how the sugar and fatty acid side chain moieties are attached, shortening of the fatty acid side chain occurs through iterative peroxisomal β-oxidation using the genes *acox-1*, *maoc-1*, *dhs-28*, and *daf-22*. Further modification occurs in the lysosomal related organelles (LROs) where CEST enzymes work to attach 4' modified head groups made from derivatives of amino acid metabolism. Some modified ascarosides then return for further side-chain processing in the peroxisome.

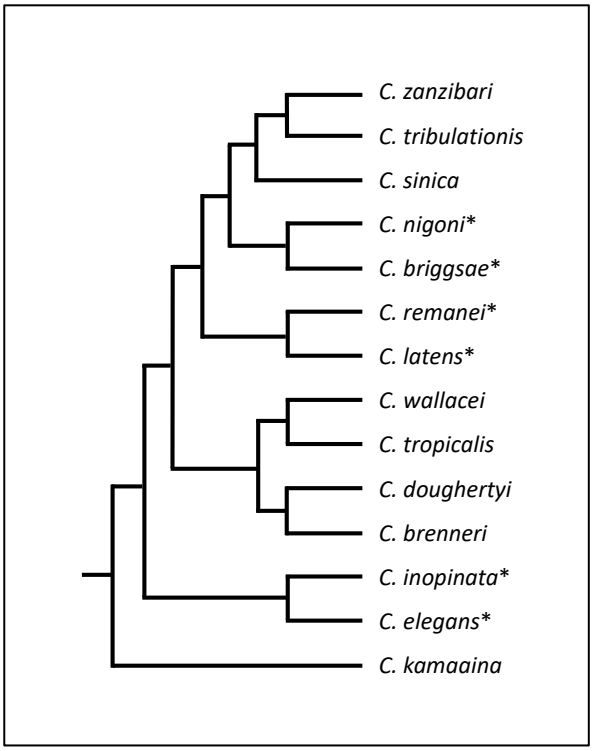
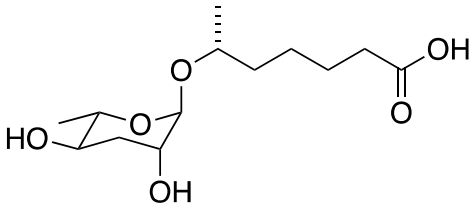
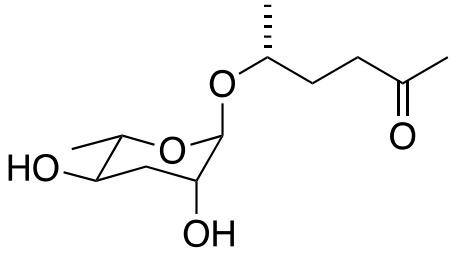
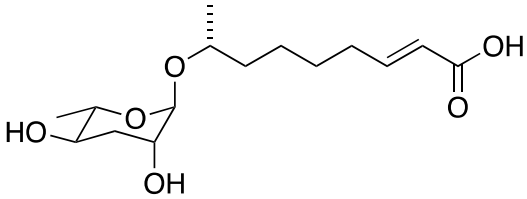
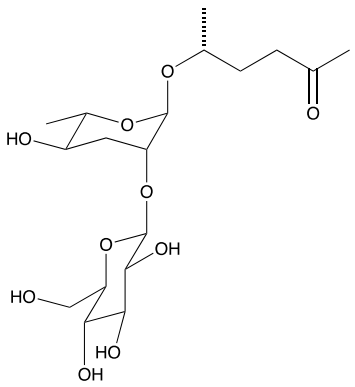
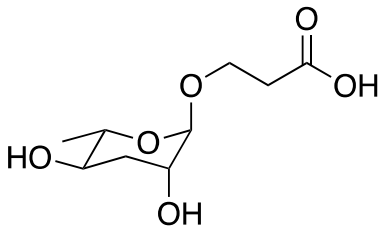
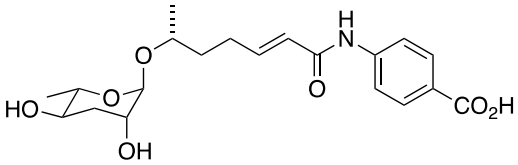
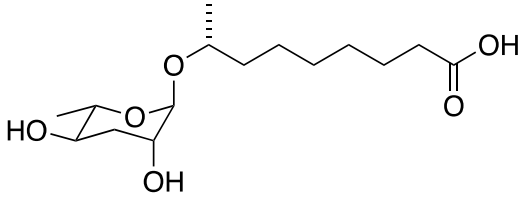
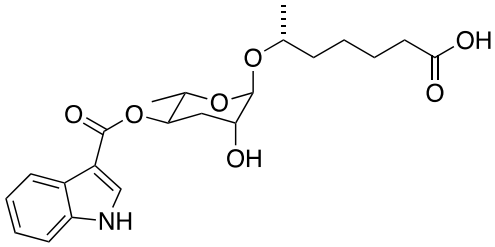
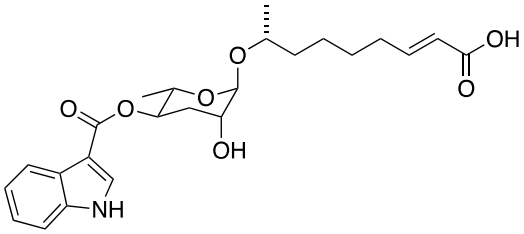
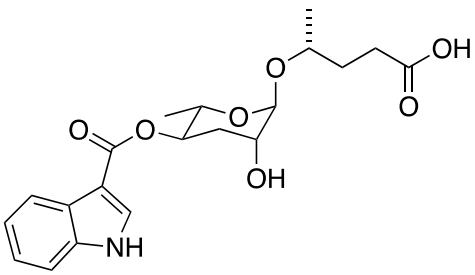
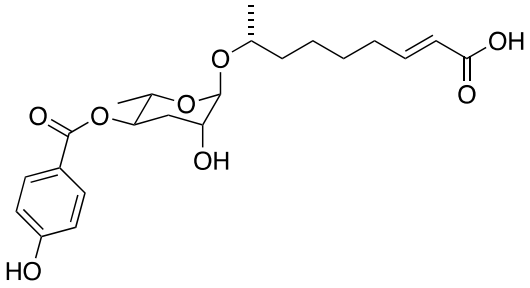
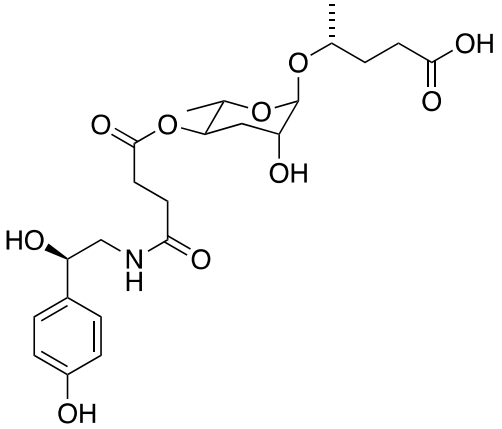
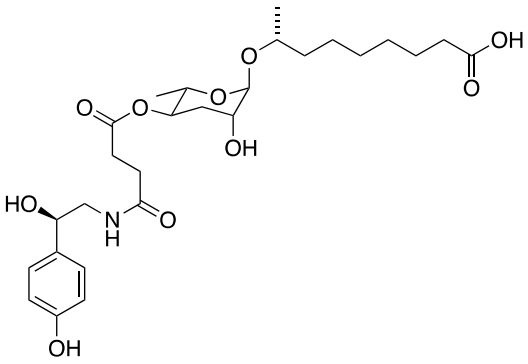
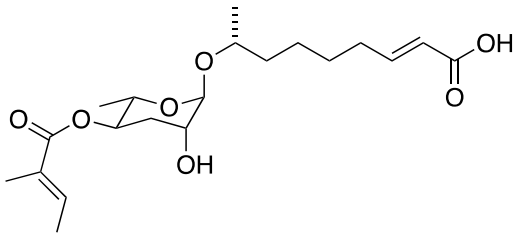


Figure 1.4 The elegans subgroup of the *Caenorhabditis* genus. Notation is made of any species in which ascaroside profiles have been studied.

Table 1.1 Ascarosides in *C. elegans* that have a specific, defined function.

Ascaroside Name	Structure	Known Function(s) in *C. elegans*	References
ascr#1		• Very weak dauer signal	• Jeong *et al.* 2005
ascr#2		• Main component of dauer pheromone in high concentrations • Signal of population density • Male attractant in low concentrations • Effects a starvation response in adults	• Butcher *et al.* 2007 • Srinivasan *et al.* 2008 • Kaplan *et al.* 2011 • von Reuss *et al.* 2012 • Ludewig *et al.* 2013
ascr#3		• Main component of dauer signal in high concentrations • Signal of food availability • Male attractant in low concentrations • Most common ascaroside in hermaphrodites • Effects a starvation response in adults	• Butcher *et al.* 2007 • Srinivasan *et al.* 2008 • Kaplan *et al.* 2011 • von Reuss *et al.* 2012 • Izrayelit *et al.* 2012 • Ludewig *et al.* 2013 • Aprison and Ruvinsky 2017
ascr#4		• Component of male attractant signal	• Srinivasan *et al.* 2008 • Kaplan *et al.* 2011

ascr#5		• Component of dauer signal	• Butcher *et al.* 2008
ascr#8		• Component of dauer signal in high concentrations • Male attractant in low concentrations	• Pungaliya *et al.* 2009 • Kaplan *et al.* 2011
ascr#10		• Main component of hermaphrodite attraction signal • Most common ascaroside in males	• Aprison and Ruvinsky 2017 • Izrayelit *et al.* 2012
icas#1		• General worm attractant and aggregation signal	• Srinivasan *et al.* 2012
icas#3		• General worm attractant and aggregation signal	• Izrayelit *et al.* 2012 • Srinivasan *et al.* 2012
icas#9		• Component of dauer signal in high concentrations • Hermaphrodite attractant in low concentrations	• Butcher *et al.* 2009a • Srinivasan *et al.* 2012

hbas#3		• General worm attractant and aggregation signal	• von Reuss *et al*. 2012
osas#9		• Released by starved L1s	• Artyukhin *et al*. 2013
osas#10		• Released by well-fed L1s	• Artyukhin *et al*. 2013
mbas#3		• Non-food-sensitive dispersal signal	• Zhang *et al*. 2017

1.11 References

Aprison EZ, Ruvinsky I. 2016. Sexually antagonistic male signals manipulate germline and soma of *C. elegans* hermaphrodites. Curr Biol. 26(20):2827-2833. doi:10.1016/j.cub.2016.08.024.

Aprison EZ, Ruvinsky I. 2017. Counteracting ascarosides act through distinct neurons to determine the sexual identity of *C. elegans* pheromones. Curr Biol. 27(17):2589-2599.e3. doi:10.1016/j.cub.2017.07.034.

Artyukhin AB, Yim JJ, Srinivasan J, Izrayelit Y, Bose N, von Reuss SH, Jo Y, Jordan JM, Baugh LR, Cheong M, Sternberg PW, Avery L, Schroeder FC. 2013. Succinylated octopamine ascarosides and a new pathway of biogenic amine metabolism in *Caenorhabditis elegans*. J Biol Chem. 288(26):18778-18783. doi:10.1074/jbc.C113.477000.

Artyukhin AB, Zhang YK, Akagi AE, Panda O, Sternberg PW, Schroeder FC. 2018. Metabolomic "dark matter" dependent on peroxisomal β-oxidation in *Caenorhabditis elegans*. J Am Chem Soc. 140(8):2841-2852. doi:10.1021/jacs.7b11811.

Bargmann CI, Horvitz HR. 1991. Control of larval development by chemosensory neurons in *Caenorhabditis elegans*. Science. 251(4998):1243-1246. doi:10.1126/science.2006412.

Bartley JP, Bennett EA, Darben PA. 1996. Structure of the ascarosides from *Ascaris suum*. J Nat Prod. 59(10):921-926. doi:10.1021/np960236+.

Bento G, Ogawa A, Sommer RJ. 2010. Co-option of the hormone-signaling module dafachronic acid – DAF-12 in nematode evolution. Nature. 466(7305):494-497. doi:10.1038/nature09164.

Bergame CP, Dong C, Sutour S, von Reuss SH. 2019. Epimerization of an ascaroside-type glycolipid downstream of the canonical β-oxidation cycle in the nematode *Caenorhabditis nigoni*. Org Lett. 21(24):9889-9892. doi:10.1021/acs.orglett.9b03808.

Bose N, Ogawa A, von Reuss SH, Yim JJ, Ragsdale EJ, Sommer RJ, Schroeder FC. 2012. Complex small-molecule architectures regulate phenotypic plasticity in a nematode. Angew Chem Int Ed Engl. 51(50):12438-12443. doi:10.1002/anie.201206797.

Brenner S. 1974. The Genetics of *Caenorhabditis elegans*. Genetics. 77:71-94.

Butcher RA, Fujita M, Schroeder FC, Clardy J. 2007. Small-molecule pheromones that control dauer development in *Caenorhabditis elegans*. Nat Chem Biol. 3(7):420-422. doi:10.1038/nchembio.2007.3.

Butcher RA, Ragains JR, Kim E, Clardy J. 2008. A potent dauer pheromone component in *Caenorhabditis elegans* that acts synergistically with other components. Proc Natl Acad Sci USA. 105(38):14288-14292. doi:10.1073/pnas.0806676105.

Butcher RA, Ragains JR, Clardy J. 2009a. An indole-containing dauer pheromone component with unusual dauer inhibitory activity at higher concentrations. Org Lett. 11(14):3100-3103. doi:10.1021/ol901011c.

Butcher RA, Ragains JR, Li W, Ruvkun G, Clardy J, Mak HY. 2009b. Biosynthesis of the *Caenorhabditis elegans* dauer pheromone. Proc Natl Acad Sci USA. 106(6):1875-1879. doi:10.1073/pnas.0810338106.

The *C. elegans* Sequencing Consortium. 1998. Genome sequence of the nematode *C. elegans*: a platform for investigating biology. Science. 282(5396):2012-2018. doi:10.1126/science.282.5396.2012.

Cassada RC, Russell RL. 1975. The dauerlarva, a post-embryonic developmental variant of the nematode *Caenorhabditis elegans*. Dev Biol. 46(2):326-342. doi:10.1016/0012-1606(75)90109-8.

Chasov JR, So WK, Chan CM, Chow KL. 2007. The species, sex, and stage specificity of a *Caenorhabditis* sex pheromone. Proc Natl Acad Sci USA. 104(16):6730-6735. doi:10.1073/pnas.0608050104.

Choe A, von Reuss SH, Kogan D, Gasser RB, Platzer EG, Schroeder FC, Sternberg PW. 2012a. Ascaroside signaling is widely conserved among nematodes. Curr Biol. 22(9):772-780. doi:10.1016/j.cub.2012.03.024.

Choe A, Chuman T, von Reuss SH, Dossey AT, Yim JJ, Ajredini R, Kolawa AA, Kaplan F, Alborn HT, Teal PEA, Schroeder FC, Sternberg PW, Edison AS. 2012b. Sex-specific mating pheromones in the nematode *Panagrellus redivivus*. Proc Natl Acad Sci USA. 109(51):20949-20954. doi:10.1073/pnas.1218302109.

Chute CD, Srinivasan J. 2014. Chemical mating cues in *C. elegans*. Seminars in Cell & Developmental Biology. 33:18-24. doi:10.1016/j.semcdb.2014.06.002.

Coburn C, Allman E, Mahanti P, Benedetto A, Cabreiro F, Pincus Z, Matthijssens F, Araiz C, Mandel A, Vlachos M, Edwards SA, Fischer G, Davidson A, Pryor RE, Stevens A, Slack FJ, Tavernarakis N, Braeckman BP, Schroeder FC, Nehrke K, Gems D. 2013. Anthranilate fluorescence marks a calcium-propagated necrotic wave that promotes organismal death in *C. elegans*. PLoS Biol. 11(7):e1001613. doi:10.1371/journal.pbio.1001613.

Coburn C, Gems D. 2013. The mysterious case of the *C. elegans* gut granule: death fluorescence, anthranilic acid, and the kynurenine pathway. Front Genet. 4. doi:10.3389/fgene.2013.00151.

Cook SJ, Jarrell, TA, Brittin CA, Wang Y, Bloniarz AE, Yakovlev MA, Nguyen KCQ, Tang LT-H, Bayer EA, Duerr JS, *et al*. 2019. Whole-animal connectomes of both *Caenorhabditis elegans* sexes. Nature. 571(7763):63-71. doi:10.1038/s41586-019-1352-7.

Curtis BJ, Kim LJ, Wrobel CJJ, Eagan JM, Smith RA, Burch JE, Le HH, Artyukhin AB, Nelson HM, Schroeder FC. 2020. Identification of uric acid gluconucleoside-ascaroside conjugates in *Caenorhabditis elegans* by combining synthesis and MicroED. Org Lett. 22(17):6724-6728. doi:10.1021/acs.orglett.0c02038.

Dolke F, Dong C, Bandi S, Paetz C, Glauser G, von Reuss SH. 2019. Ascaroside signaling in the bacterivorous nematode *Caenorhabditis remanei* encodes the growth phase of its bacterial food source. Org Lett. 21(15):5832-5837. doi:10.1021/acs.orglett9b01914.

Dong C, Dolke F, von Reuss SH. 2016. Selective MS screening reveals a sex pheromone in *Caenorhabditis briggsae* and species-specificity in indole ascaroside signaling. Organic & Biomolecular Chemistry. 14(30):7217-7225. doi:10.1039/C6OB01230B.

Edison AS. 2009. *Caenorhabditis elegans* pheromones regulate multiple complex behaviors. Curr Opin Neurobiol. 19(4):378-388. doi:10.1016/j.conb.2009.07.007.

Faghih N, Bhar S, Zhou Y, Dar AR, Mai K, Bailey LS, Basso KB, Butcher RA. 2020. A large family of enzymes responsible for the modular architecture of nematode pheromones. J Am Chem Soc. 142:13645-13650. doi:10.1021/jacs.0c04223.

Falke JM, Bose N, Artyukhin AB, Rödelsperger C, Markov GV, Yim JJ, Grimm D, Claassen MH, Panda O, Baccile JA, Zhang YK, Le HH, Jolic D, Schroeder FC, Sommer RJ. 2018. Linking genomic and metabolomic natural variation uncovers nematode pheromone biosynthesis. Cell Chem Biol. 25(6):787-796.e12. doi:10.1016/j.chembiol.2018.04.004.

Fouquey C, Polonsky J, Lederer E. 1958. Structure Chimique de L'Ascarylose. Bull Soc Chim Biol (Paris). 40(2–3):315–325.

Golden JW, Riddle DL. 1982. A pheromone influences larval development in the nematode *Caenorhabditis elegans*. Science. 218(4572):578-580. doi:10.1126/science.6896933.

Golden JW, Riddle DL. 1984a. A *Caenorhabditis elegans* dauer-inducing pheromone and an antagonistic component of the food supply. J Chem Ecol. 10(8):1265-1280. doi:10.1007/BF00988553.

Golden JW, Riddle DL. 1984b. The *Caenorhabditis elegans* dauer larva: developmental effects of pheromone, food, and temperature. Dev Biol. 102(2):368-378. doi:10.1016/0012-1606(84)90201-X.

Golden JW, Riddle DL. 1984c. A pheromone-induced developmental switch in *Caenorhabditis elegans*: temperature-sensitive mutants reveal a wild-type temperature-dependent process. Proc. Natl. Acad. Sci. USA. 81(3):819-823.

Golden JW, Riddle DL. 1985. A gene affecting production of the *Caenorhabditis elegans* dauer-inducing pheromone. Mol Gen Genet. 198(3):534-536. doi:10.1007/bf00332953.

Goodman M. 2006. Mechanosensation. WormBook. doi:10.1895/wormbook.1.62.1.

Green JS, Brown M, Dobosiewicz M, Ishida IG, Macosko EZ, Ahang X, Butcher RA, Cline DJ, McGrath PT, Bargmann CI. 2016. Balancing selection shapes density-dependent foraging behavior. Nature. 539(7628):254-258. doi:10.1038/nature19848.

Hermann GJ, Schroeder LK, Hieb CA, Kershner AM, Rabbitts BM, Fonarev P, Grant BD, Priess JR. 2005. Genetic analysis of lysosomal trafficking in *Caenorhabditis elegans*. Mol Biol Cell. 16(7):3273-3288. doi:10.1091/mbc.e05-01-0060.

Izrayelit Y, Srinivasan J, Campbell SL, Jo Y, von Reuss SH, Genoff MC, Sternberg PW, Schroeder FC. 2012. Targeted metabolomics reveals a male pheromone and sex-specific ascaroside biosynthesis in *Caenorhabditis elegans*. ACS Chem Biol. 7(8):1321-1325. doi:10.1021/cb300169c.

Jeong P-Y, Jung M, Yim Y-H, Kim H, Park M, Hong E, Lee W, Kim YH, Kim K, Paik Y-K. 2005. Chemical structure and biological activity of the *Caenorhabditis elegans* dauer-inducing pheromone. Nature. 433(7025):541-545. doi:10.1038/nature/03201.

Jezyk PF, Fairbairn D. 1967. Ascarosides and ascaroside esters in *Ascaris lumbricoides* (Nematoda). Comparative Biochemistry and Physiology. 23(3):691-705. doi:10.1016/0010-406X(67)90334-9.

Joo H-J, Yim Y-H, Jeong P-Y, Jin Y-X, Lee JE, Kim H, Jeong S-K, Chitwood DJ, Paik Y-K. 2009. *Caenorhabditis elegans* utilizes dauer pheromone biosynthesis to dispose of toxic peroxisomal fatty acids for cellular homeostasis. Biochemical Journal. 422(1):61-71. doi:10.1042/BJ20090513.

Joo H-J Kim K-Y, Yim Y-H, Jin Y-X, Kim H, Kim M-Y, Paik Y-K. 2010. Contribution of the peroxisomal *acox* gene to the dynamic balance of daumone production in *Caenorhabditis elegans*. J Biol Chem. 285(38):29319-29325. doi:10.1074/jbc.M110.122663.

