Caenorhabditis elegans as a Model for Host-Microbe-Drug Interactions

Aurian P. Garcia Gonzalez

University of Massachusetts Medical School

Let us know how access to this document benefits you.

Follow this and additional works at: https://escholarship.umassmed.edu/gsbs_diss

Part of the Organismal Biological Physiology Commons, and the Systems and Integrative Physiology Commons

Repository Citation

This material is brought to you by eScholarship@UMassChan. It has been accepted for inclusion in GSBS Dissertations and Theses by an authorized administrator of eScholarship@UMassChan. For more information, please contact Lisa.Palmer@umassmed.edu.
CAENORHABDITIS ELEGANS AS A MODEL FOR
HOST-MICROBE-DRUG INTERACTIONS

A Dissertation Presented

By

Aurian Priscilla García González

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

April 30, 2019

MD/PhD Program
CAENORHABDITIS ELEGANS AS A MODEL FOR

HOST-MICROBE-DRUG INTERACTIONS

A Dissertation Presented
By
Aurian Priscilla García González
This work was undertaken in the Graduate School of Biomedical Sciences
MD/PhD Program
Under the mentorship of

____________________________________________________________________
A.J. Marian Walhout, PhD; Thesis Advisor

____________________________________________________________________
Dohoon Kim, PhD; Member of Committee

____________________________________________________________________
Michael Lee, PhD; Member of Committee

____________________________________________________________________
Amy Walker, PhD; Member of Committee

____________________________________________________________________
Erik C. Andersen, PhD; External Member of Committee

____________________________________________________________________
Christopher Sassetti, PhD, Chair of Committee

Mary Ellen Lane, Ph.D.,
Dean of the Graduate School of Biomedical Sciences
April 30, 2019
To my mother, who encouraged me to be confident and to always care for others, for staying on the phone with me through all my late nights in the lab – making sure I’d always finish work and arrive home safe, of course...

and to my father, who with a few simple tools (including a paperclip on occasions), and an old car that wouldn’t start, taught me how to diagnose and solve problems systematically, for teaching me to appreciate the reward of a job well done...

¡Gracias!
ACKNOWLEDGEMENTS

Marian, when we met in 2012, I knew there could be no better mentor and role model for me than you. Sure, I went through some phases, and even tried to go to Job’s lab (gasp!), but I was instantly fascinated by your enthusiasm for gene regulatory networks. Over the years, you’ve defended me fiercely, believed in me and supported me through many challenging times. I hope you find your efforts have paid off. Because of you I know better how to ask a good scientific question and how to design experiments that will answer those questions. You have made me into a scientist.

Shaleen, you have been my rock and you’ve brought peace to my life. Back in the starting days of “Team Drugs”, you were so patient with my temper tantrums and my obsessive attention to detail. We worked so well as a team and balanced each other out. Now I get to share my life with you, and I cannot thank you enough for all your unconditional love and support.

Juan, during my early days in the lab, when I could do little more than press buttons and swap plates in the robot, you invested countless hours in helping me be better. You taught me how to write and communicate science, and you guided me through choosing a project and looking at the bigger picture.

Ash, you were so instrumental to my development in the lab. Without your extraordinary creativity, this project would not exist. You were patient when my wet-lab skills suggested I couldn’t possibly execute this project – I mean, who gets
only one live worm after bleaching? Your kindness and mentorship got me through countless instances of self-doubt. You also showed me that, despite being in a professional setting, we are in the end all people and should care for each other as such. I have tried to keep your spirit of remembering special occasions and taking care of each other in the lab, although I must admit that part of me is grateful that most lab members can’t see how little it compares to what you used to do.

Walhout lab members: you have made every day fun. Thank you for helping me through it all. You all made me feel special. Nana, you have been such an exceptional bay mate. We’ve shared so many stories and ideas for experiments. Céd, the newest addition to “Team Drugs” and our “on-call” Wizard for all things science: in the year that we have worked together, you have taught me so much about teamwork and creativity. Your genuine interest in my wellbeing and success is heartwarming and inspiring. Olga (& Elga), thank you for helping me get through the endless struggles of failed GC/MS experiments. You retained your interest in my project and pushed through every troubleshooting attempt. Your work ethic and scientific rigor motivate me to be a better scientist. And to all the other lab members: Jote, Gabbs, Melissa, Sushila, HeiFei, Hang, Yong-Uk, Yan, Shivani, Zeynep, Safak and Amy, thank you for being my family for the past five+ years. Rev. Brentowitz (aka baby Brent), thank you for being the baby brother I never wanted. You inspire me to be ridiculous and annoy people. And to former lab members: Alex, Emma, Leslie and John, thank you for your mentorship and advice when I got started in the lab.
To all my “Team Drugs Jr.” girls: Mackenzie, Riya, Izzy and Yomi, it was an honor to teach you and watch you grow into budding scientists. Thank you for volunteering your time to help me do more experiments, and for not complaining about the boring ones. Yomi, thank you for being patient when I expected too much from you.

Andre and Carissa, you saved my tammy project! I was so fortunate to have your help and support in completing the last portion of my thesis project. Your skills, diligence and interest in my project were the magical touch I needed to reach the finish line.

To all the administrative and technical support staff, past and present, thank you for helping me with paperwork, instrumentation, reagents, etc., and running the show “behind the scenes” so I could work smoothly every day.

Members of the Program in Systems Biology, thank you for the impromptu mentorship and experimental advice. Greeting you all around the halls brought me joy and I will miss you all. Special thanks to the Mitchell lab, and to Amir, for being such great “lab neighbors” and for sharing the excitement of microbe-drug interactions. Exchanging ideas and collaborating with your team has been amazing and I am eager to see where your projects go next.

To my medicine mentors, in particular Dr. Beth Murphy, thank you for caring about me and supporting my learning and development throughout the years. You have been an extraordinary teacher and physician role model, and I genuinely appreciate your patience all those times when it was clear that my medical
knowledge went down the drain: I still don’t know which heart valve corresponds to which murmur. And to all the patients I took care of during my graduate school years, thank you for allowing me to learn from you and for sharing your stories. You made a big impact on my life. Caring for you was a constant source of motivation to put my skills to good use, in the lab and beyond.

To all my amazing friends, and in particular to my fellow science gals, Raz and Miri, for sharing this journey with me. We’ve supported each other through the struggles of failed experiments, or through countless life dilemmas, and I can’t wait to see what we do next.

To my family, we’re not done yet so don’t get too excited. I’m not graduating until 2021. Don’t worry, I’ll remind you when you ask me again in Christmas, just like I have for the past seven years... It never gets old. Thank you for believing in me, despite not quite understanding why I care so much about worms.

To C. elegans, my spirit animal, for peacefully agreeing to be manipulated, bleached and tortured with drugs in the pursuit of my project.

To all of you, and to the many I unintentionally forgot, thank you from the bottom of my heart.
The microbes that inhabit the human body, our microbiota, greatly influence our physiology and propensity for disease. For instance, the gut microbiota metabolizes compounds from our diet to provide important nutrients. Similarly, the microbiota has the potential to impact drug response; directly by metabolizing drugs, or indirectly by providing metabolites to the host. The complexity of the mammalian microbiota, and the limited throughput of such models, prohibit a systematic interrogation of specific interactions between microbes and host drug response. Here, I use C. elegans and its bacterial diet as a suitable model with the scalability and genetic tractability to address these questions. In Chapter II, I describe host-bacteria-drug interactions involving the anti-pyrimidine drugs 5-FU and FUDR. In brief, we identified two main mechanisms by which bacteria affect the C. elegans response to anti-pyrimidines: (1) metabolic conversion into FUMP by uridine phospho-ribosyltransferase (upp) and (2) dietary supplementation of uracil. Chapter III will focus on a selective estrogen-receptor modulator, TAM, with no clear target in bacteria or C. elegans. I will describe my work characterizing a bacteria-dependent response to TAM involving fatty acid metabolism. Lastly, the Appendix will summarize my efforts to expand the sample space of tested host-microbe-drug interactions.
# TABLE OF CONTENTS

**REVIEWER PAGE** .................................................................................................................. ii

**DEDICATION** ......................................................................................................................... iii

**ACKNOWLEDGEMENTS** ......................................................................................................... iv

**ABSTRACT** ............................................................................................................................ viii

**TABLE OF CONTENTS** ........................................................................................................... ix

**LIST OF FIGURES** .................................................................................................................. xii

**LIST OF ABBREVIATIONS** .................................................................................................... xiv

**COPYRIGHT MATERIAL** ........................................................................................................ xv

## CHAPTER I: INTRODUCTION

Preface ........................................................................................................................................ 1

Microbiota effects on human health and disease ................................................................. 1

Host-microbe metabolism: diet and xenobiotics ................................................................. 3

*C. elegans* and its bacterial diet: an interspecies model .................................................... 6

Studying host-microbe-drug interactions in *C. elegans* ...................................................... 7

## CHAPTER II: BACTERIAL METABOLISM AFFECTS THE *C. ELEGANS* RESPONSE TO CANCER CHEMOTHERAPEUTICS

Preface ........................................................................................................................................ 12

Summary .................................................................................................................................... 13

Introduction ............................................................................................................................... 13

Materials and Methods .......................................................................................................... 17

*C. elegans* strains .................................................................................................................. 17

Bacterial strains ....................................................................................................................... 17

*C. elegans* phenotypic assays ............................................................................................... 18

Chemotherapeutic drugs ......................................................................................................... 18
### CHAPTER III: MICROBIAL FATTY ACIDS INFLUENCE TAM TOXICITY

#### Preface
- 52

#### Summary
- 52

#### Introduction
- 53

#### Materials and Methods
- 55
  - *C. elegans* strains
  - 55
  - Bacterial strains
  - 55
  - Chemotherapeutic drugs
  - 55
  - *C. elegans* dose-response curves
  - 56
  - Bacterial mutant genetic screens
  - 57
  - Bacterial growth curves
  - 57
  - *C. elegans* metabolic gene RNAi
  - 58
  - Fatty acids supplementation
  - 59
  - *C. elegans* fatty acids measurements
  - 59
  - Temperature shift assay
  - 59
Results ............................................................................................................... 60
  Bacteria modulate TAM toxicity ................................................................. 60
  Fatty acid metabolism protects against TAM toxicity............................... 63
  Bacterial fatty acids influence TAM toxicity ............................................. 67
Conclusions ..................................................................................................... 74

CHAPTER IV: DISCUSSION
  Preface ......................................................................................................... 78
  Microbial xenobiotic metabolism: challenges and opportunities ............ 78
  Improving and expanding the C. elegans-bacteria-drug model ................ 80
  Challenges and limitations of C. elegans as a model host ....................... 86

APPENDIX: EXPANDING THE HOST-MICROBE-DRUG MODEL .................. 88

BIBLIOGRAPHY .............................................................................................. 95
LIST OF FIGURES

CHAPTER I
1.1: C. elegans-bacteria-drug model toolkit ......................................................... 8
1.2: Mechanisms of host-microbiota-drug interactions ........................................ 10

CHAPTER II
2.1: Reported mechanism of action for CPT, 5-FU and FUDR............................. 15
2.2: Screen of chemotherapeutics in C. elegans fed two bacteria........................ 26
2.3: Bacterial metabolism is required to modulate FUDR efficacy....................... 28
2.4: Genetic screens for bacterial genes involved in host drug efficacy.............. 32
2.5: C. elegans response to CPT, 5-FU and FUDR when fed mutants............... 34
2.6: Four classes of bacterial mutants influence drug efficacy............................ 37
2.7: Bacterial nucleotide metabolism affects host drug efficacy......................... 40
2.8: Nucleobase supplementation in different bacterial mutants...................... 42
2.9: Flux balance analysis (FBA) of E. coli mutants.............................................. 44
2.10: Phenotypes of a Comamonas mutant that resembles E. coli...................... 46
2.11: Model for bacterial modulation of anti-pyrimidine response..................... 49

CHAPTER III
3.1: Comamonas increases TAM toxicity in C. elegans...................................... 60
3.2: Comamonas, but not E. coli, mutants change TAM toxicity......................... 63
3.3: C. elegans metabolic genes modulate the response to TAM........................ 64
3.4: Fatty acid synthesis and elongation decrease TAM toxicity......................... 66
3.5: TAM levels do not change upon pck-2 or dhs-19 knockdown..................... 67
3.6: Fatty acid supplementation reverts TAM toxicity....................................... 68
3.7: Experimental design for metabolomics after TAM treatment....................... 69
3.8: Fatty acid measurements after TAM treatment and elo-6 RNAi............... 69
3.9: Comamonas changes fatty acid composition in C. elegans......................... 71
3:10: Membrane composition and temperature change TAM toxicity........... 73
3.11: Model of bacteria-dependent TAM toxicity................................. 74

CHAPTER IV

4.1: Mechanisms of host-microbe-drug interactions characterized............ 82
4.2: Pyrimidine synthesis in bacteria, C. elegans and human....................... 85

APPENDIX

5.1: Pilot screen of published drugs against twenty-two bacteria............... 92
5.2: High-throughput host-microbe-drug interactions screen....................... 93
LIST OF ABBREVIATIONS
(sorted alphabetically)

5-FU: 5-fluorouracil
CFA: cyclopropane fatty acid synthase
CLM: clomiphene
CPA: cyclopropane fatty acids
CPT: camptothecin
DMSO: dimethyl sulfoxide
dTMP: deoxythymidine monophosphate
ER: estrogen receptor
FA: fatty acid
FBA: flux balance analysis
FdUMP: 5-fluoro-2'-deoxyuridine 5'-monophosphate
FUDR: 5-fluoro-2'-deoxyuridine; floxuridine
FUMP: 5-fluorouridine monophosphate
GMP: guanine monophosphate
GWAS: genome-wide association study
MIC: minimum inhibitory concentration
mmBCFA: monomethyl branched-chain fatty acid
MUFA: monounsaturated fatty acid
NGM: nematode growth media
PAC: paclitaxel
PUFA: polyunsaturated fatty acid
SERM: selective estrogen receptor modulator
TAM: tamoxifen
TMAO: trimethylamine N-oxide
TOR: toremiphene
TYMS: thymidylate synthase
RTK: receptor tyrosine kinase
UMP: uridine monophosphate
COPYRIGHT MATERIAL

Some content or figures have been previously published as described below. According to the publisher’s copyright policies, as an author of these articles, I retain the right to publish these contents in a thesis dissertation. Specific attributions are listed in the Preface section accompanying each chapter.


Other published work during graduate study, not discussed in this thesis:

CHAPTER I: Introduction

PREFACE

Some content in the section titled “C. elegans as a model system for host-microbe interactions”, as well as Fig. 1.2, have been adapted from: García-González AP, Walhout AJM (2017) Worms, bugs and drugs: Caenorhabditis elegans as a model for host-microbe-drug interactions. Curr. Opin. Sys. Biol. 6, 46-50.

