DOXORUBICIN INACTIVATION BY THE HUMAN GUT MICROBIOME

IDENTIFYING DRUG-MICROBIOME INTERACTIONS:

THE INACTIVATION OF DOXORUBICIN

BY THE GUT BACTERIUM *RAOULTELLA PLANTICOLA*

By AUSTIN YAN, B. Arts Sc.

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McMaster University
Department of Biochemistry and Biomedical Sciences
Hamilton, Ontario, Canada

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AUTHOR: Austin Yan, B. Arts Sc. (McMaster University)

SUPERVISOR: Dr. Gerard D. Wright

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Lay Abstract

The collection of microbes in the human intestinal tract, referred to as the gut microbiome, can modify therapeutic agents and change the efficacy of drug treatments. Identifying these interactions between drugs and the microbiome will help the study of drug metabolism, provide explanations for treatment failure, and enable more personalized health care. For this project, a platform was developed to isolate gut bacteria from human fecal samples and characterize bacteria that are capable of inactivating various antibiotics and anticancer drugs. Through this platform, the gut bacterium *Raoultella planticola* was found to inactivate doxorubicin, a commonly used anticancer drug. These results suggest that doxorubicin may be inactivated in the gut and demonstrates how this platform can be used to identify drug-microbiome interactions.

Abstract

The human gut microbiota contributes to host metabolic processes. Diverse microbial metabolic enzymes can affect therapeutic agents, resulting in chemical modifications that alter drug efficacy and toxicology. These interactions may result in ineffective treatments and dose-limiting side effects, as shown by bacterial modifications of the cardiac drug digoxin and chemotherapy drug irinotecan, respectively. Yet, few drug-microbiome interactions have been characterized.

Here, a platform is developed to screen for drug-microbiome interactions, validated by the isolation of a gut bacterium capable of inactivating the antineoplastic drug doxorubicin. Two hundred gut strains isolated from a healthy patient fecal sample were cultured in the presence of antibiotic and antineoplastic drugs to enrich for resistance and possible inactivation. *Raoultella planticola* was identified for its ability to inactivate doxorubicin anaerobically through whole cell and crude lysate assays. This activity was also observed in other Enterobacteriaceae and resulted in doxorubicin inactivation by the removal of its daunosamine sugar, likely mediated by a molybdopterin-dependent enzyme. Other potential drug-microbiome interactions were identified in this screen and can be analyzed further.

This platform enables the identification of drug-microbiome interactions that can be used to study drug pharmacology, improve the efficacy of therapeutic treatments, and advance personalized medicine.

Acknowledgements

This project would not be possible without my supervisor, Dr. Gerry Wright, who took on an Arts & Science student with limited computer or microbiology background and transformed me, project by project, into the database-curating, microbiota-culturing student I am today. Thank you for your lifelong commitment to meaningful mentorship, interdisciplinary learning, and keeping sticks on the ice.

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I have had the great pleasure of living in five different houses since joining the Wright lab and meeting the housemates who kept me sane by reminding me to sleep, going on adventures, and joining in house dinners even when conversations would inevitably trend towards fecal transplants. Among many: Jeremy, Steven, Yvonne, Glenda, Chris #3, Gabriel, Ken, Derek, Xing, and Rachel. (and Bubble Tea).

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Above all, I thank my parents and my lovely sister Janice for your unconditional love and patience for all that I do and your unrelenting encouragement for me to do my best, and then better. I dedicate this thesis, and everything else I do, to you.

"Then let us all do what is right, strive with all our might toward the unattainable, develop as fully as we can the gifts God has given us, and never stop learning."

Ludwig van Beethoven

Table of Contents

List of Figures

List of Tables

List of Abbreviations

ActD	actinomycin D
BBE	bacteroides bile esculin
BEEF	a rich media broth used for culturing gut isolates
BHI	brain heart infusion
BSA	bovine serum albumin
BSM	bifidobacterium selection media
CEF	cefalexin (cephalexin)
CFU	colony forming units
cPMP	cyclic pyranopterin monophosphate
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide phosphate
DOX	doxorubicin
EDTA	ethylenediaminetetraacetic acid
gDNA	genomic deoxyribonucleic acid
GTP	guanosine triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high-performance liquid chromatography
L-cys	L-cysteine
LB	lysogeny broth
LC/MS	liquid chromatography–mass spectrometry
kb	kilobase
MAC	MacConkey media
MIC	minimum inhibitory concentration
Moco	molybdopterin cofactor
MPT	molybdopterin
MRS	de Man, Rogosa, and Sharpe media
NAD^+	nicotinamide adenine dinucleotide (oxidized)
NADH	nicotinamide adenine dinucleotide (reduced)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
OD_{600}	optical density measured at a wavelength of 600 nanometres
PCR	polymerase chain reaction
RIF	rifampin
RNA	ribonucleic acid
rpm	revolutions per minute
rDNA	ribosomal deoxyribonucleic acid
subsp.	Subspecies
TMAO	trimethylamine N-oxide
TSB	trypticase soy broth
VAN	vancomycin
var.	varietas / variety

Declaration of Academic Achievement

Austin Yan conducted the majority of experiments, data collection, and analyses presented in this thesis. This report was also prepared by Austin.