Kanzaki N, Tsai IJ, Tanaka R, Hunt VL, Liu D, Tsuyama K, Maeda Y, Namai S, Kumagai R, Tracey A, Holroyd N, Doyle SR, Woodruff GC, Murase K, Kitazume H, Chai C, Akagi A, Panda O, Ke HM, Schroeder FC, Wang J, Berriman M, Sternberg PW, Sugimoto A, Kikuchi T. 2018. Biology and genome of a newly discovered sibling species of *Caenorhabditis elegans*. Nature Communications. 9(1):3216. doi:10.1038/s41467-018-05712-5.

Kaplan F, Srinivasan J, Mahanti P, Ajredini R, Durak O, Nimalendran R, Sternberg PW, Teal PEA, Schroeder FC, Edison AS, Alborn HT. 2011. Ascaroside expression in *Caenorhabditis elegans* is strongly dependent on diet and developmental stage. PLoS One. 6(3):e17804. doi:10.1371/journal.pone.0017804.

Le H, Wrobel CJ, Cohen SM, Yu J, Park H, Helf MJ, Curtis BJ, Kruempel JC, Rodrigues PR, Hu PJ, Sternberg PW, Schroeder FC. 2020. Modular metabolite assembly in *Caenorhabditis elegans* depends on carboxylesterases and formation of lysosome related organelles. eLife. 9:e61886. doi:10.7554/eLife.61886.

Ludewig AH, Schroeder FC. 2012. Ascaroside signaling in *C. elegans*. WormBook. 1-22. doi:10.1895/wormbook.1.155.1.

Ludewig AH, Izrayelit Y, Park D, Malik RU, Zimmermann A, Mahanti P, Fox BW, Bethke A, Doering F, Riddle DL, Schroeder FC. 2013. Pheromone sensing regulates *Caenorhabditis elegans* lifespan and stress resistance via the deacetylace SIR-2.1. Proc Natl Acad Sci USA. 110(14):5522-5527. doi:10.1073/pnas.1214467110.

Markov GV, Meyer JM, Panda O, Artyukhin AB, Claaßen M, Witte H, Schroeder FC, Sommer RJ. 2016. Functional conservation and divergence of *daf-22* paralogs in *Pristionchus pacificus* dauer development. Mol Biol Evol. 33(10):2506-2514. doi:10.1093/molbev/msw090.

Meyer SLF, Johnson G, Dimock M, Fahey JW, Huettel RN. 1997. Field efficacy of *Verticillium lecanii*, sex pheromone, and pheromone analogs and potential management agents for soybean cyst nematode. J Nematol. 29(3):282-288.

Miller MB, Bassler BL. 2001. Quorum Sensing in Bacteria. Annual Review of Microbiology. 55(1):165-199. doi:10.1146/annurev.micro.55.1.165.

Noguez JH, Conner ES, Zhou Y, Ciche TA, Gagains JR, Butcher RA. 2012. A novel ascaroside controls the parasitic life cycle of the entomophathogenic nematode *Heterorhabditis bacteriophora*. ACS Chem Biol. 7(6):961-966. doi:10.1021/cb300056q.

Panda O, Akagi AE, Artyukhin AB, Judkins JC, Le HH, Mahanti P, Cohen SM, Sternberg PW, Schroeder FC. 2018. Biosynthesis of Modular Ascarosides in *C. elegans*. Angew Chem Int Ed Engl. 56(17):4729-4733. doi:10.1002/anie.201700103.

Phani V, Khan MR, Dutta TK. 2021. Plant-parasitic nematodes as a potential threat to protected agriculture: current status and management options. Crop Protection. 144:105573. doi:10.1016/j.cropro.2021.105573.

Pungaliya C, Srinivasan J, Fox BW, Malik RU, Ludewig AH, Sternberg PW, Schroeder FC. 2009. A shortcut to identifying small molecule signals that regulate behavior and development in *Caenorhabditis elegans*. Proc Nat Acad Sci USA. 106(19):7708-7713. doi:10.1073/pnas.0811918106.

Rabbitts BM, Ciotti MK, Miller NE, Kramer M, Lawrenson AL, Levitte S, Kremer S, Kwan E, Weis AM, Hermann GJ. 2008. *glo-3*, a novel *Caenorhabditis elegans* gene, is required for lysosome-related organelle biogenesis. Genetics. 180(2):857-871. doi:10.1534/genetics.108.093534.

Ragsdale EJ, Müller MR, Rödelsperger C, Sommer RJ. 2013. A developmental switch coupled to the evolution of plasticity acts through a sulfatase. Cell. 155(4):922-933. doi:10.1016/j.cell.2013.09.054.

Regnier FE, Law JH. 1968. Insect pheromones. Journal of Lipid Research. 9(5):541-551. doi:10.1016/S0022-2275(20)42699-9.

Schaedel ON, Gerisch B, Antebi A, Sternberg PW. 2012. Hormonal signal amplification mediates environmental conditions during development and controls irreversible commitment to adulthood. PLoS Biol. 10(4):e1001306. doi:10.1371/journal.pbio.1001306.

Srinivasan J, Kaplan F, Ajredini R, Zachariah C, Alborn HT, Teal PEA, Malik RU, Edison AS, Sternberg PW, Schroeder FC. 2008. A blend of small molecules regulates both mating and development in *Caenorhabditis elegans*. Nature. 454(7208):1115-1118. doi:10.1038/nature07168.

Srinivasan J, von Reuss SH, Bose N, Zaslaver A, Mahanti P, Ho MC, O'Doherty OG, Edison AS, Sternberg PW, Schroeder FC. 2012. A modular library of small molecule signals regulates social behaviors in *Caenorhabditis elegans*. PLoS Biol. 10(1):e1001237. doi:10.1371/journal.pbio.1001237.

Stepek G, Buttle DJ, Duce IR, Behnke JM. 2006. Human gastrointestinal nematode injections: are new control methods required? Int J Exp Pathol. 87(5):325-341. doi:10.1111/j.1365-2613.2006.00495.x.

Stupp GS, von Reuss Sh, Izrayelit Y, Ajredini R, Schroeder FC, Edison AS. 2013. Chemical detoxification of small molecules by *C. elegans*. ACS Chem Biol. 8(2):309-313. doi:10.1021/cb300520u.

Sulston JE, Horvitz HR. 1977. Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*. Dev Biol. 56(1):110-156. doi:10.1016/0012-1606(77)90158-0.

Sulston JE, Schierenberg E, White JG, Thomson JN. 1983. The embryonic cell lineage of the nematode *Caenorhabditis elegans*. Dev Biol. 100(1):64-119. doi:10.1016/0012-1606(83)90201-4.

von Reuss SH, Bose N, Srinivasan J, Yim JJ, Judkins JC, Sternberg PW, Schroeder FC. 2012. Comparative metabolomics reveals biogenesis of ascarosides, a modular library of small-molecule signals in *C. elegans*. J Am Chem Soc. 134(3):1817-1824. doi:10.1021/ja210202y.

Wharam B, Weldon L, Viney M. 2017. Pheromone modulates two phenotypically plastic traits – adult reproduction and larval diapause – in the nematode *Caenorhabditis elegans*. BMC Evolutionary Biology. 17(1):197. doi:10.1186/s12862-017-1033-9.

Wyatt TD. 2009. Fifty years of pheromones. Nature. 457(7227):262-263. doi:10.1038/457262a.

Yim JJ, Bose N, Meyer JM, Sommer RJ, Schroeder FC. 2015. Nematode signaling molecules derived from multimodular assembly of primary metabolic building blocks. Org Lett. 17(7):1648-1651. doi:10.1021/acs.orglett.5b00329.

Zhang X, Feng L, Chinta S, Singh P, Want Y, Nunnery JK, Butcher RA. 2015. Acyl-CoA oxidase complexes control the chemical message produced by *Caenorhabditis elegans*. Proc Natl Acad Sci USA. 112(13):3955-3960. doi:10.1073/pnas.1423951112.

Zhang X, Wang Y, Perez DH, Jones Lipinsky RA, Butcher RA. 2018. Acyl-CoA oxidases fine-tune the production of ascaroside pheromones with specific side chain lengths. ACS Chem Biol. 13(4):1048-1056. doi:10.1021/acschembio.7b01021.

Zhang YK, Sanchez-Ayala MA, Sternberg PW, Srinivasan J, Schroeder FC. 2017. Improved synthesis for modular ascarosides uncovers biological activity. Org Lett. 19(11):2837-2840. doi:10.1021/acs.orglett.7b01009.

Zhao L, Zhang X, Wei Y, Zhou J, Zhang W, Qin P, Chinta S, Kong X, Liu Y, Yu H, Hu S, Zou Z, Butcher RA, Sun J. 2016. Ascarosides coordinate the dispersal of a plant-parasitic nematode with the metamorphosis of its vector beetle. Nature Communications. 7(1):12341. doi:10.1038/ncomms12341.

Zhou Y, Wang Y, Zhang X, Bhar S, Jones Lipinski RA, Han J, Feng L, Butcher RA. 2018. Biosynthetic tailoring of existing ascaroside pheromones alters their biological function in *C. elegans*. eLife. 7:e33286. doi:10.7554/eLife.33286.

Zwaal RR, Mendel JE, Sternberg PW, Plasterk RHA. 1997. Two neuronal G proteins are involved in chemosensation of the *Caenorhabditis elegans* dauer-inducing pheromone. Genetics. 145(3):715-727.

Chapter 2

O-ACYLTRANSFERASES IN *C. ELEGANS*

2.1 Abstract

One approach to understand the functions of gene families is to systematically study the phenotypes of loss-of-function mutations. *C. elegans* molecular genetics has advanced to the point that we can examine not only individual genes, but entire gene families. The 64-member O-acyltransferase (*oac*) gene family in *C. elegans* has been relatively under-studied. Here, we define the *oac* gene family as the 60 named *oac* genes as well as four other genes – *bus-1*, *rhy-1*, *ndg-4*, and *nrf-6* – that share the characteristic acyltransferase 3 and nose resistant-to-fluoxetine (NRF) protein domains. In addition to obtaining the four existing mutants, we used gene editing to make mutant strains for 56 additional genes encoding O-acyltransferases proteins in order to study their longevity and metabolomics. We discovered that one gene, *bus-1*, limits lifespan. We also detected changes in production as well as secretion in signaling small molecules in many mutants, which could be downstream of these genes.

2.2 Introduction

The nematode *Caenorhabditis elegans* has been used as a model organism since the 1970s (Ankeny 2001). It was the first multicellular, eukaryotic organism to have a fully annotated genome; it has a fully described neural connectome; it is hermaphroditic and transparent, which allows for easy genetic and microscopy studies; it is easily cultured; and it has a short lifespan (The *C. elegans* Sequencing Consortium 1998; Cook *et al* 2019; Brenner 1974). Especially important for its use as a model organism is its basal genetic and cellular similarity to higher organisms (reviewed by Ankeny 2001; Apfeld and Alper 2018).

C. elegans has approximately 20,000 genes (The *C. elegans* Sequencing Consortium 1998; Yoshimura *et al* 2019). However, of these 19,987 genes, only 52.3% (10,450 genes) have been named (Harris *et al*. 2020). While many genes are named for phenotypes directly studied in *C. elegans*, genes are also often named due to homology with researched genes in other model organisms or to establish or emulate protein domains (Tuli *et al* 2018). Within all 10,450 *C. elegans* genes that have been named, only 4,098 (39.2%) have any described phenotype or other information derived from nematode-based *in-vivo* or *in-vitro* experiments, excluding RNAseq and RNAi information (Harris *et al*. 2020).

O-acyltransferases are a subset of the acyltransferase class of enzymes that catalyze the transfer of acyl groups onto hydroxyl groups. This contrasts them with N-acyltransferases which transfer acyl groups onto amines. O-acyltransferases are ubiquitous throughout the cellular environment and are used in many various pathways ranging from bacterial growth of biofilms to Wnt signaling in flies to the conversion of cholesterol to cholesteryl ester in humans (Röttig and Steinbüchel 2013; Logan and Nusse 2004; Spector *et al* 1979). The most commonly studied form of O-acyltransferases are the membrane-bound O-acyltransferases, which include ACAT1 and PORCN and their homologs in *Drosophila* and other model organisms (Chang et al 1993; Chang *et al*. 2011).

C. elegans has several named families of membrane-bound O-acyltransferases, such as *mboa* (membrane-bound O-acyltransferase), *mom* (more of MS), and *oac* (O-acyltransferase) (Harris *et al*. 2020). The *mboa* gene family (10 genes), and the *mom* gene family (5 genes), all contain a

membrane-bound O-acyltransferase domain (MBOAT) (InterPro IPR004299; Pfam PF03062); and are related to genes in the Wnt and Hedgehog pathways (Lee *et al* 2008; Logan and Nusse 2004). However, the largest yet least-studied class of O-acyltransferases in *C. elegans* is the aptly named *oac* gene family, which contains a different protein domain, the acyltransferase 3 domain (InterPro IPR002656; Pfam PF01757). The OAC class of enzymes was named due to its predicted ability to transfer acyl groups other than amino-acyl groups, but has otherwise been unstudied in *C. elegans*.

To fully allow us to understand the *C. elegans* organism, we need to systematically create mutants and examine gene families rather than relying on a shotgun approach. In this paper, we chose the under-studied O-acyltransferase gene family to research homology, create strains, and study potential functions. To this end, we compared the protein sequences and created protein trees to understand how the genes/proteins were related and found that four additional genes – *bus-1*, *rhy-1*, *ndg-4*, and *nrf-6* – should be included in the *oac* gene family. We then created at least one isogenic mutant strain for each protein-coding *oac* for which there was not already an existing complete loss-of-function mutant – several of these mutants are also revertible (Wang *et al*. 2018; Le *et al*. 2020). Although there are several strains available that contain an *oac* mutation as well as numerous other mutations, we felt that these were less useful for identifying the functions of *oac* genes than individual mutant strains (Panda *et al*. 2018). We also studied the lifespan and metabolomics of the *oac* gene class as first phenotypic analyses of these mutants.

2.3 Results

Phylogenetic Analysis of the *oac* Gene/Protein Family

The *oac* gene family in *C. elegans* comprises 64 genes (Fig 2.1a). Four of these genes – *oac-18*, *oac-33*, *oac-47*, and *oac-60* – are pseudogenes. The 60 protein-coding *oac* genes are generally defined as having an active acyltransferase 3 domain. This is in contrast to the other classes of O-acyltransferases in *C. elegans* that use distinct domains to catalyze their acyltransferase activity.

The acyltransferase 3 domain is highly conserved across species. Proteins containing an acyltransferase 3 domain have been studied in several species of bacteria where they aid in cell surface modifications related to lysosome and bacteriophage resistance (Bera *et al.* 2005; Pearson *et al.* 2020). The domain is most numerous in nematodes and fruit flies while mammals have only one known protein with an acyltransferase 3 domain (Zhu *et al* 2007). In most nematode species, there are only a handful of *oac* genes (*Pristionchus pacificus* has 5; *Brugia malayi* has 2), but there is a marked increase in the *Caenorhabditis* genus (*C. briggsae* has 22; *C. remanei* has 36) which culminates in the expansion to 60 *oac* genes in *C. elegans* (Harris *et al.* 2020). The acyltransferase 3 domain, however, has been scantly studied in eukaryotes as it was lost in humans during the evolution of the great apes, but proteins with this domain have been predicted to be multi-pass transmembrane proteins with acyltransferase activity (Zhu *et al* 2007).

Three additional genes were found to include this *oac*-specific acyltransferase-3 domain: *bus-1*, *rhy-1*, and *nrf-6*. These genes were named prior to the designation of the *oac* gene class. *bus-1* is a gene that is expressed in the rectal epithelial cells, and *bus-1* mutants are unaffected by the nematode rectal pathogen *Microbacterium nematophilum*, which causes tail-swelling, hence the

name bacterially unswollen (Gravato-Nobre and Hodgkin 2008). A regulator of the hypoxia-inducible factor HIF-1 through the *egl-9* pathway, mutants of *rhy-1* exhibit some egg-laying defects (Shen *et al* 2006). Fluoxetine (Prozac) causes *C. elegans* nose-muscle contractions, yet nose resistant-to-fluoxetine (*nrf*) mutants are immune to this phenomenon (Choy and Thomas 1999). *nrf-6* mutants also display a pale-egg phenotype caused by a lack of yolk granules – this causes subsequent retarded development and partial embryonic lethality (Choy and Thomas 1999).

The OAC proteins generally fall into two categories: 1) 40 OACs have an acyltransferase 3 domain with a C-terminal SGNH hydrolase domain (InterPro IPR043968; Pfam PF19040) (Fig 2.1b) and 2) 13 OACs have an acyltransferase 3 domain with an N-terminal nose resistant-to-fluoxetine (NRF) domain (InterPro IPR006621; SMART SM00703) (Fig 2.1c). Four proteins, OAC-46, OAC-48, OAC-54, and RHY-1, contain only an acyltransferase 3 domain while OAC-49 has two acyltransferase 3 domains in addition to a C-terminal SGNH hydrolase domain. The SGNH hydrolase domain is ubiquitous in protein domain architecture, but when coupled with an acyltransferase 3 domain it is thought to be involved in carbohydrate modification (Pearson *et al*. 2020). OAC-50 contains only a NRF domain; the only other protein to include a NRF domain, outside those already mentioned, is NDG-4. Although the NRF domain was named for the *nrf* gene family, no NRF proteins other than NRF-6 contain this domain. *ndg-4* mutants are named for their resistance to nordihydroguaiaretic acid, a lipoxygenase inhibitor (Shreffler *et al* 1995). *ndg-4* functions in a similar capacity to *nrf-6* and exhibits a pale-egg phenotype (Choy *et al*. 2006). We therefore define the *oac* gene family to include the named *oac* genes as well as *bus-1*, *rhy-1*, *nrf-6*, and *ndg-4*.

In general, membrane-bound O-acyltransferases have conserved histidine residues required for functional enzymatic activity (Hofmann 2000; Ma *et al* 2018). We find that all the OACs that have an acyltransferase 3 domain likewise have a conserved histidine residue in the acyltransferase 3 domain (Fig 2.1c). This is the only amino acid conserved throughout the entire protein class.

We also looked at the broader OAC family using phylogenetic analysis (Fig 2.2). We rooted our tree using the carboxylesterase CEST-2. The 60 proteins generally break down into two groups by their non-acyltransferase 3 domain architecture. The proteins that contain only an acyltransferase 3 domain do not cluster and are therefore thought to have independently lost their second protein domain. BUS-1 is the only protein that crosses groupings as it contains a SGNH domain yet clusters with proteins that have a NRF domain. Some genes that occur in tandem naturally sort together, such as the cluster of OAC-21, OAC-22, and OAC-23; otherwise, there is little sorting by chromosomal location.

Strain Construction

To study the numerous *oac* genes, we obtained knockout strains for all 60 of these protein-coding genes. *bus-1(e2678)*, *ndg-4(sa529)*, *nrf-6(sa525)*, and *rhy-1(ok1402)* were already available through the Caenorhabditis Genomics Center (CGC) and we made mutants for the other 56. We used two different CRISPR/Cas9 methods for our strain creation. The first method used a single guide RNA to create large several-hundred base pair deletions. The second method used an inserted sequence to introduce early stop codons. Using these methods, we created 95 alleles representing 56 genes (Tables 2.1, 2.2, and 2.3).

Phenotypes of *oac* Mutants

One strains, *oac-46(sy1747)*, was found to be homozygous lethal. Otherwise, none of the *oac* strains showed any obvious Petri-plate observable phenotypes except *oac-16(sy1342)*, which exhibited a mobility defect where the worms did not move far from where they hatched, even to obtain nearby food (data not shown). As *oac-16(sy1341)* did not have a mobility defect, a third allele, *oac-16(sy1471)* was made using a different CRISPR method. *oac-16(sy1471)* also did not have a mobility defect, so we infer that an off-target or background mutation causes the mobility phenotype in *oac-16(sy1342)*.

As loss of *ndg-4(sa529)* has been implicated in lifespan extension, we investigated the lifespans of the other *oac* mutant strains (Brejning *et al* 2014). Lifespan assays (Fig 2.3a) indicated that several genes have non-wildtype lifespans. We found that *oac-23(sy1384)* and *oac-26(sy1386)* had drastically shortened lifespans, mostly caused by high death rates during the larval and early adult stages. *bus-1(e2678)*, on the other hand, displayed a 55% increase in lifespan compared to wild-type N2 (Fig 2.3b).

Metabolomics

The expansion of *oac* genes in nematode worms, particularly within the *Caenorhabditis* genus, coupled with the putative O-acyltransferase activity of OAC proteins, led us to test whether any of the *oac* genes were involved in ascaroside pheromone biosynthesis. Corroborating earlier metabolomic studies of million-mutation project strains that contained *oac* mutations, metabolite screening of *oac* mutants did not detect abolishment of any simple or modular ascarosides (Panda *et al*. 2018). This led us to believe that OAC proteins are not enzymes responsible for

modular ascaroside biosynthesis, confirmed by enzyme activity of the CEST proteins, which were discovered during the course of this work (Le *et al*. 2020).

We did, however, notice differences in certain ascaroside production levels. For example, ascr#1 production was higher in both the endo- and exo-metabolome of *oac-37* and *oac-48* (Fig 2.4a). Interestingly, we detected differences in secretion of certain modular ascarosides in many mutants. For example, icas#1 was largely found from the exo-metabolome of *oac-1*, *oac-4*, *oac-41* and *oac-57*, while it was mostly kept in the worm body of *oac-20*, *oac-23*, and *oac-37* (Fig 2.4b). Another example is osas#10 – while production of osas#10 was mostly uninterrupted, little was secreted in the case of *oac-1* and *oac-4*, in contrast to *oac-37* and *oac-48*, where only trace amounts were detected from the endo-metabolome (Fig 2.4c). Together, even though we do not consider OAC proteins as enzymes for modular ascaroside biosynthesis, these results suggest they play a role in the production and secretion of both simple and modular ascarosides.

2.4 Discussion

oac Gene Class is Defined by Acyltransferase 3 Domain

The *oac* gene class can generally be defined as encoding a multi-pass transmembrane acyltransferase 3 protein domain. Thus, the previously described acyltransferase genes *bus-1*, *rhy-1*, and *nrf-6*, which also contain an acyltransferase 3 domain are also a part of this gene/protein family.

The acyltransferase 3 domain, similar to other O-acyltransferase domains, contains a highly conserved histidine residue that is preserved in every gene that has an active acyltransferase 3

domain. This conservation suggests this amino acid is crucial for O-acyltransferase enzymatic function.