INTRODUCTION

Microbiota effects on human health and disease

There are at least a few hundred different species of microbes inhabiting our body’s epithelial surfaces [1]. They are collectively known as our microbiota. The Human Microbiome Project extensively characterized the complex microbial communities in our skin and gut, as well as other niches [1]. Most follow up studies on the microbiota have focused on characterizing the identity of microbes inhabiting different niches and how their composition differs between cohorts with different disease states or interventions [2-7]. Functional associations between microbial communities and human physiology are scarce.

The challenges in studying the microbiota, and its effect on host physiology, greatly mirror the experience following the sequencing of the human genome.
Sequencing more human genomes has improved our understanding of population genetic diversity, as well as identified rare and common variants. However, genome-wide association studies (GWAS) identify candidate disease-causing genes or variants but, without functional validation, GWAS often fails to provide mechanistic information connecting genes to phenotypes [8].

Similarly, while we have abundant evidence for microbiota changes under different conditions, much less is known about how specific microbes contribute to host physiology. Deriving functional associations between microbes and host phenotypes is hindered by the complexity of our microbiota. Despite these challenges, we have some clues about the role of the microbiota in host health based on studies in germ-free mice [9]. Compared to animals with a microbiota, germ-free mice have several phenotypic defects, notably in immune regulation [10,11]. Interestingly, microbial gut colonization alone does not revert immune dysregulation. A human-derived microbiota, when introduced into germ-free mice, does not revert immune dysregulation and, in fact, mimics germ-free conditions [12]. Thus, colonization of a host-evolved microbial community contributes to organismal health and physiology.

In humans, innate or acquired disruption of the microbiota, dysbiosis, is also associated with disease [13,14]. Microbiota dysregulation is broadly linked to inflammation and immunodeficiency, notably inflammatory bowel diseases [14,15]. Furthermore, many disease features can be ameliorated by manipulating the microbiota [16]. Similarly, broad-spectrum antibiotic treatment causes disruption of
the gut microbiota and, when combined with other risk factors, leads to *Clostridium difficile* infection [17]. One study in mice linked the presence of specific microbes, and their metabolism of bile acids into antimicrobial compounds, with resistance to *C. difficile* [18]. Fecal microbiota transplants, where the gut microbiota of a healthy donor is transplanted to the patient, have a high success rate in the treatment of *C. difficile* infection [19]. Evidence of endogenous microbes with anti-*C. difficile* activity as well as the curative rate of fecal microbiota transplants support dysbiosis as a cause for post-antibiotic colitis. The examples listed above highlight the importance of mapping functional associations between individual microbes and host phenotypes, especially because the use of pre- and probiotics, as well as microbiota transplants, facilitate direct modulation of the gut microbiota to maximize host health.

**Host-microbe metabolism: diet and xenobiotics**

In addition to their role in disease, the gut microbiota is also a significant contributor to our metabolic capacity [20-21]. Compounds taken orally, like nutrients or toxins from our food, or drugs, are often metabolized by the gut microbiota before entering systemic circulation [22-25]. Microbial metabolism is responsible for the digestion of many dietary compounds with various roles in host physiology. Dietary polyphenols, found in tea, wine, grapes and cranberries are anti-inflammatory compounds that prevent metabolic disease [26]. Subsequent studies linked the protective effects of polyphenols to the gut bacterium *Akkermansia muciniphila*. Polyphenol exposure increases the abundance of *A.*
muciniphila within the microbiota, and mice with heat-killed A. muciniphila lose the protective effect of polyphenols [27,28]. Conversely, one study suggests that the gut microbiota contributes to an observed link between the phosphatidylcholine-derived metabolite trimethylamine N-oxide (TMAO) and increased risk of atherosclerosis [29]. This study identified TMAO as a candidate metabolite increased in a patient cohort with cardiovascular disease. Mechanistic study in mice revealed that TMAO synthesis requires the intestinal gut flora: antibiotic-treated mice showed decreased TMAO after phosphatidylcholine supplementation. Furthermore, mice with high risk factors for atherosclerosis but with a depleted or absent microbiota show lower disease features than animals with an unaltered microbiota [29]. In summary, the microbiota has been reported to cause beneficial or harmful effects on host health in response to dietary compounds.

Analogous to their role in the metabolism of dietary compounds, gut microbes also encounter medications and modify their effects on the host [23,24]. The microbiota influence host drug responses in several ways: directly, through the metabolism of drugs; or indirectly, by modulating host factors involved in drug response. Drug metabolism by the microbiota can involve broad enzymatic reactions; for instance, microbial β-glucuronidases reverse host glucuronidation of a broad range of xenobiotics [30,31]. Glucuronidation facilitates compound excretion and often takes place in the liver. Bacterial reversal of host glucuronidation decreases compound detoxification, effectively re-activating
drugs. Decreased xenobiotic secretion, due to microbial β-glucuronidation, is linked to high toxicity of the chemotherapeutic irinotecan [32]. Drugs can also be modified by specific bacterial metabolic pathways. For example, two genes in the bacterium *Eggerthella lenta* efficiently inactivate the antiarrhythmic drug digoxin, limiting the drug’s therapeutic use [33]. Molecular characterizations of the two genes in *E. lenta* identified a function that strongly resembles cytochrome reductase activity [34]. Host-targeted drugs can also interfere with microbial metabolic pathways by mimicking endogenous metabolites, leading to changes in host drug responses. The anti-nucleotide gemcitabine (a fluorinated cytidine) is inactivated by the bacterial cytidine deaminase, leading to drug resistance in pancreatic cancers [35].

Bacteria also influence host drug response indirectly, for instance by modulating host physiology in the presence or absence of drug. The abundance of bacteria-derived bile acids, while unchanged after drug treatment, correlates with patient response to simvastatin, a cholesterol-lowering drug [36]. Bile acids produced by gut bacteria can help solubilize cholesterol, and related metabolites, which may relate to the observed improvement in cholesterol-lowering therapy. Antibiotic-treated mice also had lower statin efficacy relative to mice with a rich microbiota [37], supporting a role of gut microbes in mediating drug efficacy. In contrast, the chemotherapeutic cyclophosphamide elicits a strong anti-tumor immune response through the microbiota. In mice, cyclophosphamide leads to the translocation of gut bacteria to lymph nodes and the spleen, which activates T-
cells and results in anti-tumor immunotherapy [38]. Antibiotic-treated and germ-free mice also showed a diminished response to two other chemotherapeutics: oxaliplatin and CpG-oligodeoxynucleotides [39]. Taken together, bacteria in the gut microbiota can improve or worsen therapeutic outcomes through direct metabolism of drugs or through indirect effects on the host.

The ultimate goal of pharmacotherapy is to minimize drug toxicity and maximize therapeutic outcomes. Since bacteria in the microbiota can impact both drug toxicity and drug response in the host, it is worth exploring the extent of host-microbe-drug interactions systematically. Identifying the molecular mechanisms responsible for microbe-drug interactions can provide new microbial therapeutic targets designed to be co-administered to maximize a desired drug response [40]. Understanding how the microbiota modulate drugs can also improve drug design; compounds can be selected to capitalize on or avoid microbial metabolism, depending on the desired outcome.

**C. elegans and its bacterial diet: an interspecies model**

Mammalian models have a complex microbiota and are not amenable for high-throughput, systematic studies. One reasonable approach to gain broad biological insight into host-microbe metabolism could come from studies in model organisms cultured with a defined, or monoxenic, microbiota.

The nematode Caenorhabditis elegans was introduced as a model system in the early 1970’s [41,42] and has recently emerged as a model to study host-microbe interactions [43-45]. C. elegans is a genetically tractable host with a short
life cycle of two to three days, and is amenable to high-throughput screens. Bacteria act as a food source and microbiota of *C. elegans*. In the wild, *C. elegans* inhabits rotting vegetation and feeds on a rich bacterial community, many bacteria also inhabiting the animal's intestine [46,47]. The *C. elegans* gut microbiota composition differs from the environment's, suggesting complex mechanisms driving microbiota formation [48].

In the laboratory, *C. elegans* is most often maintained on a monoxenic diet of *Escherichia coli* OP50 [49]. Several studies have linked bacteria-derived metabolites to host phenotypes in *C. elegans*. For example, our group previously discovered that feeding *C. elegans* with *Comamonas aquatica* DA1877 (*Comamonas*) led to accelerated development, decreased fecundity and shorter lifespan, relative to *E. coli* OP50 [50]. Extensive bacterial genetics led to the discovery that most dietary phenotypes are rescued by supplementing *E. coli* OP50 with vitamin B12 [51]. Other studies have also used bacterial genetics to explore how bacteria contribute to *C. elegans* lifespan [52]. Bacteria can also protect *C. elegans* from pathogens by promoting immune responses, preventing harmful colonization or supplementing protective metabolites [53-55].

**Studying host-microbe-drug interactions in *C. elegans***

This project aims to develop the use of *C. elegans* and its bacterial diet as a model for identifying and characterizing host-microbe drug interactions. *C. elegans* is currently used in high-throughput small-molecule screens because genetic tools facilitate the discovery of drug targets [56-58]. Thus, in order to test
C. elegans as a model for host-microbe-drug interactions, I combined existing tools and strategies used in C. elegans and the bacteria it consumes (Fig. 1.1). Previous work exemplifies using the C. elegans-bacteria interspecies model to study host-microbe-drug interactions. The antidiabetic drug metformin increases lifespan in C. elegans by modulating folate metabolism in E. coli. Using genetics in both host and bacteria, as well as combinatorial drug treatment and targeted metabolomics, this study unraveled a mechanism of action of metformin through the microbiota [59].

**FIGURE 1.1: C. elegans-bacteria-drug model toolkit.** Summary of permutations to each parameter within the host-microbe-drug model system, according to the tools already in use with C. elegans.

Many studies on host-microbe-drug interactions take a “forward” approach: they identify a potential microbe-driven drug phenotype and later characterize the mechanisms involved. Because of the complexity of microbial communities in
mammalian systems, this is often laborious and time-consuming. In contrast, this project takes a “reverse” approach, beginning with individual microbes and asking whether and how they contribute to host drug response.

At the start of the project, I envisioned a subset of mechanisms of host-microbe-drug interactions that I can capture using C. elegans (Fig. 1.2). Bacteria can metabolize drugs into active or inactive forms or produce metabolites in response to drug. Lastly, bacteria can supplement drug-independent metabolites that can influence host drug response. Two classes of known host-microbiota-drug interactions are likely excluded from our model: (1) microbial metabolism of drugs following host metabolism, and (2) microbiota immunoregulation of the host. In C. elegans, bacteria act as microbiota but are also consumed as food, limiting the possibility that compounds secreted into the intestinal lumen will be further metabolized by the microbiota and re-enter C. elegans tissues. However, it is important to consider that bacteria may impact drug delivery. One study on drug delivery methods in C. elegans measured the amount of two compounds inside animals exposed to drug in a variety of methods where solid or liquid medium and live or dead bacteria were combined and tested. Liquid growth and dead bacteria, among all other combinations, showed the largest amount of drug inside C. elegans [60]. If findings on the two compounds are generalizable, bacteria have a global inhibitory effect on drug delivery in C. elegans. While bacteria likely have differential uptake of some compounds over others, whether this will have drug-specific consequences for drug bioavailability in C. elegans remains unclear.
Second, immunotherapies are not suitable to test in this model since *C. elegans* does not possess an adaptive immune system. Therefore, the role of the gut microbiota in host immunomodulation, and how it drives successful immunotherapies, could be underestimated in the *C. elegans*-bacteria model. Despite these limitations, it is reasonable to expect many drugs to be testable based on shared homology of known targets in humans. I will refer to *C. elegans* drug response in terms of efficacy: how much compound is necessary to elicit an abnormal phenotype. Thus, I can characterize bacterial influence on host drug response as “increasing” or “decreasing” drug efficacy, when animals require less or more drug, respectively, to elicit an abnormal phenotype.

**FIGURE 1.2: Mechanisms of host-microbiota-drug interactions.** Cartoon of some mechanisms by which bacteria can modulate host drug response. The two first mechanisms (left to right) require bacteria come into contact with the drug, the rightmost mechanism is independent of drug.

Another important consideration in the design of a model system for host-microbe-drug interactions in *C. elegans* is to discern the role of bacteria as diet or microbiota. The molecular mechanisms that we elucidated can suggest dietary or
metabolic/microbiota contributions by the bacteria. To complement these findings, I can use killed bacteria, which should retain dietary properties but loose microbiota function.

The following chapters will describe my work combining bacterial genetics with a genetically tractable host to elucidate the mechanisms behind a few host-microbe-drug interactions. To achieve this goal, I used two bacterial diets for which we already have genetic tools, *E. coli* and *Comamonas*, along with a handful of cancer chemotherapeutics. Cancer chemotherapeutics are a suitable starting point because many are cytotoxic agents, with conserved mechanisms of actions in bacteria. Chemotherapeutics often causes gastrointestinal side effects, as a consequence of dysbiosis and ensuing inflammation [61]. Since chemotherapeutics also affect bacteria, their efficacy on the host is likely affected by the microbiota [62].
CHAPTER II: Bacterial metabolism affects the 
C. elegans response to cancer chemotherapeutics

PREFACE

The contents of this chapter are published as:


Ashlyn D. Ritter produced the data in Fig. 2.2a. We also received an unpublished bacterial lysate protocol from Erik C. Andersen, which I used to generate the data for Fig. 2.3b. L. Safak Yilmaz performed flux balance analysis in Fig. 2.9. I performed all other experiments with technical help from Shaleen Shrestha.

For the research in this chapter, I was supported by the NIH Ruth L. Kirschstein National Research Service Award (NRSA) Individual Predoctoral Fellowship under the National Institutes of Health (GM122393).
SUMMARY

The human microbiota greatly affects physiology and disease. However, the contribution of bacteria to the response to chemotherapeutic drugs remains poorly understood. Caenorhabditis elegans and its bacterial diet provide a powerful system to study host-bacteria interactions. Here, we used this system to study how bacteria affect the C. elegans response to chemotherapeutics. We find that different bacterial species can increase the response to one drug yet decrease the effect of another. We perform genetic screens in two bacterial species using three chemotherapeutic drugs, 5-fluorouracil (5-FU), 5-fluoro-2'-deoxyuridine (FUDR) and camptothecin (CPT). We find numerous bacterial nucleotide metabolism genes that affect drug efficacy in C. elegans. Surprisingly, we find that 5-FU and FUDR act through bacterial ribonucleotide metabolism to elicit their cytotoxic effects in C. elegans, rather than by thymineless death or DNA damage. Our study provides a blueprint for characterizing the role of bacteria in the host response to chemotherapeutics.