In addition to an acyltransferase 3 domain, the NRF protein domain is also commonly found in OAC proteins. There is only one additional gene that encodes a protein with a NRF domain – *ndg-4* – and this gene is also added to the broader *oac* gene/protein family.

Creation of *oac* Strains Allows for Further Research

Complete loss-of-function alleles are the gold standard. Our creation of null mutants for the entire *oac* gene family allows for future studies of individual genes or the family as a whole. O-acyltransferases are used in many different ways throughout the cells of eukaryotic animals, and now we have the tools to elucidate their functions. It is particularly interesting to study why these genes expanded in *C. elegans*.

Longevity of *bus-1(e2678)*

One key finding with respect to *oac* gene phenotypes is the 55% lifespan increase associated with the loss of *bus-1* function. This result is consistent with the fact that *bus-1* mutants are also known to have resistance to at least one type of pathogenic bacteria (Gravato-Nobre and Hodgkin 2008). The exact nature of the *bus-1* lifespan increase, however, merits further inquiry as it could give us a better understanding of the complexities of the aging process in *C. elegans*. *bus-1* has putative homologs across the *Caenorhabditis* genus.

Metabolomics of *oacs*

Although there is an expansion of *oac* genes in *C. elegans*, they do not appear to have any direct effects on ascaroside pheromone biosynthesis; instead, they affect ascaroside production and secretion levels. Differences in ascaroside pheromone production are likely due to downstream ascaroside biosynthesis enzymes and secretion pathways that are regulated by *oac* genes.

It may be possible that the *oac* genes affect other types of pheromone signals given off by worms, such as volatile pheromones, or specialized metabolism.

2.5 Figures and Tables

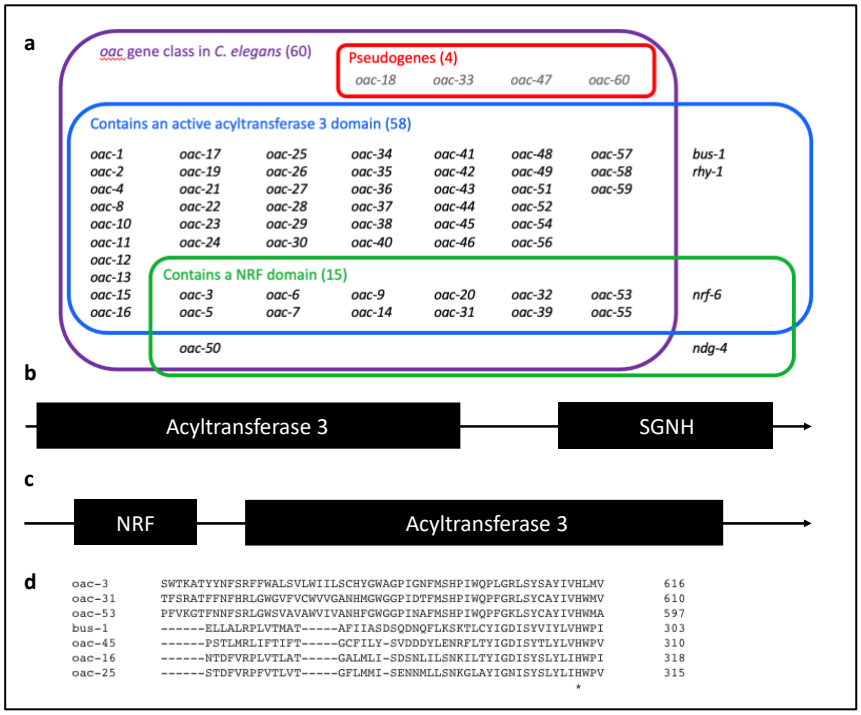


Figure 2.1 The *oac* gene family is defined by an acyltransferase 3 domain. (a) The genes of the broader *oac* gene family separated by name and protein domain architecture. Typically, proteins encoded by *oac* genes have two protein architectures: (b) an acyltransferase 3 domain toward with a C-terminal SGNH domain or (c) an acyltransferase 3 domain with a N-terminal nose resistant-to-fluoxetine (NRF) domain. (d) Here, we show a subset of *oac* genes with acyltransferase 3 domains. All proteins with an acyltransferase 3 domain have a universally conserved histidine residue, noted, likely a key catalytic site based on the importance of conserved histidines in other O-acyltransferase proteins across species. No other single residue is wholly conserved throughout the broader *oac* gene family.

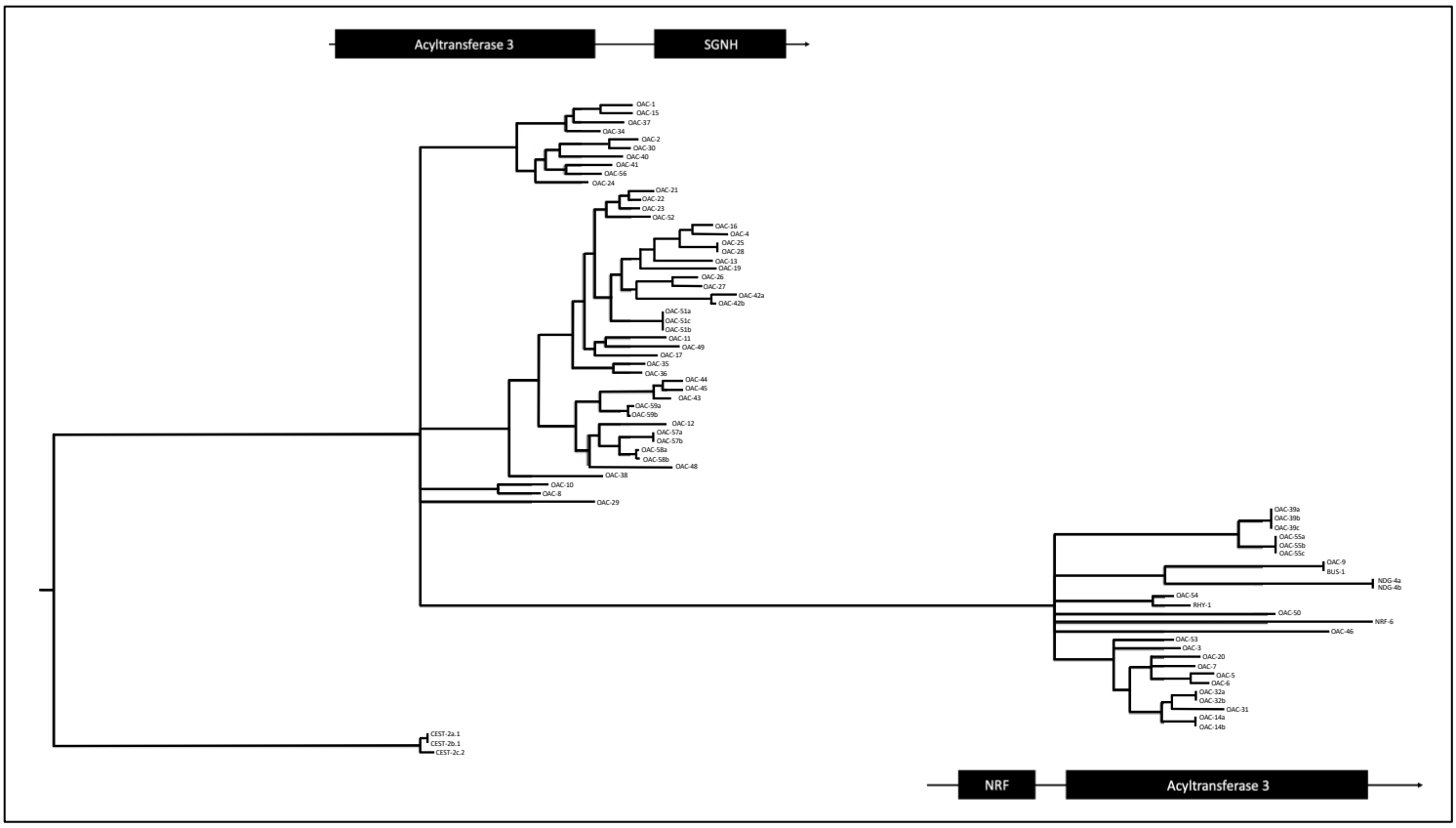


Figure 2.2 Phylogenetic tree of OAC proteins in *C. elegans*. Consensus tree from Bayesian inference analysis of the proteins with an O-acyltransferase 3 and/or NRF domains in *C. elegans* after standard deviation of split frequencies dropped below 0.05. The OAC proteins generally sort into two groups: those that contain a C-terminal SGNH domain (top group) and those that contain a N-terminal nose resistant-to-fluoxetine domain (bottom group). The exceptions to this are OAC-46 and OAC-48, which have only an acyltransferase 3 domain but sort with the SGNH group; RHY-1 and OAC-54, which have only an acyltransferase 3 domain but sort with the nrf group; and BUS-1, which has an SGNH domain but sorts with the NRF domain group. Length of branches indicates evolutionary distance between proteins.

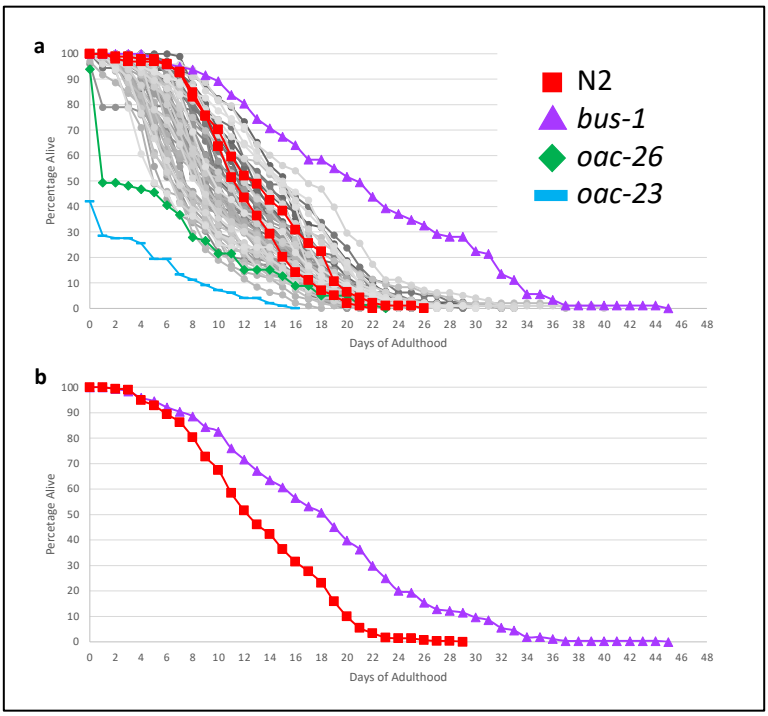


Figure 2.3 Longevity of *oac* mutant strains. (a) Longevity assays showed that most *oac* mutant strains were comparable with wild-type N2, shown in red. *oac-23(sy1384)* (blue) and *oac-26(sy1386)* (green) have drastically reduced lifespans owing mostly to high death rates during larval and early adult stages. (b) *bus-1(e2678)* (purple) shows a dramatically lengthened lifespan of 55% increase over that of N2.

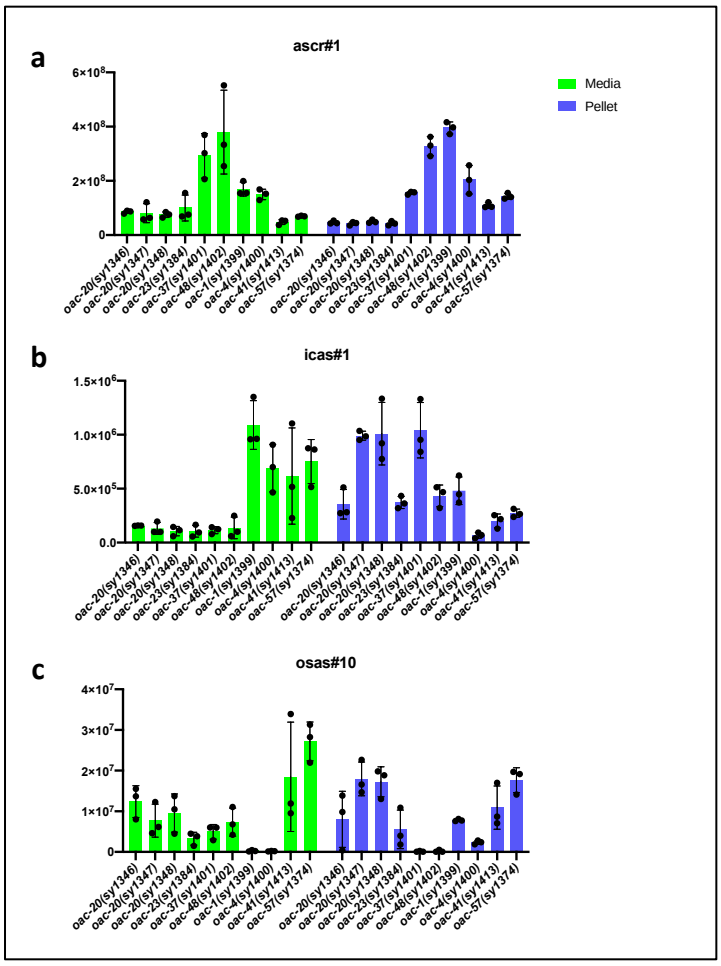


Figure 2.4 Ascaroside levels in *oac* mutant strain media and pellets. Although ascaroside biosynthesis is not affected by *oac* genes, they instead affect production and secretion levels of ascarosides. For example, (a) ascr#1 production is higher in both the endo- and exometabolomes of *oac-37*and *oac-48*. (b) icas#1 is largely found in the exometabolomes of *oac-1*, *oac-4*, *oac-41*, and *oac-57*, whereas it is largely found in the endometabolome of *oac-20*, *oac-23*, and *oac-37*. (c) osas#10 is barely secreted in *oac-1* or *oac-4* but is barely retained in the worm in *oac-37* and *oac-48*.

Table 2.1 95 alleles representing 56 *oac* genes. Strains were made using either a deletion or knock-in CRISPR/Cas9 method.

Gene	Strain	Description
oac-1(sy1399)	PS8547	605 bp Deletion
oac-2(sy1218)	PS8201	Triple-Stop Knock-In
oac-2(sy1219)	PS8202	Triple-Stop Knock-In
oac-3(sy1329)	PS7536	387 bp Deletion
oac-3(sy1053)	PS7577	1,428 bp Deletion
oac-4(sy1400)	PS8548	5 bp Deletion
oac-5(sy1333)	PS7556	854 bp Deletion
oac-5(sy1334)	PS7791	1,231 bp Deletion
oac-6(sy1326)	PS7466	1,899 bp Deletion
oac-7(sy1327)	PS7490	546 bp Deletion
oac-8(sy1284)	PS8365	Triple-Stop Knock-In
oac-8(sy1285)	PS8366	Triple-Stop Knock-In
oac-9(sy1324)	PS7592	1,129 bp Deletion
oac-10(sy1339)	PS7908	875 bp Deletion
oac-10(sy1340)	PS8110	942 bp Deletion
oac-11(sy1407)	PS8310	1438 bp Deletion
oac-12(sy1378)	PS8439	Triple-Stop Knock-In
oac-12(sy1379)	PS8440	Triple-Stop Knock-In
oac-13(sy1367)	PS8386	708 bp Deletion
oac-13(sy1368)	PS8387	481 bp Deletion
oac-14(sy1343)	PS7593	544 bp Deletion
oac-14(sy1344)	PS7696	868 bp Deletion
oac-14(sy1345)	PS7697	404 bp Deletion
oac-15(sy1369)	PS8388	579 bp Deletion
oac-15(sy1370)	PS8389	835 bp Deletion
oac-16(sy1341)	PS8113	1,255 bp Deletion
oac-16(sy1342)	PS8108	746 bp Deletion
oac-16(sy1471)	PS8577	Triple-Stop Knock-In
oac-17(sy1405)	PS8299	419 bp Deletion
oac-17(sy1406)	PS8300	1,056 bp Deletion
oac-19(sy1741)	PS9361	Triple-Stop Knock-In
oac-20(sy1346)	PS7950	757 bp Deletion
oac-20(sy1347)	PS7956	561 bp Deletion
oac-20(sy1348)	PS8109	988 bp Deletion
oac-21(sy1566)	PS8897	Triple-Stop Knock-In
oac-22(sy1286)	PS8367	Triple-Stop Knock-In
oac-22(sy1287)	PS8368	Triple-Stop Knock-In
oac-23(sy1384)	PS8517	Triple-Stop Knock-In
oac-23(sy1385)	PS8518	Triple-Stop Knock-In
oac-24(sy1246)	PS8250	Triple-Stop Knock-In
oac-24(sy1247)	PS8251	Triple-Stop Knock-In

oac-25(sy1620);oac-28(sy1621)	PS9143	8 bp insert; 8 bp insert
oac-26(sy1386)	PS8519	Triple-Stop Knock-In
oac-27(sy1475)	PS8714	Triple-Stop Knock-In
oac-27(sy1476)	PS8715	Triple-Stop Knock-In
oac-28(sy1734)	PS9351	8 bp insert
oac-29(sy1335)	PS7787	896 bp Deletion
oac-30(sy1577)	PS8934	Triple-Stop Knock-In
oac-30(sy1578)	PS8935	Triple-Stop Knock-In
oac-31(sy1328)	PS7559	591 bp Deletion
oac-32(sy1330)	PS7558	1,764 bp Deletion
oac-34(sy1290)	PS8394	Triple-Stop Knock-In
oac-34(sy1291)	PS8395	Triple-Stop Knock-In
oac-35(sy1390)	PS8538	Triple-Stop Knock-In
oac-35(sy1391)	PS8539	Triple-Stop Knock-In
oac-36(sy1411)	PS8580	Triple-Stop Knock-In
oac-36(sy1412)	PS8581	Triple-Stop Knock-In
oac-37(sy1401)	PS8549	Triple-Stop Knock-In
oac-38(sy1256)	PS8265	Triple-Stop Knock-In
oac-38(sy1257)	PS8266	Triple-Stop Knock-In
oac-39(sy1321)	PS7428	546 bp Deletion
oac-40(sy1258)	PS8301	Triple-Stop Knock-In
oac-40(sy1259)	PS8302	Triple-Stop Knock-In
oac-41(sy1413)	PS8582	Triple-Stop Knock-In
oac-41(sy1414)	PS8583	Triple-Stop Knock-In
oac-42(sy1433)	PS8632	Triple-Stop Knock-In
oac-42(sy1434)	PS8633	Triple-Stop Knock-In
oac-43(sy1381)	PS8480	Triple-Stop Knock-In
oac-44(sy1431)	PS8630	Triple-Stop Knock-In
oac-44(sy1432)	PS8631	Triple-Stop Knock-In
oac-45(sy1742)	PS9362	Triple-Stop Knock-In
oac-46(sy1747)	PS9372	Triple-Stop Knock-In
oac-48(sy1402)	PS8550	Triple-Stop Knock-In
oac-49(sy1380)	PS8479	Triple-Stop Knock-In
oac-50(sy1336)	PS7716	559 bp Deletion
oac-50(sy1337)	PS7790	927 bp Deletion
oac-50(sy1338)	PS7730	782 bp Deletion
oac-51(sy1358)	PS8482	Triple-Stop Knock-In
oac-51(sy1359)	PS8483	Triple-Stop Knock-In
oac-51(sy1388)	PS8536	Triple-Stop Knock-In
oac-51(sy1389)	PS8537	Triple-Stop Knock-In
oac-52(sy1288)	PS8369	Triple-Stop Knock-In
oac-52(sy1289)	PS8370	Triple-Stop Knock-In
oac-53(sy1331)	PS7652	483 bp Deletion
oac-53(sy1332)	PS7687	592 bp Deletion
oac-54(sy1322)	PS7465	1,138 bp Deletion

oac-55(sy1323)	PS7717	1,030 bp Deletion
oac-56(sy1372)	PS8527	Triple-Stop Knock-In
oac-56(sy1373)	PS8528	Triple-Stop Knock-In
oac-57(sy1374)	PS8529	Triple-Stop Knock-In
oac-57(sy1375)	PS8530	Triple-Stop Knock-In
oac-58(sy1376)	PS8531	Triple-Stop Knock-In
oac-58(sy1377)	PS8532	Triple-Stop Knock-In
oac-59(sy1260)	PS8303	Triple-Stop Knock-In
oac-59(sy1261)	PS8304	Triple-Stop Knock-In

Table 2.2 List of all strains made by deletion method.

Gene	Strain	Left Flanking Sequence	Right Flank Sequence	Forward Primer	Reverse Primer
oac-1(sy1399)	PS8547	GGTCGGCAGCATTAGAGACT	GCCAGTTTTTTTTGTTTTCA	GTCGGGTGCTTAAGAGAACT	AAAGGTGATGAGCCACTACA
oac-3(sy1329)	PS7536	GACGAGAAATAATGGCGTAA	ACTCCGGAATGCACAAAAGA	TCAACATGGCATGCGTTTCC	GTCCTGCAATTGGAGTCCAA
oac-3(sy1053)	PS7577	GACTAACACGTAAAGCCGTT	TCAGTGGACACATTTTTTCT	TCAACATGGCATGCGTTTCC	GTCCTGCAATTGGAGTCCAA
oac-4(sy1400)	PS8548	CTTTTAAAGATTCTTCGTCC	GGATTCCTCATGTGCATGCT	CGCTAAATACGGCCCGCTTT	ACTCGCCTCGTAATCACAGT
oac-5(sy1333)	PS7556	TTTTCTGCGTTACTTTGTTA	ACAAGAGGATCAGAAGAGGA	AACTCCAGCCGGACGCGTTT	TATGCATATAGCTGCCAGCG
oac-5(sy1334)	PS7791	TTCTGCGTTACTTTGTTAGC	ACTCAAAGTGTCACAAATCA	AACTCCAGCCGGACGCGTTT	TATGCATATAGCTGCCAGCG
oac-6(sy1326)	PS7466	TACCAACTACATTTTTCTTC	TTTTCAGTATCTTCTCCCGT	GTGGCTGAGTTATCTGCAGA	AGGCCAGACGGGAGAAGTTG
oac-7(sy1327)	PS7490	GCATTTGGTAAACTCCCCTC	CTGGATTTATAAAATGCTTG	GGAAGCTGATAGCTGGGAGG	CACAAGCCATCCAATCACCG
oac-9(sy1324)	PS7592	TGAAACATGTAAAGAACTAT	CTTTGTACGTTGTATCAGCT	CCTCATCAGCCTTCGTCAGC	ATGGGACACACGTTGATGGG
oac-10(sy1339)	PS7908	TGTGTTTTTTCAGTGTATTT	CCTACGAGAAATGGTACCTA	GAACATGGATTCCGCGTGGA	GATCCTCATTGGCAATGGTC
oac-10(sy1340)	PS8110	AACGAGATGGAAGAACAAAT	TTTCCTTTATTTCCGTTAAA	GAACATGGATTCCGCGTGGA	GATCCTCATTGGCAATGGTC
oac-11(sy1407)	PS8310	AATAAAGGCCGTCTGATAAA	CGACTTTCAGGCTGTGAAGC	CACTTTATCTGCTTGTCATC	CCAAGGCGTTCTGGAATAAT
oac-13(sy1367)	PS8386	AATAGGGTTTAACGCTGTGT	TTCTCGTTAAGCTAGTTTTA	GGCACTTTGAAGCCGTTCCT	AGTAGATAGGCCAGTGGAGC
oac-13(sy1368)	PS8387	GTTACGGCTAAGCGATCGGA	TGGACATTTTCACCCATACA	GGCACTTTGAAGCCGTTCCT	AGTAGATAGGCCAGTGGAGC
oac-14(sy1343)	PS7593	TGAAAGCAAACTTGTACGCT	GTTTTTTTGAAAATTCTTCT	TTGAGCACTTCAGGTGCAGT	AACATTCCCCATCCAAGACG
oac-14(sy1344)	PS7696	GAAAGCAAACTTGTACGCTA	TGACCTGGATCATGTTTTAC	TTGAGCACTTCAGGTGCAGT	AACATTCCCCATCCAAGACG
oac-14(sy1345)	PS7697	ACTGAAAGAGCTCAAAATTT	GGGGTTTTTCGTAAGTGCTT	TTGAGCACTTCAGGTGCAGT	AACATTCCCCATCCAAGACG
oac-15(sy1369)	PS8388	GAAATTGGAATTTAAGTGCG	TTTCCTAATAACCCAGTTGG	GTAGTGGGTGACCTGTCGGC	TTGAACTGTGCCCGGATGTG
oac-15(sy1370)	PS8389	GAAATTGGAATTTAAGTGCG	CTGTGCTCTTTCTCTGTATT	GTAGTGGGTGACCTGTCGGC	TTGAACTGTGCCCGGATGTG
oac-16(sy1341)	PS8113	AGCTAAACCGAAGCGTCTAG	TATCTCATATTCCCTATATC	CTGCCTCGGTTGCTTCCAAG	GATAGTCCGGCTTTTCCGCG
oac-16(sy1342)	PS8108	AGCAGCTAAACCGAAGCGTC	AGTTGACTTCTTTGGAAGCT	CTGCCTCGGTTGCTTCCAAG	GATAGTCCGGCTTTTCCGCG
oac-17(sy1405)	PS8299	AGTAAGTTCCACTGAGTCTA	GCTCGCCATCGCCGTGAACA	CTAGATGCCAGCTTGTTTTG	AGATACTCTTTGTCTTGGCG
oac-17(sy1406)	PS8300	TCAGTAAGTTCCACTGAGTC	AATTTCTATCCAATAGAGTT	CTAGATGCCAGCTTGTTTTG	AGATACTCTTTGTCTTGGCG
oac-20(sy1346)	PS7950	AACAATGACACTGAAACATG	TTTTTAAAGTTTGAAATAAT	GGGAATTACTTCGCAAACGC	AACGAACCCAAGGCTTTTGG
oac-20(sy1347)	PS7956	TCAGACCTGCCGGCGTGTTT	CAAGTTATCCCGTTGAGAAG	GGGAATTACTTCGCAAACGC	AACGAACCCAAGGCTTTTGG
oac-20(sy1348)	PS8109	CACTGAAACATGGTTGCACT	ACATGATTTTTCTCGGTTTC	GGGAATTACTTCGCAAACGC	AACGAACCCAAGGCTTTTGG
oac-25(sy1620)	PS9143	GGAAGCATCTAAACCAAAGC	GATTGGATTTGCAAGGGCTC	CGTTGCCTCGTAGCTGATTT	CTCGCTTCAGTAGCATGCAC
oac-28(sy1621)	PS9143	GGAAGCATCTAAACCAAAGC	GATTGGATTTGCAAGGGCTC	AACAATACCTCCAGCTTGCA	CTCGCTTCAGTAGCATGCAC
oac-28(sy1734)	PS9351	GGAAGCATCTAAACCAAAGC	GATTGGATTTGCAAGGGCTC	AACAATACCTCCAGCTTGCA	CTCGCTTCAGTAGCATGCAC
oac-29(sy1335)	PS7787	ATTTCCCAAAATTTTCAGCA	TTATTTTCAAGAATTTATAT	TGGTACTTTTGGGCTGAGTC	TCGGATCCATTCGTGAGAGC
oac-31(sy1328)	PS7559	TTTGGATTACGTCCAAGATG	AAATAGAAACACCAAAATTT	GCGACCGTATTGATGGCGAA	TTTGCATGCCTCGACTTGTG
oac-32(sy1330)	PS7558	GCATCCGACTTCTCTCAATG	TGCTGGGTTGTTGGAGCGAA	CTGCTACCTGCTTCTCACAC	GTTCGCTCCAACAACCCAGC
oac-39(sy1321)	PS7428	CCGTGGTCTTGTAGGAATGA	CATTGTGCCGCGATGCTTAT	CGCTGATTGGTCCTTTGTCG	TGGCTATGTAGTACGCGGGG
oac-50(sy1336)	PS7716	TCATGGGAAAGACTACGCTT	TTTCAGGTCAACGAATAATA	TGCATTCGCGAGATATCCGG	GAAGGGACGCCAAAACGGAT
oac-50(sy1337)	PS7790	TGGGAAAGACTACGCTTCAA	GGGAAAACCATCACTTGATG	TGCATTCGCGAGATATCCGG	GAAGGGACGCCAAAACGGAT
oac-50(sy1338)	PS7730	CATGGGAAAGACTACGCTTC	GATCCATCCAAAATTTCCTT	TGCATTCGCGAGATATCCGG	GAAGGGACGCCAAAACGGAT
oac-53(sy1331)	PS7652	TTCGTCGCTACTTCTACACA	GGATTTACGTGAGTTTTTTT	GCAAAGAGGAGGCCCTAAAA	ACAATCGACACCGCTCCTTA
oac-53(sy1332)	PS7687	ACCTTCGTCGCTACTTCTAC	GTGTGGTGTCGAACCAGACA	GCAAAGAGGAGGCCCTAAAA	ACAATCGACACCGCTCCTTA
oac-54(sy1322)	PS7465	ACTTTCAATAATTGTTCATA	AGGACTGTGGTTAATTTATT	TTTGACTCGTGGACAGGAAG	CGTTGAAGCACCAATGGGAA

oac-55(sy1323)	PS7717	GCGGCACGATATGCCCGGCA	CAGGTACCTACATATTTCCT	TAAGGCTATGGAGCTGCTCG	GAGACAAGGCTTTACTGGGC

Table 2.3 List of all strains made by insertion method.

Gene	Strain	Left Flanking Sequence	Right Flank Sequence	Forward Primer	Reverse Primer
oac-2(sy1218)	PS8201	AGATTTCGAAGAATCCTCCC	GCTGTACTACTTGACCATCT	TTCATTTGCTGCCTAGCCTG	GAAGCAGTGACGCTATGGAG
oac-2(sy1219)	PS8202	AGATTTCGAAGAATCCTCCC	GCTGTACTACTTGACCATCT	TTCATTTGCTGCCTAGCCTG	GAAGCAGTGACGCTATGGAG
oac-8(sy1284)	PS8365	TCCCTAAAACCTTCCCAAAT	GGGTATATTGGAGTAGATAT	GAAACGGGATGACCTGCAAG	GTAATATCCGCTTGGCTCGAC
oac-8(sy1285)	PS8366	TCCCTAAAACCTTCCCAAAT	GGGTATATTGGAGTAGATAT	GAAACGGGATGACCTGCAAG	GTAATATCCGCTTGGCTCGAC
oac-12(sy1378)	PS8439	CCGTCAAAGCGTCAGGATCT	ACAAGGGATCCGCGGAATAG	GGTTGGCTCTCTGCAGGTTG	CCATGTGTGAGTGAACAAGT
oac-12(sy1379)	PS8440	CCGTCAAAGCGTCAGGATCT	ACAAGGGATCCGCGGAATAG	GGTTGGCTCTCTGCAGGTTG	CCATGTGTGAGTGAACAAGT
oac-16(sy1471)	PS8577	AAACCGAAGCGTCTAGATTT	ACAAGGTATCCGTGGTCTCG	GCAAAAGCACTGGGAGTGAT	GATAGTCCGGCTTTTCCGCG
oac-19(sy1741)	PS9361	GCAGTTCTAGGCTTCCACTT	CTACCCTGACACCTTCCCAA	GTTTCAGATGGGTTCCTTGC	TGTTATTAAAGTACATGGTGGCTC
oac-21(sy1566)	PS8897	AACGACTAGATCTTCAAGGC	ATCCGGGCTCTCGCTATTCT	CGTTTTACTCTTTGATGCCTCCG	TAGAAACCCGGAGAGCACAAAG
oac-22(sy1286)	PS8367	TGTGTCAAACAGACCAAAAA	CTGTGGCAGAGGACTATTTT	TGCTGAAGCGAGCTGAGAATC	AACGATATTGCCCGCACCTAG
oac-22(sy1287)	PS8368	TGTGTCAAACAGACCAAAAA	CTGTGGCAGAGGACTATTTT	TGCTGAAGCGAGCTGAGAATC	AACGATATTGCCCGCACCTAG
oac-23(sy1384)	PS8517	CAGTCGACATCTTTACACAT	ACCTGGTCACTCTCAGTGGA	TAAGGAGTCTGCTCTTCACG	CAGGTTGCTGAGCACTCGAT
oac-23(sy1385)	PS8518	CAGTCGACATCTTTACACAT	ACCTGGTCACTCTCAGTGGA	TAAGGAGTCTGCTCTTCACG	CAGGTTGCTGAGCACTCGAT
oac-24(sy1246)	PS8250	CTTTGTCATTTCCGGATACC	TCATGGCGAAAAATTTAACG	TTGAATTGAAGGCCACCTACG	AGTAACCAAGCACAACGACG
oac-24(sy1247)	PS8251	CTTTGTCATTTCCGGATACC	TCATGGCGAAAAATTTAACG	TTGAATTGAAGGCCACCTACG	AGTAACCAAGCACAACGACG
oac-26(sy1386)	PS8519	TGATTGTAACACGATTACCA	GCACGGTTTCACATTGCGTC	GACACGAGCCCTGTTTTTCG	GCGGTGCACCTGATTATAGA
oac-27(sy1475)	PS8714	CCATGACAAGCCAACCTGCA	AAGCGGCTGGACCTGCAAGG	TAAGGTTTACATCCCGTGCG	ATCCTCCAATGTCGATGCCC
oac-27(sy1476)	PS8715	CCATGACAAGCCAACCTGCA	AAGCGGCTGGACCTGCAAGG	TAAGGTTTACATCCCGTGCG	ATCCTCCAATGTCGATGCCC
oac-30(sy1577)	PS8934	GAAATTTTCAGATTCCGCCG	AATCCTGCCACTCTACTATC	AGAAACCTTACCAAGTCGAAGC	GAAACAGGGAGGCAATGGAG
oac-30(sy1578)	PS8935	GAAATTTTCAGATTCCGCCG	AATCCTGCCACTCTACTATC	AGAAACCTTACCAAGTCGAAGC	GAAACAGGGAGGCAATGGAG
oac-34(sy1290)	PS8394	CGGTTACCTGATGGCCCGTA	ACCTGACACACATGAAAATC	GCCGCTTTGTCGGGTTTATC	GCAGACGTGAACTGCAATGG
oac-34(sy1291)	PS8395	CGGTTACCTGATGGCCCGTA	ACCTGACACACATGAAAATC	GCCGCTTTGTCGGGTTTATC	GCAGACGTGAACTGCAATGG
oac-35(sy1390)	PS8538	GTTGCTCAAGCGTGCCGAGA	CCCACCCATTTTTCACGTTG	GTGGCTTGGCTATACTTGTTG	TGTAGAGGCAAATGGCAGAG
oac-35(sy1391)	PS8539	GTTGCTCAAGCGTGCCGAGA	CCCACCCATTTTTCACGTTG	GTGGCTTGGCTATACTTGTTG	TGTAGAGGCAAATGGCAGAG
oac-36(sy1411)	PS8580	CAAGCGTGCCGAGACCAAGC	CATTTTTCACAGTGGTATGC	GTGGCTTGGCTATACTTGTTGTAC	GTTAGCCAGAAGTGATCCCAAG
oac-36(sy1412)	PS8581	CAAGCGTGCCGAGACCAAGC	CATTTTTCACAGTGGTATGC	GTGGCTTGGCTATACTTGTTGTAC	GTTAGCCAGAAGTGATCCCAAG
oac-37(sy1401)	PS8549	TGATATCGGGGTACCTGATG	GCTCGGAACTTGACACATTC	CTTCTGTGCCGACGCATTGT	GTTCCATCTGCCAGGTACTG
oac-38(sy1256)	PS8265	TTCCTGCCAGATGTATTTCC	TAATGGATACTTAGGAGTTG	CTCAAGGAGTTCGAGGCTTGG	GTCAGCAGGGCAGTATCTGG
oac-38(sy1257)	PS8266	TTCCTGCCAGATGTATTTCC	TAATGGATACTTAGGAGTTG	CTCAAGGAGTTCGAGGCTTGG	GTCAGCAGGGCAGTATCTGG
oac-40(sy1258)	PS8301	TGGGAAACTAATAACCGGTA	TTCGTTGGCTTCATTATTTT	GTCGAGTTTGAGATCGGTTCAAG	TGAAATAATCTGCCTGGTCGTG
oac-40(sy1259)	PS8302	TGGGAAACTAATAACCGGTA	TTCGTTGGCTTCATTATTTT	GTCGAGTTTGAGATCGGTTCAAG	TGAAATAATCTGCCTGGTCGTG
oac-41(sy1413)	PS8582	TACACGTTTTTCTACCGGAT	TTCCTGTGGCAAAATAATAA	ACAATGCTTACGTGGTTTGG	TCCGACTGATCGTGAATAACC
oac-41(sy1414)	PS8583	TACACGTTTTTCTACCGGAT	TTCCTGTGGCAAAATAATAA	ACAATGCTTACGTGGTTTGG	TCCGACTGATCGTGAATAACC
oac-42(sy1433)	PS8632	AATTCGGGGTCTCGCCATTG	CTGCAGTGCTTCTTTATCAC	GCTTCAAAGAGGCTTGACTTAC	AGGAGCCGTTTGAATCTTCG
oac-42(sy1434)	PS8633	AATTCGGGGTCTCGCCATTG	CTGCAGTGCTTCTTTATCAC	GCTTCAAAGAGGCTTGACTTAC	AGGAGCCGTTTGAATCTTCG
oac-43(sy1381)	PS8480	GATTTGCAAGCAATCCGAGG	ATTGGCCATTCTATCAGTAC	GTCTCTTTGACGGTCTGTTC	GTTCCCTGACAGCACATCTC
oac-44(sy1431)	PS8630	TTCTACCCAAACCAGTTTCC	CAATGGATACCTCGGAGTGG	CCTTCTAAACGGCAGGATTTG	ATCCAGAGAGGACAAAGAACC
oac-44(sy1432)	PS8631	TTCTACCCAAACCAGTTTCC	CAATGGATACCTCGGAGTGG	CCTTCTAAACGGCAGGATTTG	ATCCAGAGAGGACAAAGAACC
oac-45(sy1742)	PS9362	TCTACCCTAATCAGTTTCCC	AATGGGTACCTTGGAGTTGA	AGTTTCTTCAAAGCGGCAGG	GTCTTCAAATCCGGTCTTTTTACG

oac-46(sy1747)	PS9372	TCCTGTTATTATCACCGGCT	ATTGCTTCAGCCGGTCTAGA	TCACTTTCTCCTCGGATCTTC	CAATGCCAAACTCTGCTTGC
oac-48(sy1402)	PS8550	TTCGAGTCAAATAGGCCGCA	TACTGGAGAGGAAAATTATT	CGCGGATTCGCAATAATTTC	GATGCGATTGAGAGCTGGAA
oac-49(sy1380)	PS8479	TCCCGACTCAGTTTCCAAAT	GGCTATTTGGGAGTGGATCA	GGTACCCCTTTAAATTCCCC	CCCACCACGACAATAACCGA
oac-51(sy1358)	PS8482	TCTACCCAGAAGTATTTCCA	AATGGGTATCTTGGAGTTGA	CATTGTGCTTGGTGATTCTGG	GGGACTAGAGGTCTTTGACATTC
oac-51(sy1359)	PS8483	TCTACCCAGAAGTATTTCCA	AATGGGTATCTTGGAGTTGA	CATTGTGCTTGGTGATTCTGG	GGGACTAGAGGTCTTTGACATTC
oac-51(sy1388)	PS8536	GCATGGTAGTTTACACCGCT	CTGTGGAACATTGAAGATCA	CACTACCCTCGCCCGTTATC	ATTCAGTCGGTTGTTGTCATCG
oac-51(sy1389)	PS8537	GCATGGTAGTTTACACCGCT	CTGTGGAACATTGAAGATCA	CACTACCCTCGCCCGTTATC	ATTCAGTCGGTTGTTGTCATCG
oac-52(sy1288)	PS8369	GGTCTTGCTATTACAGTTGT	ACTAGGTTTTCATTTCTATC	GATCTAGTGAAGCAGAAACCGTC	TGACTAAAGAGCACGTGGGC
oac-52(sy1289)	PS8370	GGTCTTGCTATTACAGTTGT	ACTAGGTTTTCATTTCTATC	GATCTAGTGAAGCAGAAACCGTC	TGACTAAAGAGCACGTGGGC
oac-56(sy1372)	PS8527	CACTTGAACCCTAACCTATT	TGTTAATGGATTTCTCGGTG	AATGCGAGAAGACATCCAATGC	CCATGAACAGCGATGCCAAC
oac-56(sy1373)	PS8528	CACTTGAACCCTAACCTATT	TGTTAATGGATTTCTCGGTG	AATGCGAGAAGACATCCAATGC	CCATGAACAGCGATGCCAAC
oac-57(sy1374)	PS8529	TCAATTTTATTCTTCCATCT	AAAGTCTCTTTTAACAGTTT	ACCTGTTCACTCATACATGGTC	CTTTCACATTCGCCATGCTC
oac-57(sy1375)	PS8530	TCAATTTTATTCTTCCATCT	AAAGTCTCTTTTAACAGTTT	ACCTGTTCACTCATACATGGTC	CTTTCACATTCGCCATGCTC
oac-58(sy1376)	PS8531	TTCTATTCAATTCTCCCACT	TGAAGTGGCTTTTAACAGTT	CCCGTACACGGTTTAATTCTCTC	TTCGTCTTCCACTTCATCTTCC
oac-58(sy1377)	PS8532	TTCTATTCAATTCTCCCACT	TGAAGTGGCTTTTAACAGTT	CCCGTACACGGTTTAATTCTCTC	TTCGTCTTCCACTTCATCTTCC
oac-59(sy1260)	PS8303	CGGCTGGACCTTCAAGGCAT	TAGAGGGTTGGCAATTCTAT	CTCGAACAATGCCATCCCAG	AGGCGAGTAAAGTAAACCCAAG
oac-59(sy1261)	PS8304	CGGCTGGACCTTCAAGGCAT	TAGAGGGTTGGCAATTCTAT	CTCGAACAATGCCATCCCAG	AGGCGAGTAAAGTAAACCCAAG

Table 2.4 List of all strains.