INTRODUCTION

The microorganisms that inhabit our intestine, known as our gut microbiota, greatly influence our development, health and propensity to get sick [63,64]. More recently, it has become clear that the gut microbiota can also affect the response to anticancer immunotherapy [65-67]. However, the involvement of the microbiota
in the response to chemotherapeutics and the mechanisms involved remain to be elucidated.

Chemotherapeutics have been used for decades to treat a variety of human tumors. Some cancers, such as colorectal cancer, occur in physical proximity to the microbiota. Therefore, we reasoned that the efficacy of anti-colorectal cancer drugs, such as the anti-pyrimidine drugs 5-fluorouracil (5-FU) and 5-fluoro-2’-deoxyuridine (FUDR), and the topoisomerase I (topo-I) inhibitor camptothecin (CPT) may be affected by the microbiota. These drugs have different reported mechanisms of action, but are all thought to confer cytotoxicity through DNA damage (Fig. 2.1). CPT directly inhibits the topo-I-DNA complex, which leads to single-stranded DNA breaks and cell death [68], while 5-FU and FUDR produce FdUMP, which directly inhibits thymidylate synthase thereby causing thymineless cell death. In addition, products of 5-FU and FUDR may be converted into FUTP or FdUTP, leading to RNA or DNA damage, respectively [79].
FIGURE 2.1: Reported mechanism of action for CPT, 5-FU and FUDR. Schematic of reported mechanisms of action for the topo-I inhibitor CPT (left), and the anti-pyrimidines 5-FU and FUDR (right). Solid dark arrows indicate main reported mechanisms of action and gray dashed arrows indicate side pathways. TYMS – thymidylate synthase.

It is challenging to systematically delineate which anticancer drug efficacies are affected by the microbiota using patient data or mammalian model systems, such as mice, and to dissect the mechanisms involved. This difficulty is not only because of limitations in scale and cost, but also because of heterogeneity in human genotype, diet, and microbiota.

The nematode C. elegans and its bacterial diet have recently become a powerful interspecies model system to study the effects of diet and the microbiota on animal life history traits [70-72]. C. elegans has a short life cycle, is amenable
to high-throughput screens, and is easily maintained in the laboratory. In the wild, *C. elegans* has been found associated with different bacteria, which function not only as diet but also as a microbiota [48, 73-75]. We, and others, have proposed that *C. elegans* and its bacterial diet provide an apt model system to study the effects of bacteria on the animal’s physiology and gene expression [50, 51, 59, 76-77]. For instance, we previously found that, when fed a diet of *Comamonas aquatica* DA1877 (*Comamonas*), *C. elegans* develop faster, have reduced fecundity and live shorter than animals fed the standard laboratory diet of *Escherichia coli* OP50 (*E. coli*) [50]. By high-throughput forward and reverse genetics, in both the animal and these bacteria, we found that the *Comamonas* effects on *C. elegans* development and fecundity can be explained, in a large part, by the fact that this bacterial species can synthesize vitamin B12, whereas *E. coli* cannot [51, 77, 78].

Here, we used the interspecies system of *C. elegans* and its bacterial diet to ask whether different bacteria have different effects on the host response to chemotherapeutics. We exposed *C. elegans* to 11 drugs while feeding them either *E. coli* or *Comamonas* and found that four chemotherapeutics cause abnormal phenotypes in *C. elegans* at the administered dose. We tested the effects of three drugs, 5-FU, FUDR, and CPT, at a range of concentrations and used progeny production — no progeny (sterile), dead progeny (dead embryos), or live progeny — as a proxy for drug efficacy. We found that *E. coli* and *Comamonas* oppositely affect the *C. elegans* response to FUDR and CPT. By performing genetic screens
in both bacterial species, we identified numerous bacterial genes that modulate 5-FU, FUDR, and/or CPT efficacy in *C. elegans*. Altogether, we found that bacterial nucleotide metabolism networks modulate drug efficacy in *C. elegans*. Mutations in *E. coli* or *Comamonas upp*, which generates 5-fluorouridine monophosphate (FUMP) from 5-FU, reduce the efficacy of 5-FU and FUDR. However, mutations in bacterial *tdk, tmk*, or *thyA*, which are involved in dTMP synthesis, do not. These observations suggest that these drugs elicit their effects on *C. elegans* fecundity through bacterial production of FUMP, and RNA metabolism, and not through the generation of FdUMP, DNA metabolism or thymineless death. Indeed, uracil rescues 5-FU and FUDR toxicity in *C. elegans* while thymine had no effect. Altogether, these results demonstrate that bacterial metabolism can greatly affect chemotherapeutic drug efficacy in the host.

MATERIALS AND METHODS

**C. elegans strains**

N2 (Bristol) was used as the wild-type strain, and animals were maintained with *E. coli* OP50 as diet on Nematode Growth Media (NGM), as previously described [41].

**Bacterial strains**

Bacterial strains *E. coli* OP50, *E. coli* BW25113, and *Comamonas aquatica* DA1877 were grown as described [51]. *E. coli* deletion mutants [79] were grown in Luria Broth (LB) with 50 µg/mL kanamycin. *Comamonas aq.* DA1877 mutants [51]
were grown in LB containing 10 µg/mL gentamicin. One mutant in our screens, \textit{E. coli} ∆thyA, is a thymine auxotroph, and this mutant was grown in the presence of 20 µg/mL thymine (Fig. 2.7).

\textbf{C. elegans phenotypic assays}

All animals were fed the standard laboratory diet \textit{E. coli} OP50 until transferred to plates containing the respective bacteria and drug combination. For all fecundity assays, embryos were harvested from gravid animals by bleaching in sodium hypochlorite solution, washing embryos repeatedly with M9 buffer and then incubating overnight in M9 to allow hatching. Then, synchronized L1 animals were grown on NGM agar plates with the \textit{E. coli} OP50 diet for ~48 hours until the animals reached the L4 stage. Animals were then transferred to NGM agar drug plates and incubated at 20°C. Approximately 72 hours later, fecundity phenotypes were visually identified by microscopy, and brightfield images were collected using an Invitrogen™ EVOS™ FL microscope set at 2X magnification.

\textbf{Chemotherapeutic drugs}

Camptothecin (CPT) was purchased from Sigma Aldrich (C9911) and dissolved in dimethyl sulfoxide (DMSO) to 10 mM for a stock solution. 5-Fluorouracil (5-FU) was purchased from Sigma Aldrich (F6627) and dissolved at 300 mM in DMSO. 5-fluoro-2'-deoxyuridine (FUDR) was purchased from bioWORLD (40690016-1) and Sigma Aldrich (F0503) and dissolved in sterile water to 200 mM. All drug solutions were filter-sterilized using a Millex® GP filter unit of
0.22 µm and then stored at -20°C. At the start of each experiment, each stock solution was diluted as needed to 1000X the final concentration. To reach the final drug concentration, all drugs were added to a final volume of 0.1% in NGM agar kept at 55°C. All NMG + drug plates were prepared a day before use and allowed to dry overnight at room temperature.

**Bacterial powder**

For each experiment, ~250 mg of bacterial powder was generated from 2 L of bacterial culture. We started with a single colony, grown overnight and subsequently diluted 1:100 in 2 L of LB with no antibiotics and grown to log phase (OD$_{600}$nm 0.8-1.0). Then, cultures were centrifuged at 34,000 rpm for 30 min and washed three times with sterile water. Each bacterial pellet was weighed and sterile water was added to obtain approximately 1 g of wet weight per 25 mL. Bacteria were then disrupted using a Microfluidics™ M-110P lab homogenizer set to ~22,000 psi for five cycles. Disrupted cells were flash-frozen and lyophilized using a Labconco® FreeZone 2.5L benchtop freeze dryer/lyophilizer set to ~0.133 mBar and -80°C for approximately 60 hours until completely dry. Bacterial powder was dissolved in sterile water to achieve a concentration of 50 mg/mL. Plating the powder confirmed that there was no remaining growth.
**Bacterial mixing experiments**

Cultures of *E. coli* OP50 and *Comamonas aq.* DA1877 were grown overnight from a single colony and diluted to OD$_{600nm}$ of 0.5. Colony forming units (CFUs)/µL were determined by plating the diluted cultures. Cultures were mixed as ratios of CFUs.

**Bacterial mutant drug screens**

The following drug doses were used for the primary *E. coli* deletion collection screens [79]: CPT 4 µM, 5-FU 4 µM, FUDR 4 µM. For the *Comamonas aq.* DA1877 mutant collection screens the doses were: CPT 4 µM, 5-FU 4 µM, FUDR 65 µM. All primary screens were performed in 96-well NGM agar plates containing drug and antibiotics as described above. All bacterial mutants were grown from frozen glycerol stocks into one mL LB, incubated for ~16-18 hours at 37°C shaking at 200 rpm. Fifty µL of bacteria from overnight culture was added to each NGM plus drug well and plates were incubated overnight at room temperature. Approximately ten L4 *C. elegans* were added to each well and plates were incubated at 20°C for 72 hours. In the *E. coli* collection, two independent mutant clones are present for each gene, one mutant in an even number plate and the other mutant in the consecutive plate [79]. We screened each plate of the Keio collection, thus screened each mutant twice. For the second and third rounds of screening, 24-well NGM agar plates with drug and without antibiotics were used.
Genotyping bacterial mutants

All mutant hits from the Keio collection were sequence-confirmed using gene-specific primers at -200 bp and +200 bp from the start and the stop codons, respectively. All mutant hits from the Comamonas aq. DA1877 collection were sequenced to identify the disrupted gene, as described previously [51]. Gene names for the Comamonas aq. DA1877 mutants listed in Fig. 2.4b were derived from the closest E. coli gene matching the gene function.

Bacterial growth assay

All mutants were growth overnight at 37°C in 96-well deep well dishes with LB medium. Each bacterial culture was diluted 1,000-fold in 1.2 mL of Sugar Optimal Broth media containing 50 µM of each drug. 150 µL of bacterial drug culture was added to flat-bottom 96-well plates, one for each time point. All plates were incubated at 37°C, shaking at 200 rpm. The OD_{600nm} was measured every two hours for 10 hours using an Infinite® M1000 PRO plate reader.

Dose-response curves

For each experiment, each drug dose and bacteria combination was tested in duplicate. We used the following doses (µM): 5-FU (0.01, 0.025, 0.05, 0.075, 0.1, 0.25, 0.5, 0.75, 1.0, 2.5, 5.0, 10.0); and FUDR (0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 50, 100, 200). At the start of each experiment, two to five animals were placed in each well. Approximately 72 hours later, brightfield images of the entire well were collected using an Invitrogen™ EVOS™ FL microscope set at 2X
magnification. We used the Cell Counter Plugin from Fiji [86] to manually count dead embryos and live progeny.

**Nucleobase supplementation**

Each nucleobase was obtained from Sigma and diluted to 150 mM as follows: adenine was dissolved in 0.5 M HCl, guanine, thymine and uracil were dissolved in 1 M NaOH, and cytosine was dissolved in 0.1 M HCl. Each stock solution was stored in aliquots at -20°C. Forty µL of each base or solvent control was added to 30 mL NGM agar kept at 55°C before pouring for a final concentration of 200 µM of each nucleobase.

**E. coli flux balance analysis**

To predict the effect of gene deletions and uracil supplementation on the production of nucleotides by *E. coli* K-12 BW25113 (the parent strain in the Keio collection) [79], flux balance analysis (FBA) was performed with a slightly modified version of the genome-scale *E. coli* metabolic network model iJO1366 [81]. This model was originally developed for *E. coli* K-12 MG1655. Based on the differences between the BW25113 and MG1655 strains [79], eight genes used in iJO1366 are absent from *E. coli* K-12 BW25113 (*gatC, lacZ, mhpC, rhaA, rhaB, rhaD, araA, araB, araD*). To convert iJO1366 into a better representative model of *E. coli* K-12 BW25113, a total of ten reactions each of which is strictly dependent on the presence of one of these genes were eliminated from the network.
FBA requires a defined input based on nutrient exchange reactions, additional reaction constraints that describe the tested condition, and an objective function to maximize or minimize depending on the biological question. Here, metabolite uptake rates were obtained from previous modeling studies that characterized the growth of *E. coli* in LB medium [87]. To simulate uracil availability, the uracil uptake flux in the model was set to 9 mmol/g dW/hr, which corresponds to the maximum uracil production that can be achieved by the metabolic network from LB medium, and therefore represents a saturation condition that requires no *de novo* biosynthesis of uracil for any objective function. Gene deletions were represented by constraining all reactions associated with the deleted gene (*glnA*, *pyrB*, or *ndk*) to zero flux. The existence of paralogs encoding an enzyme with the same function was ignored, *i.e.*, redundancy in gene-reaction associations was not considered.

In each FBA run, the objective was set to maximize the production of one of the eight nucleotides tested (ribonucleotides: ATP, GTP, CTP, and UTP; deoxyribonucleotides: dATP, dGTP, dCTP, and dTTP) as represented by the flux in an artificially added reaction that consumed the nucleotide. Thus, eight optimizations were carried out for each one of the two uracil conditions, absent or present, resulting in 16 optimizations total. Results from gene deletions were compared with wild type results, *i.e.*, when no reactions were constrained to zero flux. The maximum achievable production of each nucleotide was calculated and reported as a percentage of wild-type production.
RESULTS

The effects of chemotherapeutic drugs on *C. elegans* fed different bacteria

We selected drugs from major classes of chemotherapeutics: alkylating agents (temozolomide), antimetabolites (5-FU; FUDR; methotrexate), anti-tumor antibiotics (doxorubicin; neocarzinostatin), a mitotic inhibitor (paclitaxel), topoisomerase inhibitors (CPT; etoposide), a hormone receptor antagonist (tamoxifen), and a receptor tyrosine kinase small-molecule inhibitor (sunitinib). For bacterial diets, we used *E. coli* and *Comamonas*, because large mutant collections are available for each species, enabling genetic screens to identify bacterial genes that may modulate drug efficacy in the nematode [51, 79]. We tested each drug at a single dose of 10 µM in animals fed each bacterial strain. Briefly, bacteria were seeded onto solid drug-containing media and incubated overnight. The next morning, 5-10 L4 larvae were transferred to drug or control plates, and phenotypic differences between drug-exposed and control animals were assayed after 72 hours of growth at 20°C.

Four of the 11 drugs tested caused abnormal phenotypes in *C. elegans*. CPT, 5-FU, and FUDR all result in impaired fecundity, from sterility (5-FU) to the production of few dead embryos (CPT) (Fig. 2.2a). Paclitaxel did not affect fecundity but rather resulted in a developmental delay (Fig. 2.2a). Interestingly, the *C. elegans* response to FUDR was different between animals fed the two bacteria: on *E. coli* the animals were completely sterile, while on *Comamonas* they
produced live progeny (Fig. 2.2a). This observation indicates that bacteria can affect chemotherapeutic drug efficacy in *C. elegans*.

**E. coli and Comamonas oppositely modulate CPT and FUDR efficacy**

Next, we titrated the three drugs that impaired *C. elegans* fecundity – CPT, 5-FU and FUDR - to determine if, and to what extent, animals display a difference in the response to these drugs on either bacterial diet. We compared fecundity of L4 animals fed each bacteria and drug combination after 72 hours and scored three traits: viable progeny, dead embryos, and sterility (Fig. 2.2b). For 5-FU, we did not observe any differences in drug efficacy between animals fed *E. coli* OP50 or *Comamonas* (Fig. 2.2b). In agreement with the initial screen (Fig. 2.2a), animals fed *Comamonas* fared much better on FUDR than animals fed *E. coli* OP50.

On *E. coli*, animals laid dead embryos when grown on 50 nM FUDR and were sterile when grown on 2.5 µM FUDR. By contrast, dead embryos were laid by animals grown on the highest concentration of FUDR tested (50 µM) when fed *Comamonas* (Fig. 2.2b). Therefore, we observed a large difference in FUDR efficacy in animals fed *E. coli* OP50 versus those fed *Comamonas*. Remarkably, we observed an opposite, albeit milder, difference in the *C. elegans* response to CPT: dead embryos were laid by animals exposed to 2.5 µM CPT on the *E. coli* OP50 diet and by animals exposed to 250 nM on *Comamonas*. Taken together, by testing two bacteria and three chemotherapeutic drugs, we observed that the same bacteria can lead to increased efficacy of one drug but decreased efficacy of a different drug.
FIGURE 2.2: Screen of chemotherapeutics in *C. elegans* fed two bacteria. (a) Fecundity and developmental phenotypes in *C. elegans* in response to chemotherapeutic drugs when fed either *E. coli* OP50 or *Comamonas*. All drugs were tested at a concentration of 10 µM. Brightfield images were taken at 2X magnification. RTK: receptor tyrosine kinase.
(b) Chemotherapeutic drug titrations in C. elegans L4 animals fed either E. coli OP50 or Comamonas. Colors correspond to phenotypes observed after 72 hours in three independent experiments, as indicated in (a). Representative images of fecundity phenotypes in C. elegans are shown. All images were taken at 2X or 4X (inset) total magnification.

**Active bacterial metabolism is required to modify FUDR efficacy**

An important question is whether differential drug efficacy is due to one bacterial species increasing it, the other one reducing it, or both. To start addressing this question, we mixed the two bacterial species in different ratios and compared the drug response in C. elegans. We found that the increase in drug efficacy elicited by Comamonas for CPT and by E. coli OP50 for FUDR is ‘dominant’, because even when either species was greatly diluted into the other, we observed enhanced drug efficacy in C. elegans (*Fig. 2.3a*). This result suggests that E. coli OP50 actively increases drug efficacy of FUDR, while Comamonas increases drug efficacy of CPT.
There are two possible mechanisms by which bacteria can increase drug efficacy. The first is a passive mechanism by which bacteria supplement metabolites that, when ingested by the host, increase drug efficacy. The second is an active mechanism in which bacteria metabolize the drug, or convert a metabolite in response to drug exposure, thereby affecting drug efficacy in the animal. We tested whether we could discriminate between these possibilities by comparing drug efficacy between *C. elegans* fed live bacteria (active metabolism) and dead bacteria (no active metabolism). We reasoned that the traditional method of UV-killing of bacteria may not be most suitable because, even though bacteria
cannot proliferate after high doses of UV they may remain metabolically active, and because UV causes DNA damage, which is related to the reported mechanism of action of these drugs (Fig. 2.1). Instead, we generated bacterial powder from each species by disrupting the cells at high-pressure followed by lyophilization. To quantify drug efficacy in C. elegans fed live bacteria versus bacterial powder, we adopted the term minimum inhibitory concentration (MIC), which is commonly used to describe antimicrobials [80]. Here, MIC refers to the minimum concentration of the chemotherapeutic drug at which we observed no live progeny. Instead of examining phenotypes after 72 hours as described above, we did so after 96 hours of placing L4 animals on each bacteria and drug combination to avoid differences caused by potential egg-hatching delays. For CPT, we did not observe a significant difference between animals fed live bacteria versus those fed bacterial powder (Fig. 2.3b, left). This result indicates that bacteria mostly modulate CPT efficacy through a passive mechanism.

In contrast, the bacterial influence on drug efficacy of FUDR is completely dependent on active bacterial metabolism. First, we found no difference in drug efficacy between C. elegans fed the two bacterial species when fed as metabolically inactive powder (Fig. 2.3b, right). Second, we observed both a decrease in drug efficacy (higher MIC) with E. coli OP50 powder and an increase in drug efficacy with Comamonas powder (lower MIC), relative to either live bacteria. From this finding, we conclude that at least two mechanisms are involved: E. coli metabolism increases FUDR efficacy in C. elegans, while Comamonas
metabolism reduces it. The latter effect indicates that bacterial metabolism not only increases drug efficacy but that it can also be protective.

**Genetic screens with two bacterial species and three drugs**

In order to gain insight into the mechanisms by which bacteria modulate the *C. elegans* response to chemotherapeutics, we performed genetic screens in both *E. coli* and *Comamonas*, and using all three drugs: CPT, 5-FU and FUDR. First, we used the *E. coli* Keio collection that comprises 3,985 strains, each with a deletion in a single, non-essential gene [79]. We found that the parental strain of this collection, *E. coli* K-12 BW25113, modulates drug efficacy in a similar manner as *E. coli* OP50, although there was a relatively small difference between animals fed *E. coli* OP50 and *E. coli* K-12 BW25113 with 5-FU (data not shown). Second, we used a collection of 5,760 *Comamonas* mutants we generated previously [51]. Each of these mutant strains harbors a single transposon insertion into its genome.

For each of the six genetic screens, we used a single dose of drug where animals fed the parental strain produced dead embryos. This dose allowed us to simultaneously screen for bacterial mutants that increased drug efficacy (when we observe sterility) or decreased drug efficacy (when we observe live progeny) (Fig. 2.4a, top). In each of the primary screens, between two and 20 percent of the bacterial mutants tested scored positively, either increasing or decreasing drug efficacy in *C. elegans*. These mutants were retested in a secondary screen with three doses of the drug (Fig. 2.4a, middle), and only those mutants that scored in at least two of the three doses were tested in a tertiary screen (triplicate and with
six concentrations of each drug, **Fig. 2.4a**, bottom). This process resulted in a final set of mutants that range between a single *Comamonas* mutant that increases FUDR efficacy to 16 *E. coli* mutants that reduce the efficacy of CPT (**Fig. 2.4a**, bottom). The identity of the disrupted genes in the *Comamonas* mutants was determined by sequencing [51]. The *E. coli* gene deletions were identified according to their coordinate in the collection and all were confirmed by sequencing.
FIGURE 2.4: Genetic screens for bacterial genes involved in host drug efficacy. (a) (Left) Schematic of genetic screens. (Right) Number of mutants, for each bacteria and drug combination, identified in each round of screening, as indicated. (b) Unique bacterial mutants identified in each genetic screen. Up/downward arrows correspond to in/decreased drug efficacy in animals fed the mutant bacteria. Arrows are colored based on listed functional categories.
Several observations support the high quality of the different screens. First, we found bacterial mutants for each drug/bacteria combination and, for each combination, except for Comamonas and 5-FU, we identified strains that increased or decreased drug efficacy in C. elegans. Second, two genes were each found twice in the Comamonas screens, including two independent mutations in upp found in the 5-FU and FUDR screens, and three independent mutations in purH, found in the FUDR screen. Third, a mutant in Comamonas purA was found in both the 5-FU and FUDR screens, while E. coli mutations in glnA were found in both the CPT and 5-FU screens. Strikingly, E. coli mutations in pdxH were also found with both CPT and 5-FU, but a deletion in this gene increased efficacy of CPT and decreased efficacy of 5-FU (Fig. 2.4b). Finally, one gene was found with both bacterial species and with multiple drugs: mutations in upp decreased the efficacy of 5-FU in Comamonas and FUDR in both bacteria (Fig. 2.4b). Altogether, we identified 44 unique bacterial genes that can be grouped into several broad functional categories, including different types of metabolism, most notably amino acid and nucleotide metabolism.
FIGURE 2.5: *C. elegans* response to CPT, 5-FU and FUDR when fed mutants. Each colored block represents bacterial mutant-drug interactions identified in ≥ 2/3 independent experiments. All doses listed are in µM.
Different classes of bacterial mutants

Due to the stringency of the six bacterial genetic screens, it is unlikely that we uncovered all genes that contribute to the *C. elegans* response to each of the three drugs, *i.e.*, we expect a considerable rate of false negatives. Therefore, we next performed a matrix experiment in which we tested animals fed each of the mutants that scored positively in the second retest (n = 98, Fig. 2.4a) against six concentrations of each of the three drugs in quadruplicate (Fig. 2.5). We reasoned that this experiment would enable us to define different classes of genes, including those genes that specifically affect the response to a single drug, and other genes that may elicit a more general change in drug response. Indeed, this experiment uncovered additional gene/drug interactions with the same set of mutants. In total, we identified 98 interactions involving 44 bacterial mutants, including 48 interactions that were observed previously (Fig. 2.4a) and 50 additional interactions.

Bacterial mutants can be grouped into four broad classes according to their effect on drug efficacy (Fig. 2.6a). Class I mutants (13 genes) have drug-specific effects on chemotherapeutic efficacy. Most of these (n = 11) specifically affect the response to CPT, which may not be surprising given that 5-FU and FUDR are thought to function through the same pathway (Fig. 2.1) [69]. Of these 11 genes, eight increase CPT efficacy and three decrease it. Class II mutants involve eight genes that affect the response to two of the three drugs. With the exception of one *(purC)*, these genes affect the response to both anti-pyrimidine drugs: five increase
their efficacy while two reduce it. Class III involves six genes that decreased the efficacy of all three drugs. Finally, class IV genes (n = 17) affect all three drugs but in an opposite way between the anti-pyrimidine drugs, 5-FU and FUDR, and the topo-I inhibitor CPT. Most of these mutants increase the efficacy of the anti-pyrimidine drugs and decrease the *C. elegans* response to CPT.

One explanation for the effects of bacterial mutants on drug efficacy in *C. elegans* could be that the growth of the microbes is affected by the mutation and that this in turn affects the response in the animal. To test this, we compared growth of all 98 bacterial mutants that passed the second round of screening (*Fig. 2.6b*) in the presence or absence of drug. Overall, we did not find a strong relationship between bacterial growth in the presence of drug and an increase in drug efficacy in *C. elegans*. We did observe that several mutants that decrease drug efficacy are slow growing, but that this is generally not affected by the presence of the drug (*Fig. 2.6b*).
FIGURE 2.6: Four classes of bacterial mutants influence drug efficacy.

(a) Network diagram for bacterial mutant drug interactions identified in matrix experiment, in which each mutant was used with each drug. Each node indicates a bacterial mutant that affects drug efficacy in *C. elegans*. Nodes are colored based on functional categories listed in Fig. 2.4b. Bacterial mutants are connected when they share the same effect on drug efficacy; edges are as follows: solid lines
indicate increased drug efficacy and dashed lines indicate decreased drug efficacy. Bacterial mutant-drug interactions were grouped into four classes: class I – mutants that affect drug efficacy of only one drug; class II – mutants that increase OR decrease drug efficacy of two drugs; class III – mutants that increase OR decrease drug efficacy of all three drugs; class IV – mutants that increase drug efficacy of one or more drugs AND decrease efficacy of other drugs. $E = E.\ coli$; $C = Comamonas$. (b) Bacterial growth assays in the presence or absence of drug for all 98 bacterial mutants in round 2 of screening, as indicated in Fig. 2.4a. All drugs were tested at a concentration of 50 µM. Each value represents OD600 nm after eight hours of incubation with DMSO (x-axis) or drug (y-axis). Data points are colored according to host drug phenotype.

**Bacterial nucleotide metabolism affects chemotherapeutic drug efficacy**

The set of bacterial mutants that we identified contains numerous genes involved in nucleotide metabolism (Fig. 2.7a). Given the considerable level of false negatives in our screens, we selected all available $E.\ coli$ mutants representing the genes in these pathways (e.g., including those mutants that were not recovered in the primary screen) and systematically tested them in triplicate as described for the matrix experiment (Fig. 2.5). We tested two independent deletion copies for each gene from the $E.\ coli$ mutant collection [79]. Altogether, we tested 37 duplicate mutant strains. The interactions are summarized in Fig. 2.7a, and include 42 that were not observed before.

Several observations can be gleaned from this experiment. First, we observed striking differences between the three drugs: the upper branch of the network shown in Fig. 2.7a, which depicts the final biosynthetic reactions of purine metabolism, is relatively specific for CPT, with most mutants increasing the efficacy
of this drug in *C. elegans*. An exception is *guaA*, which encodes guanine monophosphate (GMP) synthase that catalyzes the final step in GMP synthesis. A deletion in *guaA* results in slow bacterial growth [79] and decreases the efficacy of all three drugs. Second, the *de novo* pyrimidine biosynthesis branch of nucleotide metabolism, which converts L-glutamine into uridine monophosphate (UMP, **Fig. 2.7a**), increases the efficacy of all three drugs. Third, a deletion in the *E. coli* pyrimidine salvage pathway gene *upp*, which encodes uracil phosphoribosyltransferase that generates UMP from uracil, decreases drug efficacy for 5-FU and FUDR, but increases the efficacy of CPT (**Fig. 2.7a**).