Gene	Strain	Source
C. elegans Wildtype	N2	Brenner, 1974; Caenorhabditis Genomics Center (CGC)
oac-1(sy1399)	PS8547	This work
oac-2(sy1218)	PS8201	This work
oac-2(sy1219)	PS8202	This work
oac-3(sy1329)	PS7536	This work
oac-3(sy1053)	PS7577	This work
oac-4(sy1400)	PS8548	This work
oac-5(sy1333)	PS7556	This work
oac-5(sy1334)	PS7791	This work
oac-6(sy1326)	PS7466	This work
oac-7(sy1327)	PS7490	This work
oac-8(sy1284)	PS8365	This work
oac-8(sy1285)	PS8366	This work
oac-9(sy1324)	PS7592	This work
oac-10(sy1339)	PS7908	This work
oac-10(sy1340)	PS8110	This work
oac-11(sy1407)	PS8310	This work
oac-12(sy1378)	PS8439	This work
oac-12(sy1379)	PS8440	This work
oac-13(sy1367)	PS8386	This work
oac-13(sy1368)	PS8387	This work
oac-14(sy1343)	PS7593	This work
oac-14(sy1344)	PS7696	This work
oac-14(sy1345)	PS7697	This work
oac-15(sy1369)	PS8388	This work
oac-15(sy1370)	PS8389	This work
oac-16(sy1341)	PS8113	This work
oac-16(sy1342)	PS8108	This work
oac-16(sy1471)	PS8577	This work
oac-17(sy1405)	PS8299	This work
oac-17(sy1406)	PS8300	This work
oac-19(sy1741)	PS9361	This work
oac-20(sy1346)	PS7950	This work
oac-20(sy1347)	PS7956	This work
oac-20(sy1348)	PS8109	This work
oac-21(sy1566)	PS8897	This work
oac-22(sy1286)	PS8367	This work
oac-22(sy1287)	PS8368	This work
oac-23(sy1384)	PS8517	This work
oac-23(sy1385)	PS8518	This work
oac-24(sy1246)	PS8250	This work
oac-24(sy1247)	PS8251	This work

oac-25(sy1620);oac-28(sy1621)	PS9143	This work
oac-26(sy1386)	PS8519	This work
oac-27(sy1475)	PS8714	This work
oac-27(sy1476)	PS8715	This work
oac-28(sy1734)	PS9351	This work
oac-29(sy1335)	PS7787	This work
oac-30(sy1577)	PS8934	This work
oac-30(sy1578)	PS8935	This work
oac-31(sy1328)	PS7559	This work
oac-32(sy1330)	PS7558	This work
oac-34(sy1290)	PS8394	This work
oac-34(sy1291)	PS8395	This work
oac-35(sy1390)	PS8538	This work
oac-35(sy1391)	PS8539	This work
oac-36(sy1411)	PS8580	This work
oac-36(sy1412)	PS8581	This work
oac-37(sy1401)	PS8549	This work
oac-38(sy1256)	PS8265	This work
oac-38(sy1257)	PS8266	This work
oac-39(sy1321)	PS7428	This work
oac-40(sy1258)	PS8301	This work
oac-40(sy1259)	PS8302	This work
oac-41(sy1413)	PS8582	This work
oac-41(sy1414)	PS8583	This work
oac-42(sy1433)	PS8632	This work
oac-42(sy1434)	PS8633	This work
oac-43(sy1381)	PS8480	This work
oac-44(sy1431)	PS8630	This work
oac-44(sy1432)	PS8631	This work
oac-45(sy1742)	PS9362	This work
oac-46(sy1747)	PS9372	This work
oac-48(sy1402)	PS8550	This work
oac-49(sy1380)	PS8479	This work
oac-50(sy1336)	PS7716	This work
oac-50(sy1337)	PS7790	This work
oac-50(sy1338)	PS7730	This work
oac-51(sy1358)	PS8482	This work
oac-51(sy1359)	PS8483	This work
oac-51(sy1388)	PS8536	This work
oac-51(sy1389)	PS8537	This work
oac-52(sy1288)	PS8369	This work
oac-52(sy1289)	PS8370	This work
oac-53(sy1331)	PS7652	This work
oac-53(sy1332)	PS7687	This work
oac-54(sy1322)	PS7465	This work

oac-55(sy1323)	PS7717	This work
oac-56(sy1372)	PS8527	This work
oac-56(sy1373)	PS8528	This work
oac-57(sy1374)	PS8529	This work
oac-57(sy1375)	PS8530	This work
oac-58(sy1376)	PS8531	This work
oac-58(sy1377)	PS8532	This work
oac-59(sy1260)	PS8303	This work
oac-59(sy1261)	PS8304	This work
rhy-1(ok1402)	RB1297	Shen *et al*. 2006; Caenorhabditis Genomics Center (CGC)
bus-1(e2678)	CB5609	Gravato-Nobre *et al*. 2005; Caenorhabditis Genomics Center (CGC)
ndg-4(sa529)	JT529	Choy and Thomas 1999; Caenorhabditis Genomics Center (CGC)
nrf-6(sa525)	JT525	Choy and Thomas 1999; Caenorhabditis Genomics Center (CGC)

2.6 Materials and Methods

Protein Alignment: Protein alignments were performed using the ClustalOmega multiple sequence alignment tool with default settings. Protein sequences were obtained from WormBase WS280.

Protein Tree Formation: Bayesian analysis of the family of OAC proteins was carried out using MrBayes 3.2.6 (Huelsenbeck and Ronquist 2001). The Jukes-Cantor model was specified along with gamma distributed rates. Two MCMC runs of four chains were run for two hundred thousand generations. Convergence was determined to have occurred when the standard deviation of split frequencies dropped below 0.05. 25% of the trees were discarded as burn-in. CEST-2 proteins were determined to be a good root for the tree as it consistently formed its own branch during tree generation.

***C. elegans* Strain Creation and Maintenance:** The Bristol N2 strain was used as a wild-type for *C. elegans*. The *rhy-1*, *bus-1*, *ndg-4*, and *nrf-6* strains were obtained from the *Caenorhabditis* Genetics Center (CGC). Table 2.4 shows a list of all strains used in this paper. All nematode strains were kept on NGM agar plates seeded with *E. coli* (OP50) at 20°C.

Creation of deletion mutants was done using a modification of the method described in Köhler *et al*. (2017) in which only one guide RNA was used. Table 2.2 gives the flanking sequences around the deletions and the primers used in the creation of strains using the single-primer deletion method.

Creation of triple-stop knock-in mutants was done using the universal STOP-IN cassette method as described in Wang *et al*. (2018). Table 2.3 gives the flanking sequences around the insertions and the primers used in the creation of strains using the triple-stop knock-in method.

Longevity Assays: All worms used in lifespan analysis were raised at 20°C. Longevity assays were performed roughly as described in Larsen *et al*. (1995). The first day of presented survival curves is the first day of adulthood, and only 10 adults were kept on each 6 cm plate to avoid overcrowding or food shortages; n<100 for all assays and n<300 for N2 and *bus-1*. Worms were scored each day as alive, dead, or missing. Worms were declared dead when they neither moved, pumped, nor responded to prodding using the end of a platinum wire pick.

Liquid Nematode Culturing: Liquid worm cultures began with growing strains on 6 cm NGM plates seeded with 100µL of OP50 *E. coli* grown in Lennox Broth and incubated at room temperature. Once the food was consumed, and a majority of worms were in the L1 phase, each plate was washed with 25mL S-complete media into a 125mL Erlenmeyer flask. Each culture was fed with 1mL of OP50 and incubated at 22°C while shaking at 220 RPM. After food was depleted, approximately 70 hours, cultures were centrifuged at 1 G for 2 minutes. The supernatant was discarded, and the gravid worms were bleached with 24mL ddH_2O, 6mL bleach, and 900 µL 10M NaOH. This mixture was rocked for 3 minutes before the eggs were centrifuged at 1 G for 1 minute. The supernatant was discarded, and the egg pellet was washed twice with 30mL M9 buffer solution and finally suspended in 5mL M9 in a 50mL falcon tube. Eggs were placed on a rocker for 24 hours at room temperature and allowed to hatch. Approximately 70,000 larvae were seeded into 25mL cultures of S-complete in a 125mL Erlenmeyer flask and

incubated at 22°C and 220 RPM for an additional 72 hours. At that time, the worms were spun down for five minutes at 1 G and the pellet and media were separated and frozen. Two biological replicates were grown for each strain.

Metabolite Extraction: The supernatant and pellets were frozen under liquid nitrogen followed by lyophilization to dryness. Lyophilized worm pellets were crushed by shaking with 2.5 mm steel balls at 100 rpm for 1.5 min in 30 s pulses while chilled with liquid nitrogen (SPEX sample prep miniG 1600). Media and powdered pellet samples were extracted with 15 mL portions of methanol for 24 h at room temperature. Extracts were clarified via centrifugation at 5,250 G for 10 min. The extracts were dried with a Speedvac Vacuum Concentrator (ThermoFisher) and resuspended in 700 µL methanol. Samples were then centrifuged at 10,000 G and 4 °C to remove particulates and analyzed by HPLC-HRMS.

Mass Spectrometric Analysis: High resolution LC−MS analysis was performed on a ThermoFisher Scientific Vanquish Horizon UHPLC System coupled with a Thermo Q Exactive HF hybrid quadrupole-orbitrap high resolution mass spectrometer equipped with a HESI ion source. Metabolites were separated using a water-acetonitrile gradient on an Agilent Zorbax Eclipse XDB-C18 column (150 mm × 7 2.1 mm, particle size 1.8 µm) maintained at 40 °C. Solvent A: 0.1% formic acid in water; Solvent B: 0.1% formic acid in acetonitrile. A/B gradient started at 1% B for 5 min after injection and increased linearly to 100% B at 20 min, using a flow rate 0.5 mL/min. Mass spectrometer parameters: spray voltage 3.0 kV, capillary temperature 380 °C, probe heater temperature 300 °C; sheath, auxiliary, and spare gas 60, 20, and 2, respectively; S-lens RF level 50, resolution 240,000 at m/z 200, AGC target 3×106 . The instrument was

calibrated with positive and negative ion calibration solutions (ThermoFisher). Each sample was analyzed in positive and negative modes using an m/z range 70 to 1000.

2.7 References

Ankeny RA. 2001. The natural history of *Caenorhabditis elegans* research. Nat Rev Genet. 2(6):474-479. doi:10.1038/35076538.

Apfeld J, Alper S. 2018. What can we learn about human disease from the nematode *C. elegans*? Methods Mol Biol. 1706:53–75. doi:10.1007/978-1-4939-7471-9_4.

Bera A, Herbert S, Jakob A, Vollmer W, Götz F. 2005. Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan O-acetyltransferase OatA is the major determinant for lysozyme resistance of *Staphylococcus aureus*. Molecular Microbiology. 55(3):778–787. doi:10.1111/j.1365-2958.2004.04446.x.

Brejning J, Nørgaard S, Schøler L, Morthorst TH, Jakobsen H, Lithgow GJ, Jensen LT, Olsen A. 2014. Loss of NDG-4 extends lifespan and stress resistance in *Caenorhabditis elegans*. Aging Cell. 13(1):156-164. doi:10.1111/acel.12165.

Brenner S. 1974. The genetics of *Caenorhabditis elegans*. Genetics. 77(1):71-94.

The *C. elegans* Sequencing Consortium. 1998. Genome sequence of the nematode *C. elegans*: a platform for investigating biology. Science. 282(5396):2012-2018. doi:10.1126/science.282.5396.2012.

Chang CC, Huh HY, Cadigan KM, Chang TY. 1993. Molecular cloning and functional expression of human acyl-coenzyme A: cholesterol acyltransferase cDNA in mutant Chinese hamster ovary cells. Journal of Biological Chemistry. 268(28):20747-20755. doi:10.1016/S0021-9258(19)36846-2.

Chang CC, Sun J, and Chang TY. 2011. Membrane-bound O-acyltransferases (MBOATs). Front Biol. 6(3):177-182. doi:10.1007/s11515-001-1149-z.

Choy RKM, Thomas JH. 1999. Fluoxetine-resistant mutants in *C. elegans* define a novel family of transmembrane proteins. Molecular Cell. 4(2):143-152. doi:10.1016/S1097-2765(00)80362-7.

Choy RKM, Kemner JM, Thomas JH. 2006. Fluoxetine-resistance genes in *Caenorhabditis elegans* function in the intestine and may act in drug transport. Genetics 172(2):885-892. doi:10.1534/genetics.103.024869.

Cook SJ, Jarrell, TA, Brittin CA, Wang Y, Bloniarz AE, Yakovlev MA, Nguyen KCQ, Tang LT-H, Bayer EA, Duerr JS, *et al*. 2019. Whole-animal connectomes of both *Caenorhabditis elegans* sexes. Nature 571(7763):63-71. doi:10.1038/s41586-019-1352-7.

Gravato-Nobre MJ, Hodgkin J. 2008. The acyltransferase gene *bus-1* exhibits conserved and specific expression in nematode rectal cells and reveals pathogen-induced cell swelling. Developmental Dynamics. 237(12):3762-3776. doi:10.1002/dvdy.21792.

Harris TW, Arnaboldi V, Cain S, Chan J, Chen WJ, Cho J, Davis P, Gao S, Grove CA, Kishore R, *et al*. 2020. WormBase: a modern model organism information resource. Nucleic Acids Res. 48(D1):D762–D767. doi:10.1093/nar/gkz920.

Hofmann K. 2000. A superfamily of membrane-bound O-acyltransferases with implications for Wnt signaling. Trends in Biochemical Sciences. 25(3):111-112. doi:10.1016/S0968-0004(99)01539-X.

Huelsenbeck JP, Ronquist F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics. 17(8):754-755. doi:10.1093/bioinformatics/17.8.754.

Köhler S, Wojcik M, Xu K, Dernburg AF. 2017. Superresolution microscopy reveals the three-dimensional organization of meiotic chromosome axes in intact *Caenorhabditis elegans* tissue. Proc Natl Acad Sci USA. 114(24):e4734-e4743. doi:10.1073/pnas.1702312114.

Lee HC, Inoue T, Imae R, Kono N, Shirae S, Matsuda S, Gengyo-Ando K, Mitani S, Arai H. 2008. *Caenorhabditis elegans mboa-7*, a member of the MBOAT family, is required for selective incorporation of polyunsaturated fatty acids into phosphatidylinositol. Mol Biol Cell. 19(3):1174-1184. doi:10.1091/mbc.E07-09-0893.

Logan CY, Nusse R. 2004. The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol. 20(1):781-810. doi:10.1146/annurev.cellbio.20.010403.113126.

Ma D, Wang Z, Merrikh CN, Lang KS, Lu P, Li X, Merrikh H, Rao Z, Xu W. 2018. Crystal structure of a membrane-bound O-acyltransferase. Nature. 562(7726):286–290. doi:10.1038/s41586-018-0568-2.

Panda O, Akagi AE, Artyukhin AB, Judkins JC, Le HH, Mahanti P, Cohen SM, Sternberg PW, Schroeder FC. 2018. Biosynthesis of Modular Ascarosides in *C. elegans*. Angew Chem Int Ed Engl. 56(17):4729-4733. doi:10.1002/anie.201700103.

Pearson CR, Tindall SN, Herman R, Jenkins HT, Bateman A, Thomas GH, Potts JR, Van der Woude MW. 2020. Acetylation of surface carbohydrates in bacterial pathogens requires coordinated action of a two-domain membrane-bound acyltransferase. mBio. 11(4). doi:10.1128/mBio.01364-20.

Röttig A, Steinbüchel. 2013. Acyltransferases in Bacteria. Microbiology and Molecular Biology Reviews. 77(2):277-321. doi:10.1128/MMBR.00010-13.

Shen C, Shao Z, Powell-Coffman JA. 2006. The *Caenorhabditis elegans rhy-1* gene inhibits HIF-1 hypoxia-inducible factor activity in a negative feedback loop that does not include *vhl-1*. Genetics. 174(3):1205-1214. doi:10.1534/genetics.106.063594.

Shreffler W, Magardino T, Shekdar K, Wolinsky E. 1995. The *unc-8* and *sup-40* genes regulate ion channel function in *Caenorhabditis elegans* motoneurons. Genetics. 139(3):1261-1272.

Spector AA, Mathur SN, Kaduce TL. 1979. Role of acylcoenzyme A: cholesterol O-acyltransferase in cholesterol metabolism. Prog Lipid Res. 18(1):31-53. doi:10.1016/0163-7827(79)90003-1.

Tuli MA, Daul A, Schedl T. 2018. *Caenorhabditis* nomenclature. WormBook. doi:10.1895/wormbook.1.183.1.

Wang H, Park H, Liu J, Sternberg PW. 2018. An efficient genome editing strategy to generate putative null mutants in *Caenorhabditis elegans* using CRISPR/Cas9. G3. 8(11):3607.16. doi:10.1534/g3.118.200662.

Yoshimura J, Ichikawa K, Shoura MJ, Artiles KL, Gabdank I, Wahba L, Smith CL, Edgley ML, Rougvie AE, Fire AZ, *et al*. 2019. Recompleting the *Caenorhabditis elegans* genome. Genome Res. 29(6):1009-1022. doi:10.1101/gr.244830.118.

Zhu J, Sanborn JZ, Diekhans M, Lowe CB, Pringle TH, Haussler D. 2007. Comparative genomics search for losses of long-established genes on the human lineage. PLOS Computational Biology. 3(12):e247. doi:10.1371/journal.pcbi.0030247.

Chapter 3

A NEW METHOD FOR CRISPR/CAS9 IN *C. BRIGGSAE*

Adapted from Cohen SM, Sternberg PW. 2019. Genome editing of *Caenorhabditis briggsae* using CRISPR/Cas9 co-conversion marker *dpy-10*. microPublication Biology. doi:10.17912/micropub.biology.000171.

3.1 Introduction

Genomic editing of the roundworm *Caenorhabditis elegans* using the CRISPR/Cas9 system has allowed for widespread creation of null mutants – vital for scientific understanding of this model organism. A closely-related nematode species, *Caenorhabditis briggsae*, is emerging as an alternative model organism to better understand how findings in *C. elegans* can broaden and develop the larger field of nematology (Gupta *et al.* 2007). To that end, we have developed an effective and efficient co-conversion CRISPR/Cas9 system for use in *C. briggsae* using the gene *dpy-10*.

3.2 Results and Discussion

We modified the universal STOP-IN cassette method, as described by Wang *et al.* (2018), for use in *C. briggsae* (Wang *et al.* 2018). Using the wildtype AF16 strain, we tested the method by choosing the gene *Cbr-dpy-10* because of its readily observed predicted phenotype. *Cbr-dpy-10* is a predicted one-to-one ortholog of *C. elegans dpy-10*, which encodes a protein important for cuticle development and has a phenotype characterized by short, fat animals relative to wild type (Brenner 1974).

A universal STOP-IN allele of *Cbr-dpy-10*, *sy1387*, was generated and confirmed by genotype sequencing. The expected phenotype was subsequently observed, consistent with the creation of

a null allele (Figure 3.1). Surprisingly, *sy1387* is dominant. Injections of 20 animals produced 15 successful injections; 247 animals from these 15 injections (F1) were singled out and allowed to self-fertilize to produce F2. 81% of the F1 Dumpy progeny were homozygous as evidenced by their segregation of only Dumpy progeny – one of these candidates became *sy1387*. 2% of the F1s did not produce progeny. 17% of the 247 F1 Dumpy progeny were heterozygotes and had a mixture of Dumpy and non-Dumpy progeny; these non-Dumpy F2s only produced non-Dumpy progeny. This discovery of a dominant mutation will allow for more effective use of this as a co-conversion marker when screening for other mutations. We used *Cbr-dpy-10* as a potential co-CRISPR marker for a second target that will be described elsewhere. We used PCR to detect insertion of a STOP-IN cassette at this other locus; we screened 39 Dumpy strains to obtain 11 candidates for our target gene, from which we have three STOP-IN alleles.

3.3 Figures

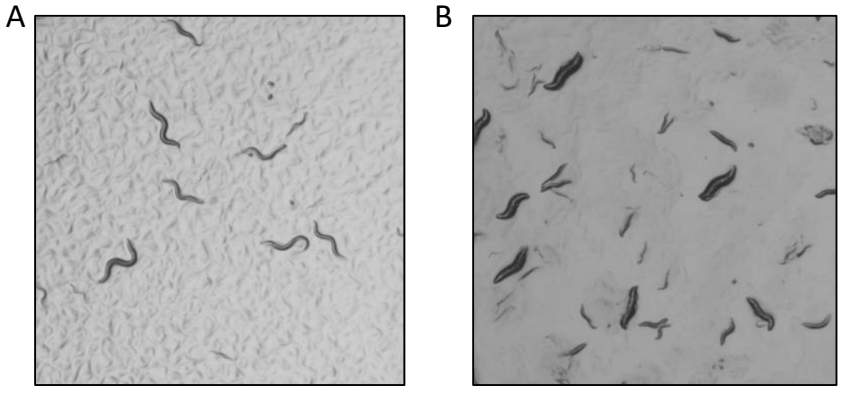


Figure 3.1 Wildtype versus *Cbr-dpy-10* in *C. briggsae*. (A) Wildtype AF16 worms as compared with (B) *Cbr-dpy-10*, which shows the Dumpy phenotype.

3.4 Materials and Methods

We used the universal STOP-IN cassette method essentially as described in Wang *et al.* (2018). Potential guide sequences were followed by a 5'-NGG-3' PAM site and were close to the start codon ATG of the target gene. The guide sequence for *Cbr-dpy-10* used in this protocol was ATTCGCGTCAGATGATGTAC, located at the beginning of the gene's second exon. To detect the stop-in insertion into *Cbr-dpy-10*, we used forward primer GAAAAACAACGGCAGAGACG and reverse primer TCCGCTTCCATAAGCACCAC.

In *sy1387*, the second exon of *Cbr-dpy-10* (shown below) was changed with the 41 basepair insert highlighted in red. This caused the early introduction of a stop codon (underlined) and a subsequent frameshift.

CTATCGAACATTCTCGCTGACAACGAACTATTCGCGTCAGATGATGGAAGTTTGTCCAGAGCAGAGGT
GACTAAGTGATAAGCTAGCTACCGGTGTGTCACAGGTCTTCAAATCGGATTTAGTTTGTTCTCATTCGT
TATCGTTTGTGCCCTTCCTATTATGTACAATCATGTCCAGAACACAATTACTTATGTTGACAGAGAAAT
AGTGGCTTATTGCGAA

All CRISPR/Cas9 system reagents were ordered from IDT except for the Cas9 protein, which was kindly provided by Tsui-Fen Chou. Sequences were downloaded from WormBase.

Strain Generated:

PS8520 *Cbr-dpy-10* (*sy1387*) II

3.5 References

Brenner S. 1974. The genetics of *Caenorhabditis elegans*. Genetics. 77(1):71–94.

Gupta BP, Johnsen R, Chen N. 2007. Genomics and biology of the nematode *Caenorhabditis briggsae*. WormBook. doi: 10.1895/wormbook.1.136.1.

Wang H, Park H, Liu J, Sternberg PW. 2018. An Efficient Genome Editing Strategy to Generate Putative Null Mutants in *Caenorhabditis elegans* Using CRISPR/Cas9. G3 (Bethesda). 8(11):3607–3616. doi: 10.1534/g3.118.200662.

Chapter 4

DAUER-INDUCING ASCAROSIDES IN *C. BRIGGSAE*

4.1 Abstract

Characterization of the functionality and formation pathways of ascaroside signaling molecules in the nematode *Caenorhabditis elegans* has been studied for several years in order to understand the worms' underlying behavioral decision-making. However, although it is known that ascarosides play a role in the behavior of other nematode species, these parallel pheromone systems have not been well-studied. Here, we show that ascarosides in the nematode *Caenorhabditis briggsae* are biosynthesized as they are in *C. elegans* and have the ability to induce the stress-resistant dauer lifestage. We show that ascr#2 is the primary component of crude dauer pheromone in *C. briggsae* in contrast to that of *C. elegans* which relies on a combination of ascr#2, ascr#3, and several other components. We further demonstrate that *Cbr-daf-22*, like its *C. elegans* ortholog *Cel-daf-22*, is necessary to produce short-chain ascarosides. Both *Cbr-daf-22* and *Cel-daf-22* mutants still produce glucosides that give us clues into the functionality of this more recently discovered class of small molecules and the broader, finely tuned system of small molecule signaling in both *C. elegans* and *C. briggsae*.