Next, we quantitatively compared the modulation of 5-FU and FUDR efficacy by each pyrimidine synthesis pathway. The dose-response curves show a ~10-fold increase in drug efficacy upon mutation of genes in the *de novo* pyrimidine biosynthesis pathway and a ~10-fold decrease in efficacy upon deletion of *upp* (**Fig. 2.7b**). These observations suggest that the bacterial conversion of 5-FU and FUDR into FUMP, an analog of UMP, by *upp*, is critical for the cytotoxic effects of both anti-pyrimidine drugs in *C. elegans*. When *upp* is deleted, this conversion is blocked or reduced, and the bacteria depend on the *de novo* UMP biosynthesis pathway. When the latter pathway is perturbed, however, the flux through the salvage pathway may be increased leading to higher levels of FUMP.
**FIGURE 2.7: Bacterial nucleotide metabolism affects host drug efficacy.** (a) Summary of the *E. coli* nucleotide biosynthesis network. Colored arrows indicate increase (upward) or decrease (downward) efficacy with CPT, 5-FU, and FUDR. Conversion products from reactions involving 5-FU and FUDR and indicated in light blue. (b) Dose-response curves of selected *E. coli* mutants. Mean and standard deviation of technical replicates in one experiment are shown.
Modulation of anti-pyrimidines by bacterial RNA rather than DNA metabolism

Remarkably, deletions in either *E. coli* upp or udk decreases anti-pyrimidine drug efficacy, while deletions in tdk, tmk, or thyA have no effect (Fig. 2.7a). This observation suggests that 5-FU and FUDR predominantly affect *C. elegans* fecundity via bacterial ribonucleotide metabolism (e.g., by generating FUMP), and not DNA metabolism (e.g., by generating FdUMP which inhibits thymidylate synthase). To test this prediction, we supplemented the animals fed different bacteria and exposed to the two drugs with each of the five nucleobases. We found that only supplementation with uracil rescues the effect of 5-FU on both *E. coli* and *Comamonas*. Uracil supplementation also rescues the effect of FUDR on *E. coli*, with no detrimental effect on *Comamonas*-fed animals (Fig. 2.8). The rescue by uracil, but not thymine, supports a model in which bacteria modulate 5-FU and FUDR efficacy through ribonucleotide- rather than DNA metabolism. The observation that thymine fails to rescue the anti-pyrimidine drug effects indicates that inhibition of thymidylate synthase may not be the principal mechanism of action [69].
FIGURE 2.8: Nucleobase supplementation in different bacterial mutants. Effect of nucleotide base supplementation at 200 µM in three independent experiments. (- = vehicle control, A = adenine, G = guanine, C = cytosine, T = thymine, U = uracil). Representative brightfield images at 2X magnification.

Flux balance analysis of bacterial nucleotide metabolism

Deletion of any gene in the de novo pyrimidine biosynthesis pathway increases efficacy of 5-FU and FUDR, with the exception of glnA, which encodes glutamine synthetase that converts L-glutamate into L-glutamine (Fig. 2.7a). One explanation is that this reaction is also upstream of purine biosynthesis. We used flux balance analysis (FBA) to computationally model nucleotide production in E.
coli ΔglnA. Interestingly, glnA deletion greatly affects purine nucleotide production but has little effect on the production of pyrimidines (Fig. 2.9). As a control, we also performed FBA with mutants of pyrB, which functions downstream of glnA in de novo pyrimidine biosynthesis (Fig. 2.7, 2.9). With these mutations, FBA predicts a reduction in pyrimidine biosynthesis, while having no effect on purines, and further predicts that uracil supplementation rescues this mutation, which is indeed what we observed (Fig. 2.9). Altogether, these observations help to explain why glnA deletion does not mimic a deletion of downstream genes in de novo pyrimidine biosynthesis. However, it does not explain why glnA deletion results in a decreased drug efficacy. Possibilities include a severe reduction in bacterial growth or an overall nucleotide imbalance.

A deletion in ndk, which encodes nucleotide diphosphate kinase, results in an increase in 5-FU and FUDR efficacy (Fig. 2.7). This enzyme is involved in the biosynthesis of all nucleotides (Fig. 2.9). However, FBA indicates that ndk mutant bacteria only affect the biosynthesis of pyrimidine nucleotides (Fig. 2.9). This is because, according to the metabolic network model, ATP and GTP can be generated by alternative pathways [81]. Thus, an increase in drug efficacy upon ndk deletion agrees with the model that bacterial synthesis of FUMP from 5-FU and FUDR is a primary mechanism of eliciting cytotoxicity in C. elegans: when ndk is deleted, FUMP can no longer be converted into FUTP and accumulates.
FIGURE 2.9: Flux balance analysis (FBA) of *E. coli* mutants. FBA was performed on the *E. coli* metabolic network model iJO1366 [81] with an objective function that maximized the production of a nucleoside triphosphate (represented by the flux of drain reactions shown as red arrows) in each simulation. Production fluxes were recalculated upon the deletion of reactions associated with each gene on the right panel. The analysis was repeated with the addition of uracil as represented by an allowed negative flux in a uracil exchange reaction. Bar graphs show production rates during gene deletion simulations as a percentage of wild type bacteria. The production rate of ribonucleotides and deoxyribonucleotides varied by less than 2%, therefore, only ribonucleotides and dTTP are shown for simplicity.

**A Comamonas mutant that increases C. elegans sensitivity to FUDR**

So far, we have found that bacterial ribonucleotide metabolism modulates the *C. elegans* response to 5-FU and FUDR. Further, we found that FUDR efficacy
is much lower when the animals are fed the *Comamonas* diet relative to a diet of *E. coli*, whereas we did not observe such a difference for 5-FU. Why does *E. coli* increase drug efficacy of FUDR relative to a diet of *Comamonas*? Or, conversely, how does *Comamonas* exert its protective effect on FUDR toxicity? We found only one *Comamonas* mutant that specifically increases FUDR efficacy, but that does not modulate the efficacy of 5-FU. This mutant harbors a transposon insertion in one of the 23S rRNA genes (Fig. 2.10a). Dose-response curves show that this mutant increases the efficacy of FUDR to the level found with *E. coli* (Fig. 2.10b), which cannot be explained by changes in bacterial growth (Fig. 2.10c). As with *E. coli*, uracil supplementation rescues progeny production in animals fed this mutant and supplemented with FUDR (Fig. 2.10d). Taken together, the protective effect of *Comamonas* on animals treated with FUDR has a genetic basis. However, the precise mechanism by which the protective effect occurs and why it is specific to FUDR (and not 5-FU) remains to be elucidated.
FIGURE 2.10: Phenotypes of a *Comamonas* mutant that resembles *E. coli*. (a) Cartoon of transposon-insertion site in the *Comamonas* 23S rRNA\(^{-}\) mutant. (b) Dose-response curves for FUDR. A representative experiment out of three biological replicates is shown. (c) Representative experiment of bacterial growth in the presence or absence of 50 µM FUDR. (d) Nucleobase supplementation; (-) = vehicle control, T = thymine, U = uracil.
CONCLUSIONS

Bacterial modulation of chemotherapeutic drug efficacy

In this study, we have shown that different bacteria can affect chemotherapeutic drug efficacy in *C. elegans*. For two of the three drugs examined in detail, we found that *E. coli* and *Comamonas* oppositely affect the response to CPT and FUDR: animals do better on *Comamonas* when challenged with FUDR, but worse when supplemented with CPT. For the third drug, 5-FU, we did not observe a difference between the two bacterial species. However, our data show that the response to each drug is affected by bacterial metabolism because we identified bacterial mutants that increase or decrease the efficacy of each of the three drugs, including 5-FU. Although we cannot yet fully explain the mechanisms that cause the bacterial differences in modulation of drug efficacy, our experiments provide insights into the mechanism of action of the different chemotherapeutic drugs.

Bacterial modulation of CPT efficacy

Our data suggest that multiple complex mechanisms in bacteria modulate drug efficacy in *C. elegans*. Some of these mechanisms are drug-specific, while others are more generic, at least for the drugs tested here. First, the efficacy of CPT is affected by bacteria, but likely in a passive way that does not require bacterial metabolism, for instance through nutritional support of nucleotides to the host. We did observe a rather striking difference between the two bacteria with
CPT where *E. coli* deletions in *upp*, *purH*, and *purA* increase CPT efficacy, while the corresponding *Comamonas* mutant strains cause a decrease (Fig. 2.6a). However, supplementing nucleobases had no effect on CPT efficacy in either *E. coli* or *Comamonas*-fed animals (data not shown). Therefore, differences in the pools of individual nucleotides do not directly affect the response to CPT, nor explain the difference in CPT efficacy between animals fed *E. coli* and *Comamonas*. The phenotypes we observed in *E. coli* and *Comamonas* nucleotide synthesis mutants likely reflect complex relationships between bacterial metabolic networks and host physiology. However, the precise mechanism by which this difference occurs remains to be elucidated.

**Bacterial modulation of 5-FU and FUDR efficacy: ribonucleotide rather than DNA metabolism**

The efficacy of FUDR is differently modulated by *E. coli* and *Comamonas* and this difference requires active metabolism in both bacteria: *E. coli* metabolism to increase and *Comamonas* metabolism to decrease drug efficacy in *C. elegans* (Fig. 2.11). With 5-FU, however, we did not observe a difference between animals fed these two bacterial species. This observation indicates that 5-FU and FUDR are differentially affected by bacteria and may not solely function in a linear pathway.
Genetic screens in bacteria revealed that both anti-pyrimidine drugs act by affecting ribonucleotide, rather than DNA metabolism, and the bacterial powder experiments demonstrate that this requires active bacterial metabolism. Our genetic data indicate that both drugs may mainly act through the production of FUMP, which is analogous to UM[82]. *E. coli* has two pathways that can synthesize UMP. The first pathway involves the conversion of glutamine into carbamoylphosphate by the *carA* protein and the subsequent multistep generation of UMP. The second pathway involves conversion of uracil into UMP, either via uridine as an intermediate, or directly by the *upp* enzyme. Mutations in the first pathway increase the efficacy of 5-FU and FUDR, while mutations in the second pathway upstream of UMP decrease efficacy and downstream (in *ndk*) increase efficacy. Altogether, these findings suggest that bacteria use the first pathway to
generate pyrimidines that support \textit{C. elegans} fecundity in the presence of anti-pyrimidines. A second pathway converts the anti-pyrimidine drugs into FUMP, which is then delivered to \textit{C. elegans}. The natural analog of FUMP, UMP, has been shown to inhibit the bacterial \textit{carA/B} enzyme \cite{83,84}, thereby blocking the \textit{de novo} pyrimidine biosynthesis pathway, in bacteria, \textit{C. elegans}, or both (Fig. 2.11). Unfortunately, it is not feasible to test this hypothesis directly by supplementing FUMP to \textit{C. elegans} fed \textit{Δupp} bacteria due to limited availability and high cost. Further, the nucleotide may not be taken up as efficiently by the animal as the nucleobase, which would make any results difficult to interpret. Interestingly, the Food and Drug Administration recently approved the use of uridine triacetate to mitigate 5-FU toxicity \cite{85}. This change supports our findings that ribonucleotide metabolism is a central mechanism of action of anti-pyrimidine drugs rather than the inhibition of thymidylate synthase by FdUMP.

**Implications for microbiota effects on chemotherapeutic drug efficacy in humans**

The human gut microbiota is highly complex and contains many bacterial species. These bacteria may affect drug efficacy even when present in small proportions, and may affect each other as well, further illustrating the complexity of potential bacteria-drug interactions. Additionally, when bacterial growth of some species is affected by chemotherapeutic drugs, the microbiota composition may change, which can further affect drug efficacy and overall health. While our study focused on a simpler metazoan system with only two bacterial species, our findings
illuminate the possible effects the microbiota may have on the efficacy of some chemotherapeutics used to treat colorectal cancer in humans. It is tempting to speculate that the treatment of such cancers may benefit from perturbations of the microbiota prior to or after administration of the drugs, such as with probiotics or with fecal transplants. Longer term, studies using additional drugs, as well as multiple bacterial species can be used to further examine the spectrum of bacteria-drug interactions. *C. elegans* and its bacterial diet provide a fruitful interspecies model system to characterize these interactions and the mechanisms involved.
CHAPTER III: Microbial fatty acids influence TAM toxicity

PREFACE

The contents of this chapter are unpublished. I performed all experiments with technical help from Yomari Rivera and Shaleen Shrestha. Olga Ponomarova performed targeted metabolomics to measure TAM in animals with metabolic gene RNAi knockdown (Fig. 3.5). Andre F. Viera and Carissa P. Olsen measured fatty acids in animals and bacteria, as shown in Fig. 3.8 and Fig. 3.9.

For the research in this chapter, I was supported by the NIH Ruth L. Kirschstein National Research Service Award (NRSA) Individual Predoctoral Fellowship under the National Institutes of Health (GM122393).

SUMMARY

Tamoxifen citrate (TAM) is one of the most common chemotherapeutic agents used to treat estrogen-receptor positive breast cancer [88]. Because of its specificity for the estrogen receptor, TAM is also broadly used to selectively activate gene silencing in many model organisms. We serendipitously discovered a bacteria-dependent effect of TAM on C. elegans development. We used bacterial and animal genetics, as well as targeted metabolomics, to characterize an off-target mechanism of action of TAM that involves fatty acid metabolism.
INTRODUCTION

As the first to encounter compounds from our diet, the gut microbiota plays an important role in our metabolism. Interactions between the microbiota and xenobiotics, like drugs, can also impact our physiology and response to medications [24]. One challenge remains in systematically identifying and characterizing such interactions between the microbiota and host drug response. We have attempted to address this problem using the nematode C. elegans along with its bacterial diet, which also acts as a microbiota, taking advantage of the scalability and genetic tractability of both host and microbe. Our first step was to test eleven chemotherapeutics against two bacterial species. We found two drugs, camptothecin and 5-fluoro-2’-deoxyuridine, with a bacteria-dependent effect [89]. We later discovered one false negative in our screen. When tested at higher doses, TAM also leads to a bacteria dependent effect. We were intrigued by these observations since TAM doesn’t have a clear drug target in C. elegans. Thus, our findings could help elucidate off-target effects of this drug in animals.

TAM is a triphenylethylene belonging to the class of drugs known as selective estrogen receptor modulator (SERM). SERMs bind to and either activate or inhibit estrogen receptors (ER), depending on the tissue, and are often used to treat ER positive breast cancers, as well as other hormone-dependent ailments [88,90]. Because of its low cost and selectivity for the ER, TAM is also often used in the spatiotemporal control of genetic engineering in mice and other model systems. For instance, the Cre DNA recombinase can be coupled to the ligand-
binding domain of ER and, upon treatment with TAM, the CreER fused protein is activated and translocates to the nucleus to initiate site-specific recombination [91-93].

In addition to its activity through hormone receptor regulation, TAM has been linked to several cellular pathways. In cancer cells, TAM inhibits acid ceramidase, disrupting sphingolipid metabolism, and increases lysosomal membrane permeability, leading to cell death [94,95]. In microorganisms, TAM causes growth inhibition through a variety of mechanisms, from changes in membrane fluidity to calcium homeostasis disruption [96]. For instance, one study in yeast used chemical genetic interactions to link TAM growth inhibition to activation of the calcineurin signaling pathway [97].

Despite having an abundance of nuclear hormone receptors relative to humans, *C. elegans* does not harbor an annotated ER [98]. Therefore, it is unclear how TAM could disrupt *C. elegans* physiology and, more importantly, why this effect is bacteria-dependent. Because of its importance in clinical use and as a research tool, we sought to characterize a mechanism of chemical toxicity of TAM in *C. elegans*. We combined genetics and targeted metabolomics to identify a role of TAM in fatty acid metabolism. First, our results suggest that bacteria mediate TAM toxicity in two ways: (1) by modulating drug bioavailability and (2) by providing different fatty acids. Second, we find that *C. elegans* fatty acid metabolism is involved in TAM toxicity.
MATERIALS AND METHODS

C. elegans strains

C. elegans N2 (Bristol) was used as the wild-type strain. Prior to all experiments, animals were maintained on Nematode Growth Media (NGM) and fed a diet of E. coli OP50, as previously described [41].

Bacterial strains

For each experiment, bacterial strains E. coli BW25113 (Keio collection background strain) [79] and Comamonas aquatica DA1877 were grown from single colony to stationary phase in Luria Broth (LB), overnight at 37°C and shaking at 200 rpm. E. coli deletion mutants [79], were grown in with 50 µg/mL kanamycin. Comamonas aq. DA1877 mutants [51] were grown in LB containing 20 µg/mL gentamicin. All Comamonas strains were concentrated 4X and all E. coli strains were concentrated 2X, to plate similar OD600 of bacteria, prior to seeding onto NGM agar + drug plates.

Chemotherapeutic drugs

Tamoxifen citrate (TAM) (S1972) was purchased from Selleckchem and dissolved to 150 mM in sterile dimethyl sulfoxide (DMSO). 5-fluorouracil (5-FU) was purchased from Sigma Aldrich (F6627) and dissolved to 300 mM in DMSO. All drug stock solutions were stored at -20°C. At the start of each experiment, stock solutions were diluted in series to 200X the final concentration and added at a final
volume of 0.5% to NGM agar heated to 55°C. NGM plates containing drug were allowed to dry overnight at room temperature before each experiment.

**C. elegans dose-response curves**

All dose-response curves were performed in 48-well plates, with a single bacterial strain tested in each plate. Each combination of drug and bacteria was tested in eight to twelve technical replicates, as listed in the corresponding figure legends, and in biological triplicate. For TAM dose-response curves, the following doses (in µM) were used in each of the following experiments, along with a vehicle (DMSO) control: Fig. 3.1b (75, 150, 300, 400, 500, 600, 750), Fig. 3.2a & 3.3a (99.12, 180.64, 329.22, 444.44, 600), Fig. 3.2b, 3.4 and 3.9d (150, 300, 600). For 5-FU, we used the following doses (µM): 0.05, 0.5, 5. Each drug stock was added at 0.5% v/v to NGM agar heated to 55°C. In all experiments involving RNAi, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at a final concentration of 2 mM. Once all additives were mixed, 0.45 mL of NGM agar with drug were aliquoted into each well and allowed to dry. The next day, 50 µL of concentrated bacterial culture were seeded onto NGM agar and drug plates and allowed to grow overnight at room temperature. N2 gravid adults were bleached to harvest embryos, then washed and allowed to hatch overnight in M9 buffer to synchronize to L1 stage. Between 50-100 animals were then plated onto each well. Once incubated at 20°C for 48 hours, animals developed beyond the L1 stage were counted manually under the dissecting microscope. The percentage of animals
that did not develop was calculated relative to the total number of animals developed in the vehicle (DMSO, 0.5% v/v) when fed each bacterial strain.

**Bacterial mutant genetic screens**

All bacteria genetic screens were performed in 96-well plates containing drug and antibiotic, while subsequent retest experiments were performed in 48-well plates without antibiotics. For the *C. aquatica* collection screen, 20 µg/mL gentamicin was added to NGM agar and, for the *E. coli* Keio collection, 50 µg/mL kanamycin was added. The following doses of TAM were used in the screen: *E. coli* (100 and 300 µM), *C. aquatica* (100 and 200 µM). We only tested one bacterial mutant for each gene in the *E. coli* Keio collection, the odd copy, compared to two mutants per each non-essential gene in the entire collection. All bacterial mutants were grown from a frozen glycerol stock into 600 µL of LB with antibiotic, as listed before, and incubated at 37°C overnight, shaking at 200 rpm. The next day, 50 µL of bacterial culture were seeded onto each well and allowed to dry overnight at room temperature. Approximately twenty L1-arrested animals were added to each well and incubated at 20°C for 72 hours. Hits were scored visually under a dissecting microscope as bacterial mutants leading to a difference in animal drug response relative to WT-fed animals. All primary hits were tested in technical triplicate in two independent experiments using the following doses (µM): 600, 300, 150, 100, and 50. Bacterial mutants that showed robust differences on host drug response were genotyped as described [51].
**Bacterial growth curves**

Bacteria were first grown overnight from single colony, in LB, at 37°C and shaking at 200 rpm. Next, each bacterial culture was diluted to 1:1000 and aliquoted into 5 mL round-bottom tubes. Drug was added at 2% v/v, for improved solubility without compromising bacterial growth. All cultures were incubated at 37°C, shaking at 200 rpm, overnight. Each culture was diluted in series and 5 µL of each diluted culture were spotted onto 15 cm LB agar plates and allowed to grow overnight at 37°C. Individual colonies were counted for each condition and the number of colony-forming units (CFUs) was calculated.

**C. elegans metabolic gene RNAi**

We tested individual 1,645 *E. coli* HT115 strains expressing dsRNA against a unique *C. elegans* metabolic gene, taken from the ORFeome library [99] or the Ahringer library [100]. We used 96-well plates and two doses of drug (µM), 300 and 600 µM, to identify mutants which increase or decrease TAM toxicity, respectively. In addition to TAM, all 96-well plates contained 50 µg/mL ampicillin and 2 mM IPTG. All subsequent retest experiments were performed in 48-well plate format, without antibiotic, using the following doses (µM): 150, 300, 600. At the start of the screen, TAM, IPTG and ampicillin were added to NGM agar heated to 55°C. A volume of 180 µL was dispensed into each well and plates were covered and allowed to dry overnight at room temperature. Simultaneously, bacteria were grown from frozen glycerol stocks in 96-well deep well plates containing 600 µL LB + 50 µg/mg ampicillin. The next day, bacteria were diluted 1:10 and allowed to
grow to approximately mid-log phase for about six hours, then concentrated 10X and seeded onto NGM agar + TAM plates, allowed to dry and incubate overnight at room temperature. Approximately twenty L1 larvae were added to each well containing NGM + TAM and the RNAi clone. Plates were incubated at 20°C for about 72 hours, and development was scored visually under the dissecting scope. Hits were scored as having developed, or not, relative to animals fed an empty vector (pL4440) control bacteria.

**Fatty acid supplementation**

A fatty acid mixture was purchased from Sigma (F7050) and added at 0.2% v.v in LB for overnight growth of bacteria as described in Bacterial strains.

**C. elegans fatty acid measurements**

Fatty acid measurements in bacteria and animals were performed as described before [101]. Briefly, lipids were extracted from whole animals using a 2:1 chloroform:methanol mixture and dried in liquid nitrogen. A calibrated phospholipid or triacylglycerol standard was added to the extracted lipids before separation by solid-phase exchange chromatography. Purified lipids were then converted into fatty acid methyl esters by incubation with methanol/2.5% H₂SO₄ before GC/MS analysis. All data are presented as percentage of the total fraction of fatty acids measured in the sample.

**Temperature-shift assay**
Animals from all previous generations were harvested in 20°C and fed a standard diet of *E. coli* OP50. Approximately 50-100 synchronized L1 animals were placed in each well in a 48-well plate, as described for Dose-response curves, and grown at either 25°C, 20°C or 15°C. Animals were incubated at the respective temperature until animals in the vehicle control condition reached the L4 stage, at approximately 30, 48, and 72 hours for 25°C, 20°C, and 15°C, respectively.

RESULTS

Bacteria modulate TAM toxicity

We serendipitously discovered that, when fed *Comamonas aquatica* DA1877 (*Comamonas*), animals showed increased developmental delay after treatment with TAM compared to animals fed *E. coli* BW25113 (*E. coli*) (Fig. 3.1).

![FIGURE 3.1: Comamonas increases TAM toxicity in C. elegans.](image)

(a) Animals treated with TAM and fed *E. coli* or *Comamonas*. Bright-field images taken at 1.25X magnification. (b) Dose-response curves for the number of animals that did not develop beyond L1 relative to the vehicle control (DMSO 0.5%) by 48 hours. Shown is the mean and standard deviation of three independent experiments, each with twelve technical replicates; p < 0.01 by paired t-test.
To understand the mechanism by which *E. coli* and *Comamonas* affect drug efficacy of TAM, we performed genetic screens in both bacteria using the *E. coli* Keio collection, with 3,985 single-gene deletion strains [79], and a *Comamonas* transposon mutagenesis collection of 5,760 strains previously generated by our group [51]. Each bacterial mutant was screened against two doses of TAM: a high dose, where WT-fed animals do not develop, to look for mutants causing decreased toxicity and a low dose, where WT-fed animals develop, to find mutants that increase toxicity. After a primary screen and three rounds of validation, we identified four mutants in *Comamonas* that change the animal’s response to TAM (Fig. 3.2a) and none in *E. coli*. Two mutants led to an increase in the number of animals that failed to develop beyond the first larval stage (L1), or increase toxicity of TAM: tadC*, a component of the type II secretion system [102,103] and exbD*, involved in TonB-dependent transport [104]. Two other mutants decrease TAM toxicity: acrR*, a transcriptional repressor of the acrA/B multidrug efflux pump [105], and a hypothetical protein with no annotated functional domains or homology. Since acrR is specifically involved in regulating multidrug efflux, it is likely that this mutant exerts its effect through decreased TAM accumulation. Interestingly, while two of our *Comamonas* gene hits have an *E. coli* ortholog, neither ∆acrR nor ∆exbD mutant *E. coli* influenced the *C. elegans* response to TAM (Fig. 3.2b). First, we wondered whether bacteria have different sensitivities to TAM. Interestingly, while *E. coli* growth was unaffected by TAM, *Comamonas* growth was reduced starting at 90 µM (Fig. 3.2c). In addition, bacterial mutant sensitivities to TAM correlate
with their effects on host drug response: mutants leading to increased host drug resistance are also resistant to TAM supplementation and vice versa, with \( exbD^- \) showing the most sensitivity to TAM, as well as the highest host drug sensitivity (Fig. 3.2c). Next, we tested each bacterial mutant for their specificity in changing host drug response. We used an anti-pyrimidine drug, 5-FU, which affects \( C. elegans \) through nucleotide metabolism \[82\], different than SERMs. None of the \( Comamonas \) mutants changed the animal’s response to 5-FU, relative to animals fed wild-type \( Comamonas \), suggesting drug specific activity on host TAM response (Fig. 3.2d). The three \( Comamonas \) mutants for which there are functional annotations, \( tadC^- \), \( exbD^- \), and \( acrR^- \), suggest transport as a mechanism to both increase and decrease toxicity of TAM in \( C. elegans \). At least one such transport mechanisms could involve the drug itself. For instance, because of its specific involvement in multidrug efflux, the observed drug resistance in animals fed \( Comamonas acrR^- \) is likely due to decreased drug bioavailability in \( C. elegans \).
Fatty acid metabolism protects against TAM toxicity

The bacterial genetic screens failed to identify the mechanisms by which TAM treatment leads to developmental delay in *C. elegans*. To characterize the
mechanism of action of TAM in C. elegans, we used a set of 1,645 RNAi clones against C. elegans metabolic genes and tested for either increased or decreased drug toxicity. We identified four genes for which RNAi knockdown led to increased drug efficacy: *dhs-19*, a short-chain dehydrogenase recently observed in lipid droplets [106]; two genes, *elo-3* and *elo-6*, involved in fatty acid (FA) elongation [107]; and *pck-2*, a paralog of human phosphoenolpyruvate carboxykinase involved in glycolysis gluconeogenesis [108] (Fig. 3.3a). Our ability to identify host genes which modulate TAM toxicity suggests that feeding an *E. coli* diet does not only exert its protective effect by limiting drug bioavailability. Next, we tested drug specificity of the metabolic gene RNAi hits similarly to the bacterial mutants. Three out of four RNAi clones did not change host drug response to 5-FU. In contrast, RNAi of *pck-2* led to increased drug efficacy of TAM and decreased drug efficacy of 5-FU (Fig. 3.3b).

**FIGURE 3.3**: *C. elegans* metabolic genes modulate the response to TAM. (a) Dose-response curves for RNAi screen hits, shown is the average and standard deviation of three independent experiment with ten technical replicates. (b) Test for drug specificity of RNAi screen hits. Dose-response curves for an anti-
pyrimidine drug, 5-FU. All data shown are the mean and standard deviation of three independent experiments, each with twelve technical replicates. ns: p ≥ 0.05, **** p < 0.0001, by paired t-test.

To further test the role of each gene identified in the screen, we selected genes involved in similar functions or belonging to the same metabolic pathway. Neither the gene paralogs of pck-2 nor a few genes immediately upstream or downstream of pck-2 showed a significant difference in TAM toxicity (Fig. 3.4a). Similarly, among the hydroxysteroid dehydrogenases, only dhs-19 RNAi showed an increase in undeveloped animals upon treatment with TAM (Fig. 3.4b). In contrast, RNAi of FA elongation genes showed varying degrees of increased drug toxicity. All but elo-7 RNAi showed a significant (p < 0.001) increase in undeveloped animals (Fig. 3.4c). Our results suggest that FA elongation may protect against TAM toxicity. We wondered whether FA synthesis also contributes to TAM response. We tested several RNAi clones against genes involved in FA synthesis. RNAi knockdown of the fatty acid desaturases fat-5, fat-6 and fat-7 led to increased TAM toxicity (Fig. 3.4d).
FIGURE 3.4: Fatty acid synthesis and elongation decreases TAM toxicity. Dose-response curves for genes involved in related pathways to the screen hits: glycolysis/gluconeogenesis (a), hydroxysteroid dehydrogenation (b), FA elongation (c) and synthesis (d). Data shown is the mean and standard deviation of two independent experiments, each with twelve technical replicates.

One explanation for the observed gene-specific effects of dhs-19 and pck-2 RNAi on TAM response is direct or indirect changes to drug uptake or metabolism. To test this, we measured TAM in developed animals exposed to 375 µM TAM for twelve hours. Neither dhs-19 nor pck-2 RNAi led to changes in the
amount of TAM observed (Fig. 3.5), suggesting that changes in drug bioavailability or metabolism are not related to the effect of dhs-19 or pck-2 on TAM response.

**FIGURE 3.5: TAM levels do not change upon pck-2 or dhs-19 knockdown.**
TAM measurement by GC/MS in animals treated with 375 µM TAM for twelve hours, shown is the mean and standard deviation of three independent experiments; ns: not significant by paired t-test.