4.2 Introduction

The nematode *Caenorhabditis elegans* has long been used as model organism not only for understanding general biological phenomena but also for the specific questions of nematode development and behavior. However, as we further explore this species, it is important to expand those findings to show they are applicable to other nematodes beyond *C. elegans*.

The closely related nematode *Caenorhabditis briggsae* has frequently been used to confirm and further investigate findings in *C. elegans* (Hillier *et al.* 2007; Gupta *et al.* 2007; Wang and Chamberlin 2002). Orthologous protein sequences are about 80% identical between *C. elegans* and *C. briggsae*, similar to the divergence between protein sequences of humans and mice (78.5%) (Stein *et al.* 2003). *C. briggsae* shares many useful traits with *C. elegans* including hermaphroditism, a fully annotated genome, transparency, and a similar and short lifecycle (Gupta *et al.* 2007; Stein *et al.* 2003). Also, the growth conditions and methods for genetic and behavioral analysis are likewise similar to those of *C. elegans* and are relatively easy to modify if they do not already exist (Baird and Chamberlin 2006). Specifically, the recent development of an easy and efficient CRISPR method in *C. briggsae* has made further investigation into the genetics and behavior of this species, both for itself and as a comparison with *C. elegans*, an obvious next step (Cohen and Sternberg 2019; Culp *et al.* 2020).

One of the most intriguing developmental stages in nematodes is the dauer diapause stage. This lifestage is thought to be vital to the survival of the species during times of inconsistent or nonexistent food supplies or other limiting conditions, and it is similar to the infective juvenile (IJ) stage of many parasitic nematodes (Fodor *et al.* 1983). The dauer lifestage in *C. elegans* and *C. briggsae* is primarily triggered by the confluence of signals indicating the abundance of food, the concentration of worms in the local area, and the local amount of various dauer-inducing pheromones (Cassada and Russell 1975; Golden and Riddle 1982; Golden and Riddle 1984). However, unlike *C. elegans*, the dauer lifestage in *C. briggsae* is not also triggered by high temperatures (Inoue *et al.* 2007). Genetic screens for dauer-constitutive and dauer-defective

mutants have identified similar genes in both species in the few cases examined, suggesting that the dauer formation pathway is generally conserved (Inoue *et al.* 2007).

Dauer pheromone was found to comprise various ascarosides, glycosides of the dideoxysugar ascarylose, named for the *Ascaris* parasitic nematodes in which they were first discovered (Jeong *et al.* 2005, Jezyk and Fairbairn 1967). These ascarosides are made internally by the worms in various types and amounts throughout their lives and affect not only dauer decisions, but also most other behaviors including mating, aggregation, development, and much more (Edison 2009; Srinivasan *et al.* 2012; Pungaliya *et al.* 2009; Wharam *et al.* 2017). Nematodes across all clades produce different ascaroside profiles indicating that many of these signals are species-specific (Choe *et al.* 2012). A broad range of species also respond to ascarosides including fungi and plants (Hsueh *et al*. 2013; Manohar *et al*. 2020).

Ascarosides in *C. elegans* are made modularly using building blocks derived from cellular waste products including the sugar ascarylose and fatty acid side chains; complexity is increased by modulating the length of the fatty acid-like side chain and attaching head or terminal groups scavenged from neurotransmitters, amino acids, and other readily available materials in the cell (von Reuss *et al.* 2012). Ascarosides require a shortened fatty-acid side chain in order to be functional; this process is done through the peroxisomal beta-oxidation pathway involving the four genes: *Cel-acox-1*, *Cel-maoc-1*, *Cel-dhs-28*, and *Cel-daf-22* (Golden and Riddle 1985; von Reuss *et al.* 2012; Butcher *et al.* 2009). Mutants that lack a gene along the beta-oxidation pathway are unable to produce any of the short-chain ascarosides that affect behavior, although they are able to register exogenous ascaroside signals (Butcher *et al.* 2009).

In *C. elegans*, further modification to the 4’ position of an ascaroside to form increasingly complex signals occurs in the gut granules (Panda *et al.* 2017). These are birefringent and autofluorescent lysosomal-related organelles (LROs) in the nematode intestine used to break down and recycle cellular waste (Hermann *et al* 2005). Mutants that lack gut granules, such as *Cel-glo-1*, *Cel-glo-3*, or *Cel-apb-3* cannot produce 4’-modified or some terminally modified ascarosides (Rabbitts *et al.* 2008; Panda *et al.* 2017; Le *et al*. 2020). *Cbr-glo-1* is a one-to-one ortholog with its counterpart in *C. elegans* and has been shown to have a similar function; specifically, mutants lack both gut granules and the ability to form 4’ modified ascarosides (Appendix A; Le *et al*. 2020).

In this study, we show that the ortholog of the essential ascaroside biosynthesis gene *Cel-daf-22*, *Cbr-daf-22*, has a similar function. We used this information, as well as the previously reported discovery of *Cbr-glo-1* to compare the crude pheromones of AF16, *Cbr-daf-22*, and *Cbr-glo-1*. Through these experiments, we found that the major component of *C. briggsae* dauer-pheromone is ascr#2. We also found anti-dauer activity in the crude pheromone of both *Cel-daf-22* and *Cbr-daf-22*. Subsequent investigation of the dauer-promoting activity of *Cel-glo-1* and *Cbr-glo-1* found that their pheromone, lacking the additional information provided by 4’ modified ascarosides, causes an irregular dauer response. Based on these findings, we postulate the existence of an anti-dauer compound, or class of compounds, that adds further complexity to the intricate and finely tuned system of ascaroside signaling in *Caenorhabditis* species.

4.3 Results

Strain Construction and Phenotype of *Cbr-daf-22*

It has been shown that a *Cbr-glo-1* loss-of-function mutant has the same phenotype as does a *Cel-glo-1* mutant: neither mutant is able to form gut granules (Appendix A; Le *et al.* 2020; Hermann *et al* 2005). Concomitantly, a *Cbr-glo-1* mutant is also unable to produce complex ascarosides that have been modified with a head group at the 4' carbon positions (Appendix A; Le *et al.* 2020). To further explore the similarities between *C. elegans* and *C. briggsae* ascaroside formation, we made two mutant strains of *Cbr-daf-22*, ortholog of *Cel-daf-22*, which controls the last step in the *C. elegans* peroxisomal beta-oxidation pathway (Figure 4.1a). These mutants are unable to form simple and modular (e.g., icas#2) short-chain ascarosides (Figure 4.1b), the phenotype associated with *Cel-daf-22* mutant metabolomes in *C. elegans* (von Reuss *et al.* 2012). However, they are able to form other 1' modifications to glucose sugars, including the previously identified small molecule classes of indole glucosides (iglu) and anthranilic acid glucosides (angl) (Figures 4.1b-d) (Coburn and Gems 2013; Stupp *et al.* 2013; Le *et al.* 2020).

C. briggsae Crude Pheromone Dauer Assays

We performed a series of dauer assays using wild-type (AF16) worms to determine how the various innate crude pheromone preparations from *Cbr-glo-1* and *Cbr-daf-22* mutants would affect wild-type worm dauer formation under a range of concentrations. Wild-type (AF16) crude pheromone created an expected dose-response curve that showed a stable increase in the number of dauers as the amounts of pheromone increases (Figure 4.2a). At the high end of pheromone concentrations, almost all worms went into dauer. This assay was repeated using crude dauer pheromone from *Cbr-daf-22* (Figure 4.2b) and *Cbr-glo-1* mutants (Figure 4.2c). As hypothesized

due to its lack of innate short-chain ascarosides, *Cbr-daf-22* crude dauer pheromone was unable to induce dauer formation at any concentration. *Cbr-glo-1* crude pheromone, however, appeared to induce dauer formation at fairly consistent levels regardless of the amount added. These data suggest that the dauer pheromone in *C. briggsae* are simple (i.e., unmodified), short-chain ascarosides.

These dauer assay experiments were also performed in *C. elegans* using N2 worms with crude pheromone from N2 (Figure 4.2d), *Cel-daf-22* (Figure 4.2e), and *Cel-glo-1* (Figure 4.2f). The same pattern occurs in these assays whereby the N2 pheromone has a typical dose-response curve, the *Cel-daf-22* pheromone induces no dauers, and the *Cel-glo-1* pheromone shows an inconsistent dauer induction effect relative to the amount added. In the *C. elegans* curve, this response is more muted than in *C. briggsae*, with the percentage of dauers being consistently lower.

Isolation of Main Dauer Pheromone Component in *C. briggsae*

The response of *Cbr-glo-1* mutants to doses of crude pheromone (Figure 4.2c) indicates that at least one major component of the *C. briggsae* dauer-inducing pheromone must be a simple ascaroside since *Cbr-glo-1* pheromone is able to induce dauer formation. As the *C. briggsae* metabolome primarily consists of ascr#2 and ascr#6.1, and their derivatives, we were able to delineate the likely major dauer pheromone component to those two simple ascarosides (Dong *et al.* 2016; von Reuss 2018). Also, *C. briggsae* has been shown to respond to *C. elegans* dauer pheromone (Fodor *et al.* 1983), primarily consisting of ascr#2 and asc#3 among others – making ascr#2 a leading candidate for the dominant dauer pheromone signal (Butcher *et al.* 2007).

The dauer pheromone curve for ascr#2 demonstrated that it was the main dauer ascaroside for *C. briggsae* (Figure 4.3a). *C. briggsae* dauer ascarosides are active as low as the nanomolar to micromolar range, consistent with the general amounts of crude pheromone or purified single ascaroside needed to produce dauer formation effects in *C. elegans* (Srinivasan *et al.* 2012) (Figure 4.4). In contrast to the dauer effects of ascr#2, ascr#6.1 prompts little to no dauer formation even at high concentrations (Figure 4.3b). In agreement with the metabolomics performed above (Figure 4.1b), ascr#2 is present in all dauer-inducing crude pheromone tested and is not present in crude pheromone that is unable to induce dauer formation (Figure 4.3c).

Comparison of *C. briggsae* and *C. elegans* crude dauer pheromones shows that AF16 pheromone elicits a slightly higher rate of dauer formation than the same amount of N2 pheromone (Figure 4.3d). However, each pheromone affects the two species similarly.

The crude pheromone for *Cbr-daf-22* shows more indole glucosides; this is similar to what is found in the general *Cbr-daf-22* metabolome (Figure 4.3e). In contrast, *Cbr-glo-1* and *Cel-glo-1* crude pheromone both show more phosphorylated ascarosides (Figure 4.3e).

Cbr-daf-22 Pheromone Causes an Anti-Dauer Effect

Although we found that ascr#6.1 had essentially no effect on dauer formation in *C. briggsa*e, the fact that, at high concentrations, it does show some marginal dauer formation indicated that the overall paucity of dauer formation in *Cbr-daf-22* mutants at all concentrations was unusual. To test for an anti-dauer effect, we combined the crude pheromones of AF16 and *Cbr-daf-22* to see if such a combination was additive or antagonistic. We found dauer formation was significantly

depressed whenever *Cbr-daf-22* pheromone was present (Figure 4.5a). When this experiment was performed in *C. elegans*, the same phenomenon was observed (Figure 4.5b).

4.4 Discussion

Comparison of Ascaroside Formation Pathways in *C. briggsae* and *C. elegans*

As *C. briggsae* and *C. elegans* are closely related evolutionarily, so too are the basic pathways with which they create their myriad of nematode-specific communication molecules, collectively called ascarosides (reviewed by von Reuss 2018). The *C. briggsae* one-to-one orthologs of *Cel-daf-22* (*Cbr-daf-22*) and *Cel-glo-1* (*Cbr-glo-1*) have been shown to be physiologically and metabolomically equivalent (Appendix A; Le et al 2020).

Cel-daf-22 and *Cbr-daf-22* mutants cannot create the biologically relevant short-chain ascarosides used by both worm species. However, they are still able to produce indole glucosides (iglu) and anthranilic acid glucosides (angl) at wildtype or lower levels. Functionally, the absence of short-chain ascarosides in *Cel-daf-22* and *Cbr-daf-22* crude pheromone causes a complete lack of dauer formation under the same conditions where increased amounts of AF16 or N2 crude pheromone causes a linear increase in dauer formation.

Cel-glo-1 and *Cbr-glo-1* mutants have previously been shown to lack gut granules and the subsequent ability to form 4’ modified ascarosides. Functionally, this lack appears to also create an imbalance in the fine-tuned system of dauer-formation communication signaling and causes *Cbr-glo-1* and *Cel-glo-1* mutant crude pheromone to cause unusual dose response curves where the amount of dauer formation appears to be independent of the amount of pheromone

encountered. As phosphorylated-ascarosides are upregulated in both *Cel-glo-1* and *Cbr-glo-1* (Figure 3e), they are possible candidates for this effect.

With these similarities of the main ascaroside-forming pathways, we can infer that other major components of the ascaroside formation pathways, such as the remainder of the beta-oxidation pathway, are likewise conserved. We also expect to see further similarities in the major components of ascaroside formation between *C. elegans* and other nematode species, especially when their ascaroside profiles overlap.

Isolation of Primary Dauer Pheromone Component in *C. briggsae*

C. briggsae releases fewer amounts and types of ascarosides than *C. elegans* and, subsequently, has seemingly fewer behaviors regulated by ascarosides. The pathways that are conserved between the two species indicate their importance to those behaviors.

We confirmed that ascr#2 is the main component of dauer pheromone in *C. briggsae*. Thus, ascr#2 acts functionally the same in both *C. briggsae* and *C. elegans*. Combined with the conserved ascaroside formation pathways, this indicates that it may be an ascaroside originally used by the evolutionary ancestor of both species. However, the two species then diverge in other dauer-inducing ascaroside signals such as ascr#3, which is another major component of *C. elegans* dauer pheromone, but which is absent in the *C. briggsae* metabolome (Butcher *et al.* 2007).

C. elegans and *C. briggsae* not only are closely related in the elegans group of the *Caenorhabditis* genus, but also have overlapping habitats (Cutter *et al.* 2006). The two species have diverged climatically and temporally with *C. briggsae* preferring warmer climates and seasons than *C. elegans* (Félix and Duveau 2012). But this divergence likely did not change their underlying communication needs when it comes to basic dauer-ascaroside messaging. Overlying yet distinct ascaroside profiles may also indicate that, in the wild, their pheromone signally may be mutually beneficial to both species using broad-strokes signals such as ascr#2. However, we know that indole-modified ascaroside biosynthesis and subsequent behavior is highly species-dependent (Dong *et al.* 2016). Further refinement using species-specific signaling would allow the worms to give the best adaptive advantages to their own brethren. These different signals also would allow the worms to ignore signals that indicate imperfect conditions for other species when conditions for their own species (temperature proclivities, for example) are ideal.

The use of ascr#2 as a dauer-inducing pheromone in *C. briggsae* also aligns with the observation that indole modifications of ascr#2 act as a sex pheromone (Dong *et al.* 2016). The unmodified pheromone may be used as an indication of negative reproductive conditions and thus a dauer-promoting signal while the further modification indicates positive reproductive conditions.

Anti-Dauer Effects of *Cbr-daf-22* and *daf-22* Dauer Pheromone

Although the absence of short-chain ascarosides and their dauer-inducing signals staves off dauer-formation under ideal developmental conditions, the almost complete lack of dauer formation in *Cbr-daf-22* and *Cel-daf-22* samples indicates something more. At high concentrations of ascarosides, some dauers are usually found, likely due to population density

indications and other factors. However, *Cbr-daf-22* and *Cel-daf-22* pheromone actively depresses dauer formation, even when in the presence of dauer-promoting crude pheromone from wild-type worms.

Our findings indicate that *Cbr-daf-22* and *Cel-daf-22* metabolomes contain either a specific anti-dauer compound or a compound that acts as a receptor antagonist. Potential candidates for either process likely come from the angl or iglu families of glucosides, as these small molecules are highly represented in *Cbr-daf-22* and *Cel-daf-22* metabolomes. The iglu family of glucosides is especially interesting as a potential anti-dauer signal as previous studies have shown these small molecules are made using indole derived from *E. coli*, the primary food source of lab-cultured worms (Stupp *et al.* 2013). It would also help explain the idea that if there is some food, there will never be 100% dauer even at extremely high concentrations of dauer pheromone (Golden and Riddle 1984).

While some glucosides are upregulated in the metabolomes of *Cbr-glo-1* and *Cel-glo-1* (iglu#1, iglu#2, angl#1, and angl#2), most glucosides are wholly absent, which together may explain the unusual dauer curves of those strains. If the finely balanced system between dauer and anti-dauer signals becomes disordered, it may cause confusion in worms that are trying to integrate a multitude of signals prior to the dauer decision.

In addition to food, population density, and pheromone, the dauer-decision system is further adjusted through the use of additional pheromones. Thus, the dauer decision is not simply made under unfavorable conditions opposite to the standard L3 reproductive stage – instead, there is an

active anti-dauer signal that adds to the complexity of the finely-tuned dauer-decision process in *C. elegans* and *C. briggsae.*

4.5 Figures and Tables

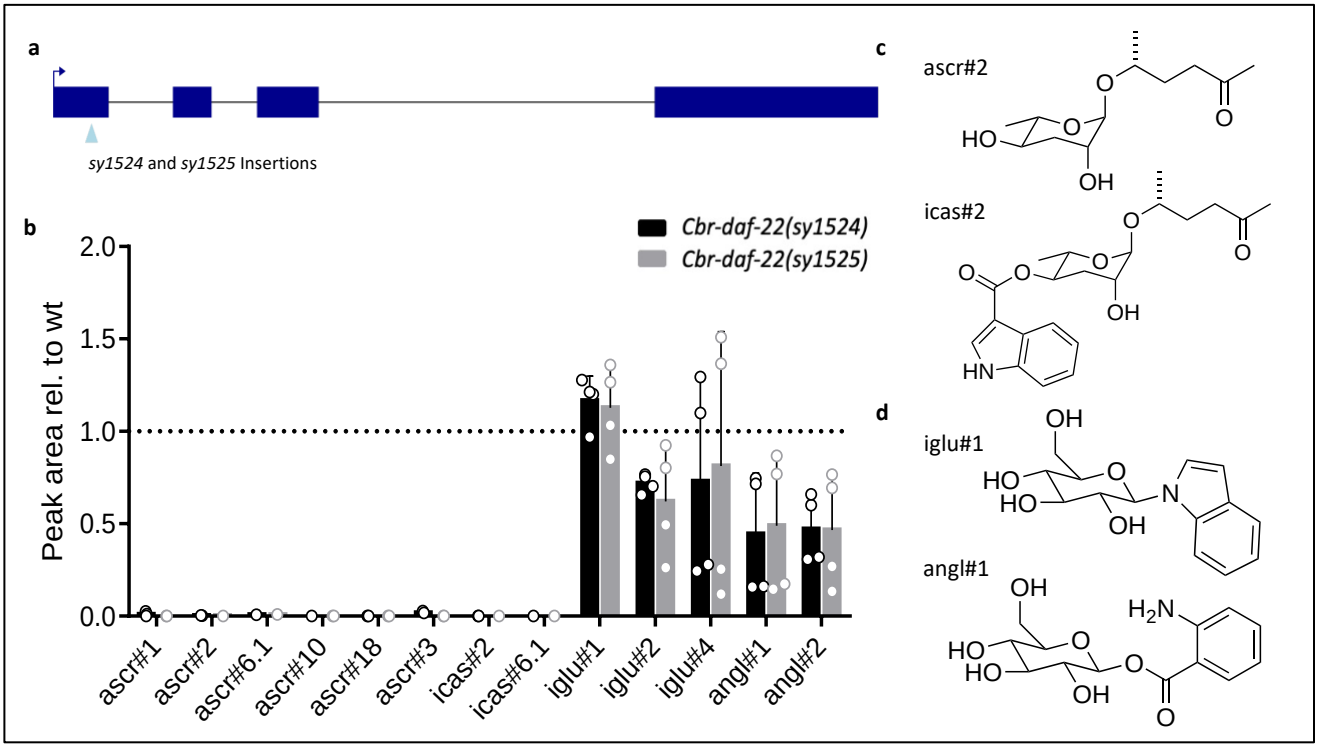


Figure 4.1 *C. briggsae daf-22* mutant and phenotypes. (a) Two *Cbr-daf-22* strains made using the CRISPR/Cas9 triple stop knock-in method to insert the full 43 basepair triple-stop insert *(sy1524)* and a complex substitution *(sy1525)* in the first exon. (b) The metabolomes of both *Cbr-daf-22* strains do not produce any (c) functional short-chain ascarosides or their 4' modifications. (d) The *Cbr-daf-22* strains are, however, still able to produce glucoside sugars modified at the 1' position including indole glucosides (iglus) and anthranilic acid glucosides (angls).