**Bacterial fatty acids influence TAM toxicity**

Both FA synthesis and elongation seem to protect against TAM toxicity. These results suggest that either the production or the utilization of FAs may be important in driving TAM toxicity. To test whether FAs are directly involved in TAM toxicity, we tested whether supplementation of a commercial FA cocktail could rescue TAM toxicity.
FA supplementation rescued TAM toxicity under both *Comamonas* diet and metabolic gene RNAi (Fig. 3.6). We reasoned that changes in FA composition in may be responsible for TAM toxicity. To avoid confounders caused by severe TAM toxicity, we analyzed FA composition in developed animals treated with a mild dose of TAM (150 µM) for twelve hours (Fig. 3.7). We observed a decrease in the monomethyl branched chain FA C17ISO upon elo-6 RNAi (Fig. 3.8), as has been documented before [107]. However, we did not observe any differences in FA composition between the TAM-treated and untreated conditions (Fig. 3.8).
FIGURE 3.7: Experimental design for metabolomics after TAM treatment. Animals were grown from in each bacterial diet and, after approximately 48 hours, when the animals reached the L4 stage, they were transferred to plates with the same bacterial diet but with or without TAM. After twelve hours of drug exposure, animals and their bacterial diet were collected.

FIGURE 3.8: Fatty acid measurements after TAM treatment and elo-6 RNAi. FA measurement by GC/MS in neutral lipids (a) and phospholipids (b) in animals treated with (+) or without (-) TAM for twelve hours. Shown is the mean and standard deviation of three independent experiments. Dashed line indicates 5% of the total fraction, values below this threshold are not shown. The experiment was performed as described in Fig. 3.7.
Since FA supplementation also rescues TAM toxicity in animals fed *Comamonas*, we sought to measure FA composition in animals treated with TAM and fed either *E. coli* or *Comamonas*. First, *E. coli*, but not *Comamonas*, contains cyclopropane FAs and provides them to *C. elegans*, where they may be used for storage, as evidenced by their enrichment in neutral lipids over phospholipids (Fig. 3.9a-b). These unique bacterial FAs are generated from unsaturated fatty acids by the enzyme cyclopropane fatty acid synthase (CFA) when cells enter stationary phase [109]. We wondered whether cyclopropane FAs could explain the differences in host drug toxicity, between *E. coli* and *Comamonas*. *E. coli* Δcfa showed no difference in the host response to TAM, relative to WT (Figure 3.9d). Therefore, the presence of cyclopropane FAs in *E. coli*, and in animals fed *E. coli*, do not explain the differences in response to TAM.

We turned our attention to fatty acids present in *Comamonas*-fed, but not *E. coli*-fed animals. Interestingly, we discovered a significant increase in the mono-unsaturated FA (MUFA) cis-vaccenic acid (C18:1n7), both in neutral lipids and phospholipids, in animals fed *Comamonas*, independent of TAM (Fig. 3.9a-b). Surprisingly, fatty acids composition of the bacterial diet was slightly different than in the animals. *Comamonas* shows a TAM-independent increase in the MUFA C16:1n7, but not C18:1n7, relative to *E. coli* (Fig. 3.9c). Therefore, *Comamonas* supplies dietary C16:1n7, which turns to C18:1n7 in *C. elegans*. One study using isotope-labeled bacteria suggests that most of the C16:1n7 and C18:1n7 FAs found in *C. elegans* are bacteria-derived, not synthesized de novo [101].
FIGURE 3.9: *Comamonas* changes fatty acid composition in *C. elegans*. FA measurement by GC/MS in neutral lipids (a) and phospholipids (b) in animals treated with (+) or without (-) TAM for twelve hours or in bacteria (c) under the same conditions. Dashed line indicates 5% of the total fraction, values below this threshold are not shown. The experiment was performed as described in Fig. 3.7.

Data shown is the mean and standard deviation of three independent experiments.

(d) Dose-response curve for *E. coli* ∆cfa strain, which cannot synthesize CFAs.
Our results suggest that *Comamonas* has higher MUFAs than *E. coli*, and these differences are reflected in the host. FAs are used for energy storage, signaling, and as the backbone for biological membranes; thus, changes in FA composition can impact physiology. For instance, in the homeoviscous adaptation model of cell membranes, increased FA saturation is linked to increased membrane fluidity [110,111]. Dynamic control of membrane fluidity allows maintenance of cellular membranes upon changes in temperature. In cold temperatures, membranes shift into a more rigid state, and the inverse is true of hot temperatures. In *C. elegans*, the FA desaturase *fat-7* gene is significantly upregulated in response to cold temperatures and leads to increased polyunsaturated FAs (PUFAs) [112]. In contrast, upon hot exposure, *fat-7* is downregulated [113]. Interestingly, when we compare membrane fatty acid saturation in animals fed the two bacteria, we observed a significant increase in MUFAs and the absence of cyclopropane fatty acids (CPAs). Taken together, animals fed *Comamonas* could have increased membrane fluidity, which could impact TAM toxicity. To test whether changes in membrane fluidity, resulting from different dietary inputs from bacteria, are related to the observed differences in TAM response, we grew animals in different temperatures and treated with TAM. Different temperatures led to bacteria-dependent changes in TAM toxicity. In *Comamonas*-fed animals, lower temperatures led to decreased toxicity of TAM, almost phenocopying *E. coli*-fed animals grown at physiologic (20°C) temperatures. In contrast, *E. coli*-fed animals showed increased TAM toxicity when
grown at higher temperatures (25°C). Lower temperature decreases TAM toxicity in *Comamonas*-fed animals while higher temperature increases TAM toxicity in *E. coli* (Fig. 3.10).

**FIGURE 3.10:** Membrane composition and temperature change TAM toxicity. (a) Fatty acids in phospholipid extracts, from Fig. 3.9a, grouped based on saturation. mmBCFAs: monomethyl branched-chain fatty acids, CPAs: cyclopropane fatty acids (b) Dose-response curves for animals grown in different temperatures and treated with TAM. All data shown is the mean and standard deviation of two independent experiments, each with twelve technical replicates.
CONCLUSIONS

Bacteria-dependent TAM toxicity

Our results suggest two mechanisms by which bacteria modulate TAM toxicity: (1) changes in drug bioavailability and (2) dietary supplementation of fatty acids (Fig. 3.11).

![Diagram of bacteria-dependent TAM toxicity]

**FIGURE 3.11: Model for bacteria-dependent TAM toxicity**

We identified four *Comamonas* transporter mutants with different effects on TAM toxicity in *C. elegans*, suggesting that drug bioavailability is a major mechanism by which bacteria modulate toxicity (Fig. 3.2a). Interestingly, none of the *Comamonas* mutants led to changes in host drug response to 5-FU (Fig. 3.2d), a drug with a different mechanism of action than TAM, suggesting that their effect is specific. This was especially surprising for the *acrR* mutant, since it is involved in the regulation of a multi-drug efflux pump. The absence of *E. coli* mutants with
an effect on TAM toxicity, including mutants in the *Comamonas* orthologs (Fig. 3.2b), as well as the absence of growth defects in the presence of TAM (Fig. 3.2c), suggest that *E. coli* may be effectively impermeable to TAM. In *C. elegans*, since bacteria influence drug delivery in solid media [114], *E. coli*’s limited drug uptake could translate into decreased drug bioavailability. Addressing drug accumulation in animals fed the two bacterial diets is challenging since dosing and exposure time are important constraints. In fact, when supplemented at doses where both *E. coli*- and *Comamonas*-fed animals experience developmental delay, there is no difference in the amount of TAM available in animals fed each bacterial species (data not shown). However, *E. coli*-fed animals must have some drug exposure since we were able to perform a metabolic gene RNAi screen. The background strain for RNAi experiments is *E. coli* and, since we identified several RNAi clones leading to increased drug efficacy, we can conclude that: (1) when fed *E. coli*, enough TAM enters the animal and (2) decreased drug bioavailability is not the only mechanism by which *E. coli* modulates TAM toxicity since we can overcome its protective effects by metabolic gene RNAi knockdown.

**Role of FA metabolism in TAM toxicity**

In addition to changes in drug bioavailability, we have several results suggesting a role of fatty acid metabolism in modulating TAM toxicity. First, we identified several *C. elegans* genes involved in fatty acid synthesis and elongation whose RNAi knockdown leads to increased TAM toxicity (Fig. 3.3a). Second, we revert TAM toxicity by supplementing a broad fatty acid cocktail (Fig. 3.6). Third,
we find that, although TAM treatment does not change fatty acid levels (Fig. 3.8 & Fig. 3.9), *Comamonas* supplies an increased fraction of MUFAs relative to *E. coli* (Fig. 3.9).

We reasoned that, by changing growth temperature of *C. elegans*, we could expose diet-dependent changes in membrane fluidity that may contribute to TAM toxicity. Specifically, cold temperature decreases membrane fluidity such that *Comamonas*-fed animals, with a higher membrane fluidity, could mimic *E. coli*-fed animals. Indeed, we observed that, when grown at 15°C, animals fed *Comamonas* phenocopied *E. coli*-fed animals in their response to TAM (Fig. 3.10). Furthermore, the reverse is also true: when grown at higher temperatures (25°C), *E. coli*-fed animals phenocopied *Comamonas*-fed animals’ TAM response (Fig. 3.10). Therefore, growth temperature has a bacteria-dependent effect on TAM response, likely through modulation of membrane fluidity. It would be interesting to measure whether there are differences in fatty acid levels between animals fed different bacteria and grown at different temperatures.

How fatty acid saturation and membrane fluidity relate to TAM toxicity may be related to the drug’s chemical structure. *In vitro*, TAM has been associated with changes in lipid membrane physicochemical properties [115-117]. The result of TAM interacting with lipid membranes does not simply mimic cholesterol; it is driven by drug localization in the inner or outer layers of the membrane and by drug concentration (Khadka *et al* 2015). According to patient data gathered by the FDA, side-effects observed in humans taking the maximum tolerated doses
include reversible neurotoxicity and changes to cardiac rhythms, but there are no conclusive mechanistic studies that characterized these side effects. Our results suggest that increased membrane fluidity increases TAM toxicity: *Comamonas* diet provides more MUFAs, but no cyclopropane fatty acids, (Fig. 3.9) and is associated with increased TAM toxicity. Decreasing membrane fluidity, by feeding cyclopropane fatty acids in *E. coli* or by decreasing growth temperature, rescues TAM toxicity.

Overall, we have identified an off-target effect for the commonly used compound TAM. In *C. elegans*, TAM disrupts fatty acid metabolism and its toxicity can be significantly modulated by changes in dietary fatty acids or growth temperature. It is worth noting that, given the broad utility of TAM as a research tool, these findings should be tested in cell lines or animal models using this drug.
CHAPTER IV: Discussion

PREFACE

Portions of the section “Challenges and limitations of C. elegans as a model host”, as well as Fig. 4.2, have been adapted from: García-González AP, Walhout AJM (2017) Worms, bugs and drugs: Caenorhabditis elegans as a model for host-microbe-drug interactions. Curr. Opin. Sys. Biol. 6, 46-50. Some content on the use of bacterial mutant collections in the section “Improving and expanding the C. elegans-bacteria-drug model” was adapted from the following submitted manuscript: Diot C, García-González AP, Walhout AJM. C. elegans and its bacterial diet: an interspecies model to explore the effects of microbiota on drug response.

DISCUSSION

Microbial xenobiotic metabolism: challenges and opportunities

Reports on microbial metabolism leading to severe drug toxicity highlight the importance of identifying when and how such interactions occur [23,24]. However, given the hundreds of species inhabiting our microbiota and the several thousand drugs currently in clinical use, it may be worth narrowing the search space. Many drugs demonstrate a high inter-individual variability that cannot be completely explained by genetic differences in drug pharmacokinetics, or pharmacogenomics [118,119]. Whenever variations in patient response to
medications cannot be explained by pharmacogenomics, microbial metabolism may be at play. Therefore, drugs can be prioritized based on inter-individual variability, and high toxicity or narrow therapeutic range. Constraining the search space for microbes may rely on maximizing functional divergence, such that, microbes with the most variable metabolic capacities can be tested as representatives of microbial contributions to host drug response. A different approach could consider medication-induced dysbiosis, where the composition of the microbiota changes in patient cohorts receiving drug treatment. While antibiotics have received much attention, other host-targeted drugs have also been associated with dysbiosis, with unknown consequences for the host [7,120]. In one study of the TwinsUK cohort, 19 out of 51 medications showed significant differences in gut microbiota composition between patients in the untreated and treated arms [7]. Studies that have characterized medication-induced dysbiosis can provide a list of candidate microbes that are enriched or depleted with drug therapy, and these microbes can be individually tested for their role in host drug response.

With a defined search space, one possible workflow for characterizing host-microbe-drug interactions is as follows: (1) identify drug and microbial subsets suitable for testing, (2) use an animal model with high scalability, such as *C. elegans*, as a first-line tool to identify interactors and (3) test positive interactions using germ-free and monoxenic mice. Once host-microbe-drug interactions are characterized, the next step is to improve personalized medicine by manipulating
the microbiota to improve drug therapy. In an extreme case, fecal microbiota transplants can help reconfigure the gut microbiota to enrich microbial communities that help maximize drug response or avoid adverse drug reactions. Alternative interventions include: (1) modulating microbial communities with “beneficial” strains in probiotics and (2) ingesting compounds that provide metabolic fuel for the gut microbiota with “prebiotics”. The use of pre- or probiotics is not currently approved by the Food and Drug Administration and has not been tested for improving patient drug responses. However, the use of probiotics is documented to improve inflammatory or infectious diseases, as well as other health outcomes [121-123]. In a different, albeit more complex, scenario, drugs targeting microbial enzymes could be co-administered to improve drug response. For example, bacterial β-glucuronidases have been shown to activate the chemotherapeutic drug irinotecan, resulting in toxicity [32]. Indeed, co-administration of irinotecan with a bacterial β-glucuronidase inhibitor decreases toxic side effects associated with the drug [137]. A following study demonstrated that bacterial β-glucuronidase inhibitor co-administration reduces toxicity of several non-steroidal anti-inflammatory agents in mice [40].

**Improving and expanding the C. elegans-bacteria-drug model**

The project outlined here provides evidence to support the use of *C. elegans* as a suitable host for the large-scale interrogation of host-microbe-drug interactions. The results I describe also highlight the benefits of a genetically tractable and monoxenic microbiota. By design, our model system not only
identifies the causal organisms responsible for host-microbe-drug interactions, but also identifies the molecular mechanisms involved. Despite only characterizing a limited number of drugs, our results show a variety of mechanisms through which bacteria can modulate host drug responses (Fig. 4.1). For the anti-pyrimidine drugs 5-FU and FUDR, I found two mechanisms that modulate host drug response: (1) bioactivation into FUMP and (2) supplementation of pyrimidines. For CPT, we found bacterial nucleotide metabolism networks that contribute to drug efficacy. Lastly, for TAM, we found two bacteria-dependent mechanisms that independently contribute to drug toxicity: (1) drug sequestration by bacteria, which influences bioavailability in the host, and (2) dietary fatty acids.