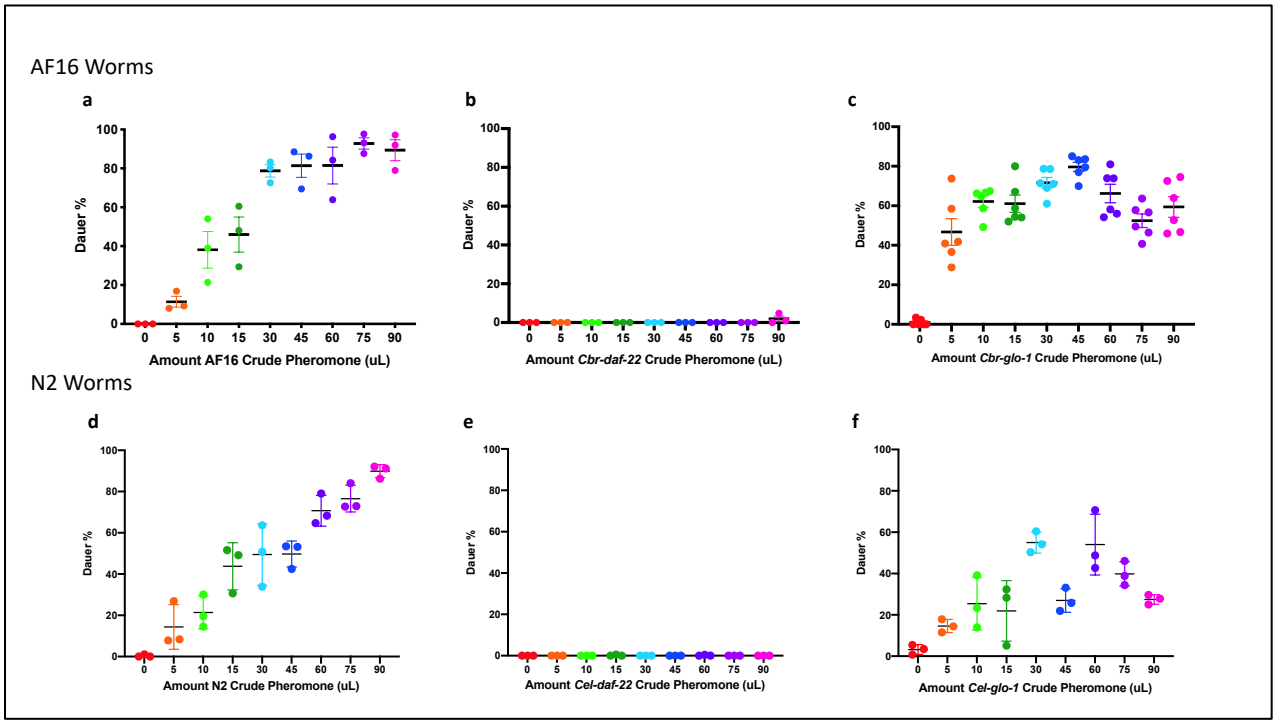


Figure 4.2 Response to doses of crude pheromone in wild-type *C. briggsae* and *C. elegans*. (a) The dauer curve of AF16 crude pheromone using AF16 worms creates a linear increase in dauer percentage with the increase in the amount of pheromone. (b) *Cbr-daf-22*(*sy1524*) crude pheromone produces no dauer-inducing effect on wild-type *C. briggsae* worms. (c) *Cbr-glo-1*(*sy1382*) crude pheromone produces a dauer curve that remains stable despite changing amounts of dauer pheromone. (d) When carried out in *C. elegans*, N2 pheromone and (e) *Cel-daf-22* pheromone produce very similar curves to their *C. briggsae* counterparts. (f) *Cel-glo-1* pheromone again produces a non-linear effect, but it is more muted than that of *C. briggsae*.

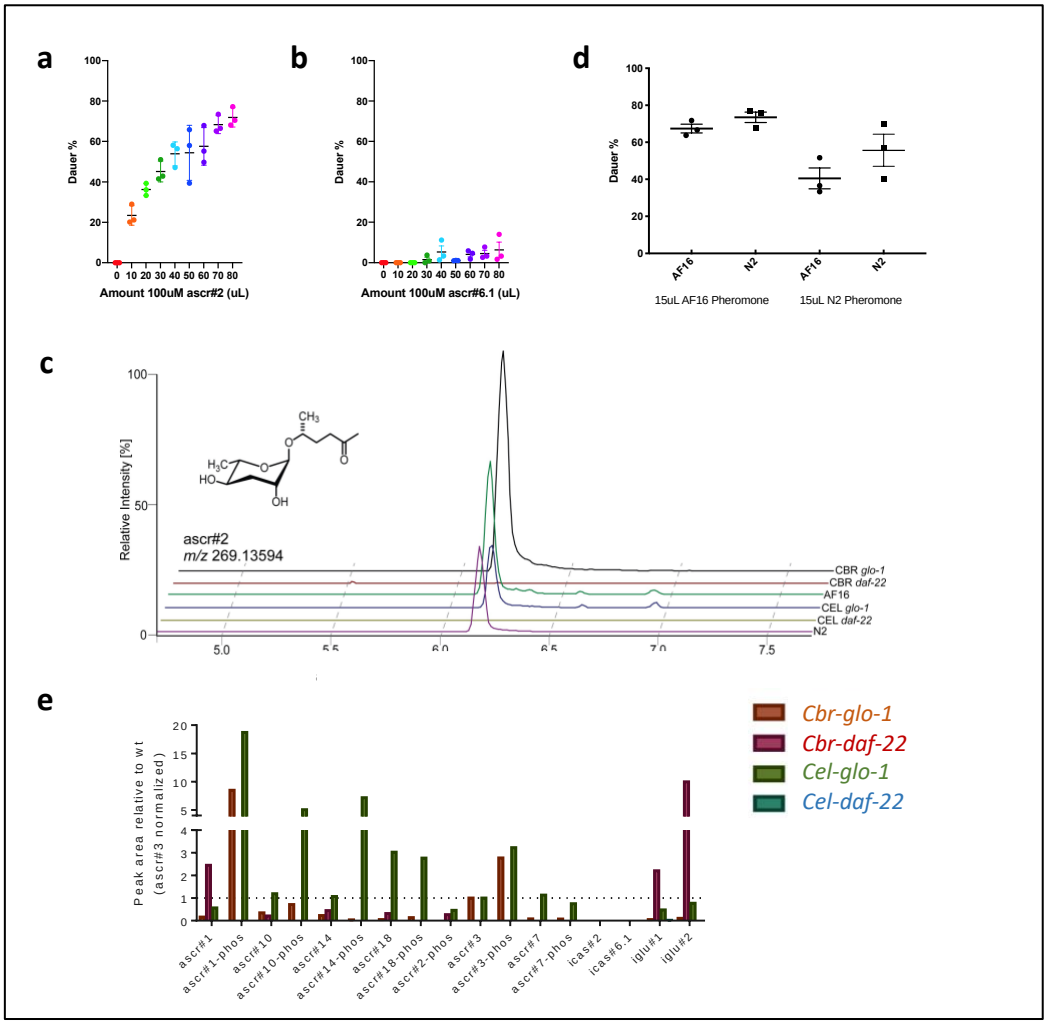


Figure 4.3 Ascarosides are used as signals to induce dauer formation. (a) ascr#2, a main component of the *C. briggsae* metabolome, induces dauer formation increasingly as the amount of ascaroside increases, and is a main component of dauer pheromone in *C. briggsae*. (b) ascr#6.1, another major component of the *C. briggsae* metabolome, does not significantly induce dauer, even at high concentrations. (c) ascr#2 is found in the crude pheromone of AF16, N2, *Cbr-glo-1*, and *Cel-glo-1*, all of which are able to induce dauer formation; ascr#2 is not found in the crude pheromone of *Cbr-daf-22* or *Cel-daf-22*. (d) Crude pheromone affects *C. elegans* and *C. briggsae* similarly except that AF16 pheromone appears to be slightly more potent than N2 pheromone. (e) Crude pheromone from *Cbr-daf-22(sy1524)* shows many glucosides as opposed to short-chain ascarosides while *Cbr-glo-1(sy1382)* crude pheromone shows many phosphorylated ascarosides.

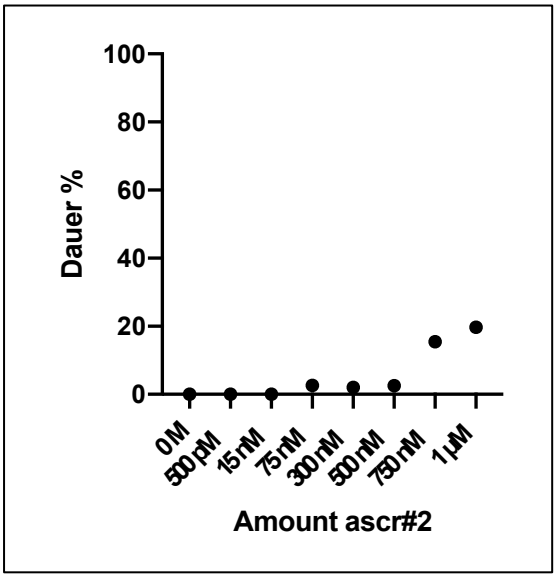


Figure 4.4 Active concentrations of ascr#2. ascr#2 begins to affect dauer formation between 500 nM and 750 nM.

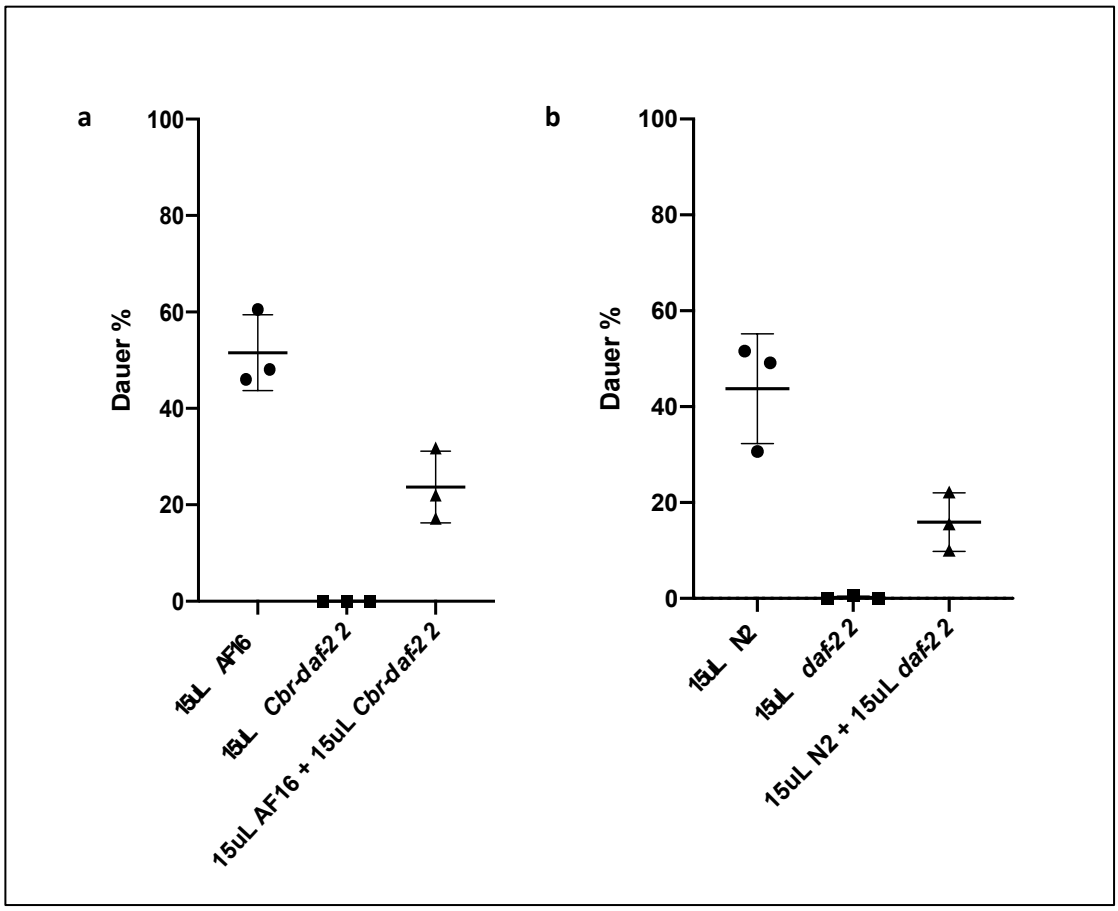


Figure 4.5 Combinations of crude pheromone are not additive. (a) When combined with wild-type *C. briggsae* crude pheromone, *Cbr-daf-22* mutant crude pheromone actively suppresses dauer formation in AF16 worms. (b) The same suppression occurs when wildtype *C. elegans* crude pheromone is combined with *daf-22* mutant crude pheromone.

Table 4.1 List of Strains

Gene	Strain	Source
C. elegans Wild-type	N2	Brenner, 1974; Caenorhabditis Genomics Center (CGC)
Cbr-daf-22(*sy1524*)	PS8777	This work
Cbr-daf-22(*sy1525*)	PS8778	This work
Cbr-glo-1(*sy1382*)	PS8515	Le *et al* 2020; Sternberg lab collection
C. briggsae Wild-type	AF16	Caenorhabditis Genomics Center (CGC)
daf-22(*ok693*)	RB859	The *C. elegans* Deletion Mutant Consortium 2012; Caenorhabditis Genomics Center (CGC)
glo-1(*zu391*)	JJ1271	Hermann *et al* 2005; Caenorhabditis Genomics Center (CGC)

4.6 Materials and Methods

***C. briggsae* and *C. elegans* Strains and Strain Maintenance:** The Indian AF16 strain was used as a wild-type for *C. briggsae* while the Bristol N2 strain was used as a wild-type for *C. elegans*. See Table 4.1 for a list of all strains used. All nematode strains were kept on NGM agar plates seeded with *E. coli* (OP50) at 20°C.

Construction of *CBR-daf-22* Mutants: Construction of the two *Cbr-daf-22* mutants was done using the *C. briggsae* modifications of the universal STOP-IN cassette method as described in Cohen and Sternberg (2019) and Wang *et al.* (2018). The guide used was AATAGTGCATTAGACGATTG; the forward primer was ATGAGCCCAACCAAGCCAAA; and the reverse primer was CGGCTGGGTATGGAAGCTTT. *Cbr-daf-22* (*sy1524*) was a successful insertion of the universal STOP-IN cassette, while *Cbr-daf-22* (*sy1525*), contains a complex substitution; both are shown below with the flanking sequences underlined.

sy1524

GTGAATAGTGCATTAGACGAGGGAAGTTTGTCCAGAGCAGAGGTGACTAAGTGATAAGCTAGCTTGTGGACTAAAATATGCCG

sy1525

AAAGAGGCTGTGAATAGTGCTAAATCCGATTTGAAGACCTGTGACACACCGGTAGCTAGCTTATCACTTGTGGACTAAAATATGCCG

Dauer Pheromone Collection: Crude pheromone of AF16, *Cbr-daf-22(sy1524)*, *Cbr-glo-1(sy1382)*, N2, *Cel-daf-22(ok693)*, and *Cel-glo-1(zu391)* was made using previously described methods (Golden and Riddle 1984; Schroeder and Flatt 2014). Briefly, 1L of liquid worm culture was grown until exhausted. The liquid culture supernatant was separated from the pellet, filtered, and then dried completely. The dried material was extracted using ethanol; the extracts were pooled, dried, and re-dissolved in 1mL of sterilized water.

Dauer Assays: Dauer assays were run using the method described in Lee *et al.* (2017). The dauer plates were prepared by adding the desired amount of crude pheromone or ascaroside to a 35x10 mm plate before adding 2 mL agarose. These plates were left to dry for one day. Prior to adding any worms, the plates were seeded with 10µL of heat-killed OP50 in the center of the plate. Ten young adult worms were placed on the plate and allowed to lay eggs for three hours. The young adult worms were removed, and the plates were seeded with an additional 10 µL of OP50 on top of the eggs. The plates were stored in an incubator at 25.5°C for 48 hours. At the 24-hour point, the plates were opened momentarily to allow for air circulation. At the 48-hour point, the number of total worms and dauer-stage worms was counted for each plate.

Liquid Nematode Culturing: Culturing began by chunking *C. briggsae* onto 10 cm NGM plates (each seeded with 800 µL of OP50 *E. coli* grown to stationary phase in Lennox Broth) and incubated at 22°C. Once the food was consumed, each plate was then washed with 25 mL of S-complete medium into a 125 mL Erlenmeyer flask, and 1 mL of OP50 *E. coli* was added (*E. coli* cultures were grown to stationary phase in Lennox Broth, pelleted and resuspended at 1 g wet mass per 1 mL M9 buffer), shaking at 220 RPM and 22°C. After 70 hours, cultures were centrifuged at 1000 G for one minute. After discarding supernatant, 24 mL H_2O was added along with 6 mL bleach, 900 µL 10 M NaOH, and the mixture was shaken for three minutes to prepare eggs. Eggs were centrifuged at 1000 G, the supernatant was removed, and the egg pellet washed with 25 mL M9 buffer twice and then suspended in a final volume of 5 mL M9 buffer in a 50 mL centrifuge tube. Eggs were counted and placed on a rocker and allowed to hatch as L1 larvae for 24 hours at 22°C. 75,000 L1 larvae were seeded in 25 mL cultures of S-complete with 1 mL of OP50 and incubated at 220 RPM and 22°C in a 125 mL Erlenmeyer flask. After 72 hours, worms

were spun at 1000 G for 5 min and spent medium was separated from worm body pellet. Separated medium and worm pellet were flash frozen over liquid nitrogen and then lyophilized. Two biological replicates were grown for each strain. Mutants were grown with parallel wildtype controls, and biological replicates were started on different days.

Metabolite Extraction: Lyophilized pellet and media samples were crushed and homogenized by shaking with 2.5 mm steel balls at 1300 RPM for 3 minutes in 30 s pulses while chilled with liquid nitrogen (SPEX sample prep miniG 1600). Powdered media and pellet samples were extracted with 10 mL methanol in 50 mL centrifuge tubes, rocking overnight at 22°C. Extractions were pelleted at 5000 G for 10 minutes at 4°C, and supernatants were transferred to 20 mL glass scintillation vials. Samples were then dried in a SpeedVac (Thermo Fisher Scientific) vacuum concentrator. Dried materials were resuspended in 1 mL methanol and vortexed for 1 min. Samples were pelleted at 10000 G for 5 minutes and 22°C, and supernatants were transferred to 2 mL HPLC vials and dried in a SpeedVac vacuum concentrator. Samples were resuspended in 100 µL of methanol, transferred into 1.7 mL Eppendorf tubes, and centrifuged at 18,000 G for 20 minutes at 4°C. Clarified extracts were transferred to HPLC vials and stored at −20°C until analysis.

Mass Spectrometric Analysis: High resolution LC-MS analysis was performed on a Thermo Fisher Scientific Vanquish Horizon UHPLC System coupled with a Thermo Q Exactive hybrid quadrupole-orbitrap high-resolution mass spectrometer equipped with a HESI ion source. 1 µL of extract was injected and separated using a water-acetonitrile gradient on a Thermo Scientific Hypersil GOLD C18 column (150 mm x 2.1 mm 1.9 um particle size 175 Å pore size, Thermo

Scientific) and maintained at 40°C. Solvents were all purchased from Fisher Scientific as HPLC grade. Solvent A: 0.1% formic acid in water; solvent B: 0.1% formic acid in acetonitrile. A/B gradient started at 1% B for 3 min, then from 1% to 100% B over 20 min, 100% for 5 min, then down to 1% B for 3 min. Mass spectrometer parameters: 3.5 kV spray voltage, 380°C capillary temperature, 300°C probe heater temperature, 60 sheath flow rate, 20 auxiliary flow rate, 2.0 spare gas; S-lens RF level 50.0, resolution 240,000, m/z range 150–1000 m/z, AGC target 3e6. Instrument was calibrated with positive and negative ion calibration solutions (Thermo-Fisher) Pierce LTQ Velos ESI pos/neg calibration solutions. Peak areas were determined using Xcalibur 2.3 QualBrowser version 2.3.26 (Thermo Scientific) using a 5 ppm window around the m/z of interest.

4.7 References

Baird SE, Chamberlin HM. 2006. *Caenorhabditis briggsae* methods. WormBook. Review.

Butcher RA, Fujita M, Schroeder FC, Clardy J. 2007. Small -molecule pheromones that control dauer development in *Caenorhabditis elegans*. Nat Chem Biol. 3(7):420-422. doi:10.1038/nchembio.2007.3.

Butcher RA, Ragains JR, Li W, Ruvkun G, Clardy J, Mak HY. 2009. Biosynthesis of the *Caenorhabditis elegans* dauer pheromone. Proc Natl Acad Sci USA. 106(6):1875-1879. doi:10.1073/pnas.0810338106.

The *C. elegans* Deletion Mutant Consortium. 2012. Large-scale screening for targeted knockouts in the *Caenorhabditis elegans* genome. G3. 2(11):1415–1425. doi:10.1534/g3.112.003830.

Cassada RC, Russell RL. 1975. The dauerlarva, a post-embryonic developmental variant of the nematode *Caenorhabditis elegans*. Dev Biol. 46(2): 326-342. doi:10.1016/0012-1606(75)90109-8.

Choe A, von Reuss SH, Kogan D, Gasser RB, Platzer EG, Schroeder FC, Sternberg PW. 2012. Ascaroside signaling is widely conserved among nematodes. Curr Biol. 22(9):772-780. doi:10.1016/j.cub.2012.03.024.

Coburn C, Gems D. 2013. The mysterious case of the *C. elegans* gut granule: death fluorescence, anthranilic acid, and the kynurenine pathway. Front Genet. 4. doi:10.3389/fgene.2013.00151.

Cohen SM, Sternberg PW. 2019. Genome editing of *Caenorhabditis briggsae* using CRISPR/Cas9 co-conversion marker *dpy-10*. microPublication Biology. doi:10.17912/micropub.biology.000171.

Culp E, Richman C, Sharanya D, Jhaveri N, van den Berg W, Gupta BP. 2020. Genome editing in the nematode *Caenorhabditis briggsae* using the CRISPR/Cas9 system. Biology Methods and Protocols. 5(1):1-5. doi:10.1093/biomethods/bpaa003.

Cutter AD, Félix M-A, Barrière A, Charlesworth D. 2006. Patterns of nucleotide polymorphism distinguish temperate and tropical wild isolates of *Caenorhabditis briggsae*. Genetics. 173(4):2021-2031. doi:10.1534/genetics.106.058651.

Dong C, Dolke F, von Reuss SH. 2016. Selective MS screening reveals a sex pheromone in *Caenorhabditis briggsae* and species-specificity in indole ascaroside signaling. Org & Biomol Chem. 14(30):7217-7225. doi:10.1039/C6OB01230B.

Edison AS. 2009. *Caenorhabditis elegans* pheromones regulate multiple complex behaviors. Curr Opin Neurobiol. 19(4):378-388. doi:10.1016/j.conb.2009.07.007.

Félix M-A, Duveau F. 2012. Population dynamics and habitat sharing of natural populations of *Caenorhabditis elegans* and *C. briggsae*. BMC Biol. 10(1):59. doi:10.1186/1741-7007-10-59.

Fodor A, Riddle DL, Nelson FK, Golden JW. 1983. Comparison of a new wild-type *Caenorhabditis briggsae* with laboratory strains of *C. briggsae* and *C. elegans*. Nematologica 29(2):203-216. doi:10.1163/187529283X00456.

Golden JW, Riddle DL. 1982. A pheromone influences larval development in the nematode *Caenorhabditis elegans*. Science. 218(4572):578-580. doi:10.1126/science.6896933.

Golden JW, Riddle DL. 1984. The *Caenorhabditis elegans* dauer larva: developmental effects of pheromone, food, and temperature. Dev Biol. 102(2):368-378. doi:10.1016/0012-1606(84)90201-X.

Golden JW, Riddle DL. 1985. A gene affecting production of the *Caenorhabditis elegans* dauer-inducing pheromone. Mol Gen Genet. 198(3):534-536. doi:10.1007/bf00332953.

Gupta BP, Johnsen R, Chen N. 2007. Genomics and biology of the nematode *Caenorhabditis briggsae*. WormBook. Review.

Hermann GJ, Schroeder LK, Hieb CA, Kershner AM, Rabbitts BM, Fonarev P, Grant BD, Preiss JR. 2005. Genetic analysis of lysosomal trafficking in *Caenorhabditis elegans*. Mol Biol Cell. 16(7):3273-3288. doi:10.1091/mbc.e05-01-0060.

Hillier LW, Miller RD, Baird SE, Chinwalla A, Fulton LA, Koboldt DC, Waterston RH. 2007. Comparison of *C. elegans* and *C. briggsae* genome sequences reveals extensive conservation of

chromosome organization and synteny. PLOS Biol. 5(7):e167. doi:10.1371/journal.pbio.0050167.

Hsueh Y-P, Mahanti P, Schroeder FC, Sternberg PW. 2013. Nematode-trapping fungi eavesdrop on nematode pheromones. Curr Biol. 23(1):83-86. doi:10.1016/j.cub.2012.11.035.

Inoue T, Ailion M, Poon SH, Kim HK, Thomas JH, Sternberg PW. 2007. Genetic analysis of dauer formation in *Caenorhabditis briggsae*. Genetics. 177(2):809-818. doi:10.1534/genetics.107.078857.

Jeong P-Y, Jung M, Yim Y-H, Kim H, Park M, Hong E, Lee W, Kim YH, Kim K, Paik Y-K. 2005. Chemical structure and biological activity of the *Caenorhabditis elegans* dauer-inducing pheromone. Nature. 433(7025):541-545. doi:10.1038/nature03201.

Jezyk PF, Fairbairn D. 1967. Ascarosides and ascaroside esters in *Ascaris lumbricoides* (Nematoda). Comp Biochem Physiol. 23(3):691-705. doi:10.1016/0010-406X(67)90334-9.

Le HH, Wrobel CJ, Cohen SM, Yu J, Park H, Helf MJ, Curtis BJ, Kruempel JC, Rodrigues PR, Hu PJ, *et al*. 2020. Modular metabolite assembly in *Caenorhabditis elegans* depends on carboxylesterases and formation of lysosome related organelles. eLife. 9:e61886. doi:10.7554/eLife.61886.

Lee JS, Shih P-Y, Schaedel ON, Quintero-Cadena P, Rogers AK, Sternberg PW. 2017. FMRFamide-like peptides expand the behavioral repertoire of a densely connected nervous system. Proc Natl Acad Sci USA. 114(50):E10726-E10735. doi:10.1073/pnas.1710374114.

Manohar M, Tenjo-Castano F, Chen S, Zhang YK, Kumari A, Williamson VM, Wang X, Klessig DF, Schroeder FC. 2020. Plant metabolism of nematode pheromones mediates plant-nematode interactions. Nature Communications. 11(1): 208. doi:10.1038/s41467-019-14104-2.

Panda O, Akagi AE, Artyukhin AB, Judkins JC, Le HH, Mahanti P, Cohen SM, Sternberg PW, Schroeder FC. 2017. Biosynthesis of Modular Ascarosides in *C. elegans*. Angew Chem Int Ed Engl. 56(17):4729-4733. doi:10.1002/anie.201700103.

Pungaliya C, Srinivasan J, Fox BW, Malik RU, Ludewig AH, Sternberg PW, Schroeder FC. 2009. A shortcut to identifying small molecules signals that regulate behavior and development in *Caenorhabditis elegans*. Proc Natl Acad Sci USA. 106(19):7708-7713. doi:10.1073/pnas.0811918106.

Rabbitts BM, Ciotti MK, Miller NE, Kramer M, Lawrenson AL, Levitte S, Kremer S, Kwan E, Weis AM, Hermann GJ. 2008. *glo-3*, a novel *Caenorhabditis elegans* gene, is required for lysosome-related organelle biogenesis. Genetics 180(2):857-871. doi:10.1534/genetics.108.093534.

Schroeder NE, Flatt KM. 2014. *In vivo* imaging of dauer-specific neuronal remodeling in *C. elegans*. J Vis Exp. (91):e51834. doi:10.3791/51834.

Srinivasan J, von Reuss SH, Bose N, Zaslaver A, Mahanti P, Ho MC, O'Doherty OG, Edison AS, Sternberg PW, Schroeder FC. 2012. A Modular Library of Small Molecule Signals Regulates Social Behaviors in *Caenorhabditis elegans*. PLOS Biol. 10(1). doi:10.1371/journal.pbio.1001237.

Stein LD, Bao Z, Blasiar D, Blumenthal T, Brent MR, Chen N, Chinwalla A, Clarke L, Clee C, Coghlan A, *et al*. 2003. The genome sequence of *Caenorhabditis briggsae*: a platform for comparative genomics. PLOS Biol. 1(2):e45. doi:10.1371/journal.pbio.0000045.

Stupp GS, von Reuss SH, Izrayelit Y, Ajredini R, Schroeder FC, Edison AS. 2013. Chemical detoxification of small molecules by *C. elegans*. ACS Chem Biol. 8(2):309-313. doi:10.1021/cb300520u.

von Reuss SH. 2018. Exploring modular glycolipids involved in nematode chemical communication. CHIMIA International Journal for Chemistry. 72(5):297-303. doi:10.2533/chimia.2018.297.

von Reuss SH, Bose N, Srinivassan J, Yim JJ, Judkins JC, Sternberg PW, Schroeder FC. 2012. Comparative metabolomics reveals biogenesis of ascarosides, a modular library of small-molecule signals in *C. elegans*. J Am Chem Soc. 134(3):1817-1824. doi:10.1021/ja210202y.

Wang H, Park H, Liu J, Sternberg PW. 2018. An efficient genome editing strategy to generate putative null mutants in *Caenorhabditis elegans* using CRISPR/Cas9. G3. 8(11):3607-3616. doi:10.1534/g3.118.200662.

Wang X, Chamberlin HM. 2002. Multiple regulatory changes contribute to the evolution of the *Caenorhabditis lin-48 ovo* gene. Genes Dev. 16(18):2345-2349. doi:10.1101/gad.996302.

Wharam B, Weldon L, Viney M. 2017. Pheromone modulates two phenotypically plastic traits – adult reproduction and larval diapause – in the nematode *Caenorhabditis elegans*. BMC Evol Biol. 17(1):197. doi:10.1186/s12862-017-1033-9.

Appendix A

A NULL MUTATION OF *CBR-GLO-1*

Relevant sections adapted from: Le HH, Wrobel CJ, Cohen SM, Yu J, Park H, Helf MJ, Curtis BJ, Kruempel JC, Rodrigues PR, Hu PJ, *et al*. 2020. Modular metabolite assembly in *Caenorhabditis elegans* depends on carboxylesterases and formation of lysosome related organelles. eLife. 9:e61886. doi:10.7554/eLife.61886.

5.1 Results and Discussion

In addition to *C. elegans* and *P. pacificus*, modular ascarosides have been reported from several other *Caenorhabditis* species (Dong et al. 2020; Kanzaki et al. 2018), including *C. briggsae* (Dong et al. 2016; von Reuss 2018). To assess whether the role of LROs in the biosynthesis of modular metabolites is conserved across species, we created two *Cbr-glo-1* (CBG01912.1) knock-out strains using CRISPR/Cas9. As in *C. elegans*, *Cbr-glo-1* mutant worms lacked autofluorescent LROs, which are prominently visible in wild-type *C. briggsae* (Figure 5.1). Comparative metabolomic analysis of the endo- and exo-metabolomes of wild-type *C. briggsae* and the *Cbr-glo-1* mutant strains revealed that biosynthesis of all known modular ascarosides is abolished in *Cbr-glo-1* worms, including the indole carboxy derivatives icas#2 and icas#6.2, which are highly abundant in wild-type C. briggsae (Figure 5.2a; Dong et al., 2016). In addition, the *C. briggsae* MS2 networks included several large *Cbr-glo-1*-dependent clusters representing modular glucosides, including many of the compounds also detected in *C. elegans*, for example iglu#4 and angl#4. As in *C. elegans*, production of unmodified glucoside scaffolds, e.g., iglu#1 and angl#1, was not reduced or increased in *Cbr-glo-1* mutants, whereas biosynthesis of most modular glucosides derived from attachment of additional moieties to these scaffolds was abolished (Figure 5.2b). Taken together, these results indicate that the role of LROs as a central

hub for the assembly of diverse small molecule architectures, including modular glucosides and ascarosides, may be widely conserved among nematodes (Figure 5.2c).

5.2 Figures

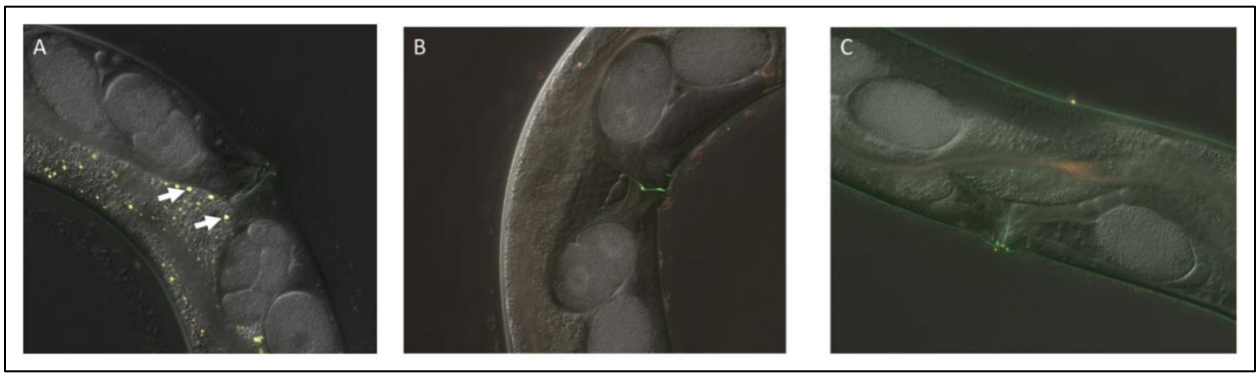


Figure 5.1 Adapted from Figure 5, Supplemental Figure 1 in Le *et al*. Gut Granules in *C. briggsae*. (**a**) *C. briggsae* WT AF16 has gut granules similar to *C. elegans* which are also both birefringent and easily tagged by Lysotracker Red (see arrows). Gut granule loss is evident in both (**b**) *Cbr-glo-1*(*sy1382*) and (**c**) *Cbr-glo-1*(*sy1383*).

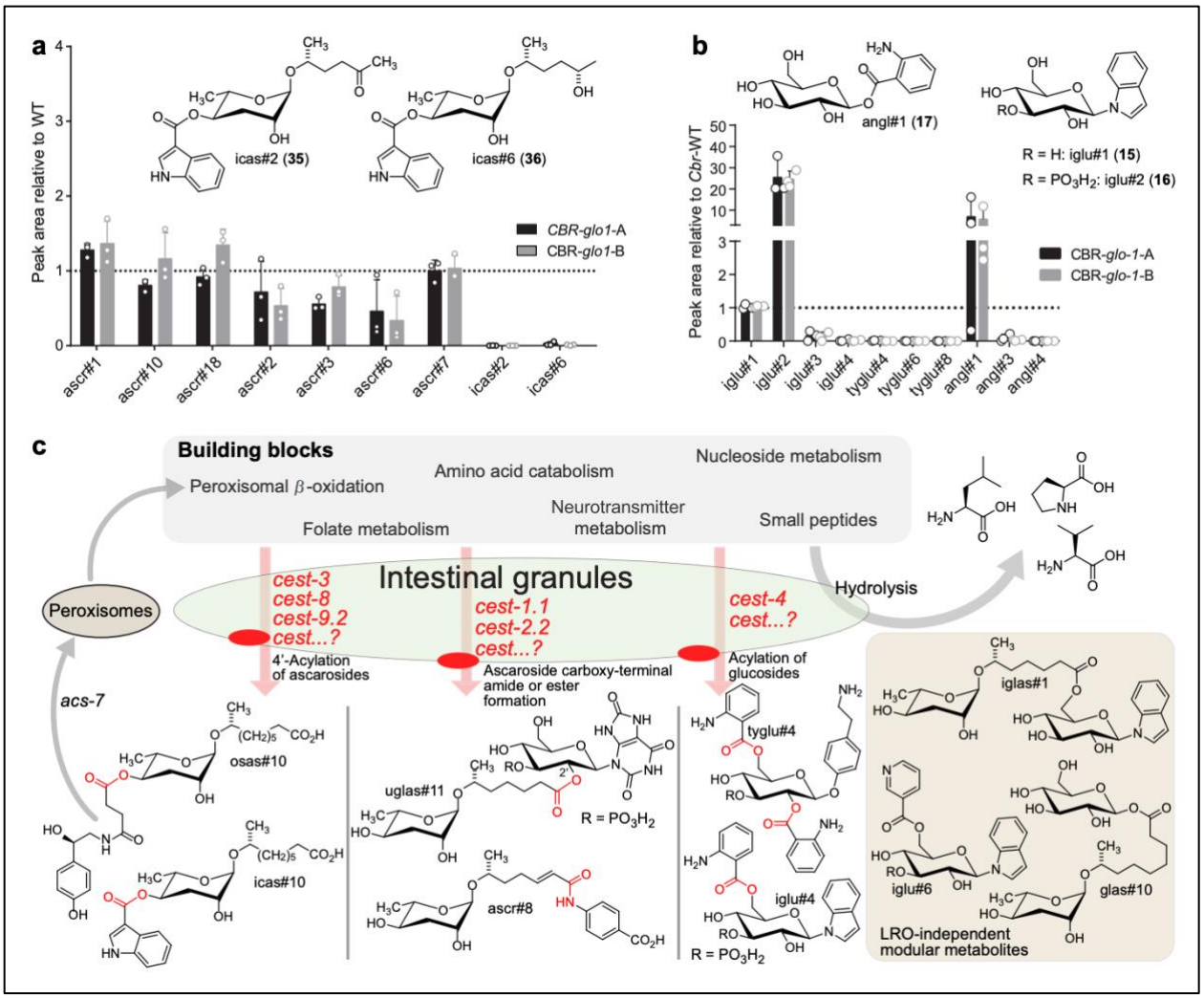


Figure 5.2 Adapted from Figure 5 in Le *et al*. Metabolome of *Cbr-glo-1*. Relative abundance of (a) simple and modular ascarosides and (b) simple and modular glucosides in the endo-metabolome of *Cbr-glo-1* mutants relative to wild-type *C. briggsae*. n.d., not detected. (**c**) Model for modular metabolite assembly. CEST proteins (membrane-bound in the LROs, red) mediate attachment of building blocks from diverse metabolic pathways to glucose scaffolds and peroxisomal *β*-oxidation-derived ascarosides via ester and amide bonds. Some of the resulting modular ascarosides may undergo additional peroxisomal *β*-oxidation following activation by *acs-7* (Dolke *et al*. 2019).

5.3 Materials and Methods

***C. briggsae* CRISPR Mutagenesis for Generation of *glo-1* Null Mutants:** The *C. briggsae glo-1* mutants *sy1382* and *sy1383* were both created using the *briggsae* adaptation of the STOP-IN cassette method as described in Cohen and Sternberg, 2019 and Wang *et al*., 2018. Both strains were made using a successful insertion of the STOP-IN cassette into the middle of the first exon using the guide AACAAATCTCCGGATGATTG. To detect the insertion, we used forward primer GGGTGACCGCCCATTTATTG and reverse primer AAAGGCGCACATCTTGCT.

***C. briggsae* Imaging:** 0.5 mL of 2 mM Lysotracker Deep Red (Thermo Fisher 1 mM stock in DMSO) was added to a 6 cm NGM plate seeded with 0.1 mL of E. coli OP50 and incubated in the dark for 24 hr at 20˚C. L4 larvae of *C. briggsae* were added to the plate and allowed to grow in the dark for 24 hr at 20˚C. To image, *C. briggsae* were transferred to an agarose pad on a glass slide with 10 mM of levamisole to immobilize the worms. Microscopic analysis was performed using a Zeiss Axio Imager Z2 florescence microscope with Apotome.

Nematode Cultures, Mixed Stage: Culturing began by chunking *C. briggsae* onto 10 cm NGM plates (each seeded with 800 mL of OP50 *E. coli* grown to stationary phase in Lennox Broth) and incubated at 22˚C. Once the food was consumed, the cultures were incubated for an additional 24 hr. Each plate was then washed with 25 mL of S-complete medium into a 125 mL Erlenmeyer flask, and 1 mL of OP50 *E. coli* was added (*E. coli* cultures were grown to stationary phase in Terrific Broth, pelleted and resuspended at 1 g wet mass per 1 mL M9 buffer), shaking at 220 RPM and 22˚C. After 70 hr, cultures were centrifuged at 5000 G for 1 min. After

discarding supernatant, 24 mL H2O was added, along with 6 mL bleach, 900 mL 10 M NaOH and the mixture was shaken for 3 min to prepare eggs. Eggs were centrifuged at 5000 G, the supernatant was removed, and the egg pellet washed with 35 mL M9 buffer twice and then suspended in a final volume of 5 mL M9 buffer in a 50 mL centrifuge tube. Eggs were counted and placed on a rocker and allowed to hatch as L1 larvae for 24 hr at 22˚C. 70,000 L1 larvae were seeded in 25 mL cultures of S-complete with 1 mL of OP50 and incubated at 220 RPM and 22˚C in a 125 mL Erlenmeyer flask. After 72 hr, cultures were fed an additional 1 mL of OP50 and incubation continued. After an additional 48 hr, worms were spun at 1000 G 5 min and spent medium was separated from worm body pellet. Separated medium and worm pellet were flash frozen over liquid nitrogen until further processing. At least three biological replicates were grown for all mutant strains. Mutants were grown with parallel wildtype controls, and biological replicates were started on different days.

Metabolite Extraction: Lyophilized pellet and media samples were crushed and homogenized by shaking with 2.5 mm steel balls at 1300 rpm for 3 min in 30 s pulses while chilled with liquid nitrogen (SPEX sample prep miniG 1600). Thus, powdered media and pellet samples were extracted with 15 mL methanol in 50 mL centrifuge tubes, rocking overnight at 22˚C. Extractions were pelleted at 5000 g for 10 min at 4˚C, and supernatants were transferred to 20 mL glass scintillation vials. Samples were then dried in a SpeedVac (Thermo Fisher Scientific) vacuum concentrator. Dried materials were resuspended in 1 mL methanol and vortexed for 1 min. Samples were pelleted at 5000 g for 5 min and 22˚C, and supernatants were transferred to 2 mL HPLC vials and dried in a SpeedVac vacuum concentrator. Samples were then resuspended in 200 mL of methanol, transferred into 1.7 mL Eppendorf tubes, and centrifuged at 18,000 G for

20 min at 4˚C. Clarified extracts were transferred to fresh HPLC vials and stored at 20˚C until analysis.

Mass Spectrometric Analysis: High resolution LC-MS analysis was performed on a Thermo Fisher Scientific Vanquish Horizon UHPLC System coupled with a Thermo Q Exactive HF hybrid quadrupole-orbitrap high-resolution mass spectrometer equipped with a HESI ion source. 1 mL of extract was injected and separated using at water-acetonitrile gradient on a Thermo Scientific Hypersil GOLD C18 column (150 mm x 2.1 mm 1.9 um particle size 175 A˚ pore size, Thermo Scientific) and maintained at 40˚C. Solvents were all purchased from Fisher Scientific as HPLC grade. Solvent A: 0.1% formic acid in water; solvent B: 0.1% formic acid in acetonitrile. A/B gradient started at 1% B for 5 min, then from 1% to 100% B over 20 min, 100% for 5 min, then down to 1% B for 3 min. Mass spectrometer parameters: 3.5 kV spray voltage, 380˚C capillary temperature, 300˚C probe heater temperature, 60 sheath flow rate, 20 auxiliary flow rate, one spare gas; S-lens RF level 50.0, resolution 240,000, m/z range 100– 1200 m/z, AGC target 3e6. Instrument was calibrated with positive and negative ion calibration solutions (Thermo-Fisher) Pierce LTQ Velos ESI pos/neg calibration solutions.

5.4 References

Cohen SM, Sternberg PW. 2019. Genome editing of *Caenorhabditis briggsae* using CRISPR/Cas9 co-conversion marker *dpy-10*. microPublication Biology. doi:10.17912/micropub.biology.000171.

Dolke F, Dong C, Bandi S, Paetz C, Glauser G, von Reuss SH. 2019. Ascaroside Signaling in the Bacterivorous Nematode *Caenorhabditis remanei* Encodes the Growth Phase of Its Bacterial Food Source. Org Lett. 21(15):5832–5837. doi:10.1021/acs.orglett.9b01914.

Dong C, Dolke F, von Reuss SH. 2016. Selective MS screening reveals a sex pheromone in *Caenorhabditis briggsae* and species-specificity in indole ascaroside signaling. Org & Biomol Chem. 14(30):7217-7225. doi:10.1039/C6OB01230B.

Dong C, Dolke F, Bandi S, Paetz C, von Reuss SH. 2020. Dimerization of conserved ascaroside building blocks generates species-specific male attractants in *Caenorhabditis* nematodes. Org Biomol Chem. 18(27):5253–5263. Doi: 10.1039/D0OB00799D.

Kanzaki N, Tsai IJ, Tanaka R, Hunt VL, Liu D, Tsuyama K, Maeda Y, Namai S, Kumagai R, Tracey A, *et al*. 2018. Biology and genome of a newly discovered sibling species of *Caenorhabditis elegans*. Nature Communications. 9(1):3216. doi:10.1038/s41467-018-05712-5.

von Reuss SH. 2018. Exploring modular glycolipids involved in nematode chemical communication. CHIMIA International Journal for Chemistry. 72(5):297-303. doi:10.2533/chimia.2018.297.

Wang H, Park H, Liu J, Sternberg PW. 2018. An efficient genome editing strategy to generate putative null mutants in *Caenorhabditis elegans* using CRISPR/Cas9. G3. 8(11):3607-3616. doi:10.1534/g3.118.200662.