Two out of four mechanisms we identified involve metabolites from bacteria that are utilized by the host. One important consideration is whether these metabolites are drug-dependent, do they change upon exposure to drug? One experiment, comparing drug responses between animals fed live bacteria to animals fed a metabolically inactive bacteria powder, suggests that bacterial metabolism does not change drug response to CPT. We later discovered that bacterial nucleotide metabolism networks may contribute to host response to CPT. In addition, TAM treatment did not change bacterial fatty acids. Taken together, endogenous nucleotides provided from bacteria to the host contribute to the observed differences in drug response to CPT and TAM between E. coli and Comamonas. A preferred alternative for identifying nutritional or microbiota contributions would be to use an axenic media, where metabolites can be
integrated and host drug responses compared against animals with a microbiota. Several chemical formulations exist for feeding *C. elegans* without the presence of bacteria. One notable challenge in the use of a chemically-defined media involves the requirement of particles in order to sustain *C. elegans* growth [138]. For identifying host-microbe-drug interactions, utilizing axenic media could limit the accessibility of drugs to the animal. Liquid culturing could improve drug absorption in *C. elegans* fed axenic media and was shown to lead to greatest drug absorption inside the animal [114].

**Figure 4.1: Mechanisms of host-microbe-drug interactions characterized.**

In order to improve the use of *C. elegans* as a model system for host-microbe-drug interactions, it is worth considering each dimension: host and microbe. *C. elegans* has several advantages as a model host, many of which were not explored here. Most notably, there are abundant genetic tools for characterizing the host contribution to the observed host-microbe-drug interactions. Genome-wide RNAi knockdown libraries [124], as well as a collection
of genome-sequenced wild isolates [125], allow for careful dissection of host
genetic factors driving drug responses. As for microbiota, our project explored two
bacterial species, for which genetic tools were available. Testing each of the two
bacterial collections against four drugs in total revealed an important limitation of
the primary genetic screens: high false positive and false negative rates. The work
described in Chapters II & III addressed high false positive rates by three
successive tests of primary hits until a high-confidence set was identified. We were
surprised to find a high rate of false negatives in our screen: upon identifying
bacterial pathways enriched in our set, we discovered many missing mutants that,
upon testing, also led to changes in host drug efficacy. The technical design of
each bacterial genetic screen could explain a high false negative rate; we
performed each primary screen with only one biological replicate and one technical
replicate. Our best estimates of false positive and false negative rate using control
bacteria ranged between one to five percent, but the results from the screen
strongly support a significant underestimation. One solution to decrease high false
positives is to increase the number of biological replicates in each screen.

It is worth expanding on the set of bacteria tested, provided that they
support C. elegans development. Given the utility of bacterial genetic tools, one
strategy could be to include bacteria for which mutant collections are already
available, like: Acinetobacter baylyi [126], Burkholderia thailandensis [127],
Klebsiella pneumoniae [128], Pseudomonas aeruginosa [129], Staphylococcus
aureus [130] and Bacillus subtilis [131]. Our results with a limited number of drugs
also revealed important trends in the effect of microbes in host drug response. For instance, in Chapter II, we identified twenty-three bacterial mutants affecting all three drugs, CPT, 5-FU and FUDR (Fig. 2.6). These bacterial mutants could be used in the future as a control for drug non-specific microbe-drug interactions. Similarly, mutants involved in drug transport, like those identified in Chapter III could serve as a preliminary test for the role of drug bioavailability in driving the observed host-microbe-drug interactions.

Improving the use of C. elegans as a first-line tool for characterizing host-microbe-drug interactions also involves identifying human homology with the mechanisms we uncover. Our results can provide testable hypotheses to be pursued in mammalian model systems. In Chapter II, I propose a mechanism of action through ribonucleotide metabolism for the anti-pyrimidine drugs 5-FU and FUDR, partly based on the role of the bacterial gene upp. Work in human cell lines showed that inhibition of UPRT, the human ortholog of upp, also leads to drug resistance to 5-FU [132]. The role of upp in drug resistance to 5-FU in human cells suggests that ribonucleotide metabolism may be a conserved mechanism of action of anti-pyrimidines (Fig. 4.2).
Figure 4.2: Pyrimidine synthesis in bacteria, C. elegans, and human. (Top) Bacterial pyrimidine synthesis through the salvage pathway, which can also convert 5-FU, or the de novo pathway, which utilizes glutamate to produce uracil monophosphate. (Bottom) Human, C. elegans and E. coli orthologs for genes involved in 5-FU bioactivation. Abbreviations: Gln = glutamine, UMP = uracil monophosphate, dUR = deoxyuridine, dTMP = deoxythymidine monophosphate.
Challenges and limitations of *C. elegans* as a model host

Several model systems attempting to characterize host-microbe interactions have emerged, each with advantages and disadvantages. *C. elegans* offers unparalleled scalability and genetic tools. One limitation in using *C. elegans* includes the technical challenges of testing human microbiota. *C. elegans* grows aerobically, and many human gut microbes grow anaerobically. A recent study overcame this technical limitation by first growing the human gut bacteria *Raoultella planticola* anaerobically in the presence of drug, before being consumed as food (aerobically) by *C. elegans* [133]. Interestingly, the experimental design of the aforementioned study highlights one feature of the *C. elegans*-bacteria-drug model: interactions between bacteria and drugs can occur *ex vivo* and manifest themselves in the host once the bacteria has been consumed. Further validation with metabolically inactive bacteria can help resolve dietary or microbiota effects.

The human gut microbiota is made up of a rich microbial community, where organisms co-exist and influence each other. In all previous studies using the *C. elegans*-bacteria-drug model, host-microbe-drug interactions are only tested using individual bacteria. While it facilitates mechanistic characterization, testing individual microbes does not mimic a true microbiota, and will not identify microbe-drug interactions that require microbial metabolic exchanges. For example, bacterial communities within the human gut have an important role for the anti-bacterial protonsil. When tested against individual bacteria, protonsil does not have anti-bacterial properties *in vitro*. *In vivo* studies, however, showed strong
antibacterial effects. Further studies found that the microbial transformation of protonsil by bacterial azo-reductases is responsible for the bioactivation of the antibacterial agent \textit{in vivo} [134,135]. In brief, bacteria can convert compounds that in turn affect other microorganisms within the microbiota.

A strength in using \textit{C. elegans} as a model host derives from the extensive genetic tools available. Considering host-microbe interactions, tools for gene knockdown are limited only to \textit{E. coli} strains. Comparing the effects of gene knockdown in the background of two different \textit{E. coli} strains led to differences in animal physiology [136], suggesting that expanding RNAi-compatible bacterial diets is a rich unexplored source of host-microbe interactions.
APPENDIX: Expanding the host-microbe-drug model

PREFACE

The contents of this chapter are unpublished. I performed all preliminary experiments to optimize the screen conditions, as shown in Fig. 5.1 and Fig. 5.2 with technical help from Yomari Rivera. I later performed the screen alongside Cédric J.P. Diot.

APPENDIX

The *C. elegans*-bacteria-drug interspecies model offers two significant advantages: genetic tractability of host and microbe and scalability of the host for high-throughput screens. The contents presented in Chapters II & III exemplify the strengths of genetic tractability: we can use genetic screens in host and microbe (or both) to characterize the mechanisms behind host-microbe-drug interactions. However, the second listed advantage, scalability for high-throughput screens, is almost neglected. To address this, I designed a large-scale screen of host-microbe-drug interactions. This involved three steps: (1) validating new bacterial diets for *C. elegans*, (2) identifying a set of compounds that maximize diversity in mechanisms of action and chemical structures and (3) optimizing the design of the screen to minimize technical noise.

First, to validate new bacterial diets for *C. elegans*, I explored three categories of bacteria: (1) aerobic and non-pathogenic human microbiota, to enhance the utility of the model system to identify relationships pertinent to human
microbiota; (2) bacteria co-isolated with *C. elegans* in the wild and shown to be ‘beneficial’ to animal development [46], since support normal animal physiology and could still provide insights into host-microbe-drug interactions; and (3) laboratory strains selected for their biomedical relevance and regularly tested in *C. elegans*, since these strains are important for human diseases and have extensive physiologic characterizations for *C. elegans* maintained on these diets. Select bacteria were tested against four growth media (Luria Broth, Nutrient Broth, Nematode Growth Media, Brain Heart Infusion Media and De Man, Rogosa and Shape Broth) and three growth temperatures (25°C, 30°C, and 37°C) to identify a set of growth conditions with maximal growth. Each combination of medium and temperature was tested in duplicate using a plate reader to measure absorbance. This allowed us to identify growth conditions and a time to stationary phase. To standardize growth conditions, we prepared bacterial glycerol stocks at different densities for each strain such that, when diluted at 1:1000 in the preferred growth medium and temperature, each culture would reach stationary phase no later than twenty-four hours later. Once bacterial growth conditions were optimized, animal feeding conditions were tested. Since bacteria may have different growth rates after plating on NGM agar, we tested different bacterial concentrations such that we could support animals for about three days before food became scarce. Optimal feeding concentrations ranged from one in fifty thousand dilution to fifty-fold concentrations. During these experiments, we tested L1 stage and L4 stage animals for development and reproduction, respectively, when fed each diet.
Interestingly, out of twenty human microbiota isolates tested only six supported *C. elegans* development, while eight supported reproduction. Several hypotheses emerged following our observations. For instance, why would two strains support animal reproduction but not development? One possibility is bacterial density or cell size, if bacteria are too big or their lawns are too dense, they may become functionally inaccessible for smaller larvae. Additionally, why do most human isolates not support *C. elegans* development nor reproduction? Since we also introduced different bacterial growth media, we re-tested each bacterial strain after washing three times with M9 buffer. None of the bacterial strains gained the ability to support animal development or reproduction suggesting that bacterial growth media was not responsible for inadequate nutrition. Broadly, bacteria could fail to support *C. elegans* development for two reasons: they are missing important nutrients or they produce toxic compounds. To differentiate between the two broad possibilities, one could mix a beneficial diet with a query diet. If animal development is rescued by supplementing the beneficial diet, nutritional deficiencies are likely responsible for the inability of the query diet to support animal development. In contrast, if animal development cannot be rescued by supplementing a beneficial diet, an active toxicity from the query diet is likely.

An important consideration is whether including additional bacteria in the screen will significantly improve information content. For instance, does testing *E. coli* and *Comamonas* provide enough information about the distribution of host drug responses and the mechanisms involved? Intuitively, a sampling of two
bacteria will not provide a comprehensive picture of host drug responses. In addition, many bacterial species within the microbiota are responsible for the known examples of microbial metabolic functions associated with changes in host physiology [21]. To test whether increasing the number of bacteria in the screen will improve information content, we tested the five drugs for which we previously observed abnormal phenotypes against twenty-two bacteria. We evaluated drug response as the minimum inhibitory concentration (MIC), the smallest dose required to inhibit *C. elegans* development, and we clustered the distribution of MICs in animals fed *E. coli* or *Comamonas*, or both. We discovered that for all five drugs there are at least two bacterial strains whose effect on host drug response was not captured by *E. coli* nor *Comamonas* (Fig. 5.1). For three out of the five drugs animals fed *E. coli* and *Comamonas* cluster with the same MIC. Between two and eleven bacteria showed different host drug responses that were not otherwise captured. Interestingly, we observed one bacterial strain that caused increased host drug resistance to FUDR than *Comamonas*. Therefore, testing more bacterial strains is informative of broader distributions of host drug responses.
FIGURE 5.1: Pilot screen with published drugs against twenty-two bacteria. Bubble chart of drug MIC clusters when animals are fed any of the twenty-two bacteria. PAC: paclitaxel; max: animals did not show an abnormal phenotype at the highest dose tested.

Next, we sought to develop a robust protocol for testing host-microbe-drug interactions at high throughput. This required technical troubleshooting of two parameters: bacterial growth conditions and design of the screen. First, we performed bacterial growth curves for an expanded set of twenty-six bacteria to determine the optimal temperature, media and time to reach stationary phase (data not shown). Second, we evaluated drug dosing, multi-well plate layout, and the optimal number of technical and biological replicates. An increased number of wells in multi-well plates could improve throughput but compromise robustness and increase technical noise. In fact, a pilot test of five drugs and doses in 96-well plates revealed a false positive rate (observed drug resistance) could reach up to
twenty-five percent. This led to the selection of 48-well plates as a suitable scale for optimizing throughput while minimizing technical robustness. When we tested technical reproducibility in 48-well plates, we observed less than two percent false positive rates for five drugs tested. Despite the low false positive rate, a large number of technical replicates is required to account for a one to five percent error rate; errors include fungal contamination of the well or failure to seed bacteria or animals. After further testing, we optimized the design of the screen as described in Fig. 5.2.

FIGURE 5.2: High-throughput host-microbe-drug interactions screen

The screen was performed in 48-well plates where each plate contained five doses of each of two drugs, and a vehicle control, against one bacterial strain, each condition tested in quadruplicate. As was described in all previous experiments, animals from previous generations were fed the standard laboratory diet, *E. coli* OP50. Once animals reached adulthood, their progeny were isolated
using hypochlorite solution and synchronized overnight. L1 larvae were then placed in the 48-well plates containing drug media and bacteria. After 72 hours, we captured organismal phenotypes like survival, development, and fecundity. We tested approximately fifty drugs against over twenty bacteria for host-microbe-drug interactions. Over thirty percent of the drugs tested led to abnormal phenotypes in animals fed at least one bacterial strain. A first step in analyzing the observed host-microbe-drug interactions will include transforming drug phenotypes into matrices that can be clustered based on similarity between drugs, to identify trends in structurally or mechanistically similar compounds; or clustered based on phenotypes observed between bacterial strains to test whether phylogenetic relationships account for the observed similarities or differences in each strains effect on host drug responses. Quantifying drug responses according to minimum inhibitory concentrations (MICs), where animals fail to develop, offers a simple way to collect quantitative information from the large screen. A preliminary analysis including three \textit{E. coli} strains, four \textit{Buttiauxella} strains and three \textit{Enterobacter} strains, revealed significant differences in host drug response profiles between closely-related strains suggesting that slight genetic differences between bacteria can impact host-microbe-drug interactions. Host-microbe-drug interactions identified in the screen will be characterized in the near future utilizing genetics and targeted metabolomics as illustrated in Chapters II & III.
BIBLIOGRAPHY