Committee members Dr. Gerry Wright (supervisor), Dr. Mike Surette, and Dr. Brian Coombes, as well as Dr. Erin Westman, provided guidance and direction throughout the project.

Jennifer Lau performed the fecal sample preparations on various isolation media described in Section 2.3.2. Colonies were picked by Austin, who performed all subsequent culturing and screening.

Genome assembly outlined in Section 3.3.6 was performed by Austin under the guidance of Nicholas Waglechner.

Dr. Kalinka Koteva helped with running and analyzing samples run on the LC/MS and micrOTOF mass spectrometers as described in Section 4.2.8 and 4.7.1.

Preparation of the source plates and scanning of the doxorubicin screen for the KEIO collection in Section 5.2.4 was done by Dr. Jean Philippe Cote. Media preparation, replica pinning, and data analysis were performed by Austin.

Chapter 1.
Introduction: Identifying drug-microbiome interactions

1.1. The gut microbiota in human health and disease

The human gut microbiota consists of the diverse microbial species in the intestine, and has co-evolved symbiotically with its host (*1*,*2*,*3*). The gut microbiota outnumbers human cells ten to one, and is estimated to contain over a thousand different bacterial species with significant variation between individuals (*4*). These microbes affect human health, and are involved in host energy metabolism, brain development, and immune system maturation (*2*,*5*,*6*). A disturbed microbiota, or dysbiosis, has been implicated in a wide range of pathologies, including inflammatory bowel disease, irritable bowel syndrome, obesity, type-1 diabetes, colon carcinoma, and more recently, autism and cardiovascular disease (*2*,*7*,*8*,*9*,*10*).

Host-microbiota studies have ranged from identifying correlations between certain bacterial strains and disease phenotypes to *in vivo* microbiota transplants in animal models demonstrating that fecal bacterial populations or specific strains can induce disease or behavioural changes (*11*,*12*,*13*). The ability for the gut microbiota to affect host health has led to the labelling of these microbes as a "forgotten organ" and the overall host-microbiota system as a "superorganism" (*14*,*15*).

Rapid advances in the field have helped to shift research from correlations to cause-and-effect studies that are expected to carry significant clinical implications (*16*). These developments have come with advent of next-generation DNA sequencing and metagenomic tools that have helped to characterize the microbiome, increasing the ability to elucidate interactions between host and microbiota (*17*,*18*). The Human Microbiome Project, initiated in 2008, has enabled population-scale data collection of hundreds of adults at various body sites, including the gastrointestinal tract, to identify individual and population-level microbiota variations (*4*,*19*,*20*).

Ongoing efforts to characterize the gut microbiota aim to demystify the underlying mechanisms of the host-microbiota relationship, particularly in relation to metabolism and disease (*21*). Understanding these mechanisms will aid in the development of therapies including prebiotics and probiotics that can selectively target and modulate the microbiota to achieve desired clinical outcomes (*22*,*23*). Identifying these mechanisms will also provide a more comprehensive map of host-microbiota interactions for the development of more effective, personalized health care treatments (*8*,*24*). This project will focus on the ability of the gut microbiota to contribute to host metabolic processes, particularly in respect to the pharmacokinetics of therapeutic agents.

1.2. The modification of therapeutic agents by the gut microbiome

The combined genomes of the gut microbiota, referred to as the gut microbiome, exceeds the human genome by a hundred-fold, and results in many potential biochemical interactions in the host-microbiota relationship (*25*). These interactions enable the microbiota to play a key role in host nutrient metabolism by breaking down energy sources that are otherwise inaccessible to mammalian enzymes (*26*). As a result, over one third of all small molecules in the bloodstream are microbial metabolites (*27*). Though linking specific metabolites to unique strains of bacteria remains a challenge due to complexities in the host-microbiota relationship, certain microbes have been implicated in taurine metabolism, while the bacterial metabolism of choline was recently attributed to a specific gene cluster (*7*,*28*). Linking these mechanisms to specific strains or genes allow for a species-oriented or function-based approach, respectively, to the study of host-microbiome interactions, allowing for population-wide applications despite individual variations in the microbiota (*28*).

These metabolic interactions not only affect nutrients, but can impact therapeutic agents, where any unforeseen bacterial modification of drugs may lead to

undesired effects, such as reduced treatment efficacy or the generation of toxic products (*25*). The study of the interactions between drugs and microbes that affect a drug's efficacy, toxicity, and delivery is referred to as pharmacomicrobiomics (*29*).

In a comprehensive review of microbial metabolic reactions, Scheline suggested that "the gut gastrointestinal microflora has the ability to act as an organ with a metabolic potential equal to or sometimes greater than that of the liver" (*30*). Unlike the liver, which is involved in compound oxidation and conjugation, the microbiota is often involved in substrate reduction and hydrolysis (*30*). A 2006 metagenomic study by Gill *et al*. identified the enrichment of xenobiotic metabolic genes in the gut microbiome, as well as gene expression that could be induced by bile salts or xenobiotic metabolites (*31*). These metabolic reactions can occur when drugs are administered orally, often the preferred route for therapeutic agents, but even intravenously administered drugs may come into contact with the gut microbiota through blood circulation and diffusion (*32*). Drugs may also be secreted into the gut for excretion and recycled back into the bloodstream as a result of enterohepatic circulation (*32*). This recycling is due to competing liver and microbial enzymes that inactivate and reactivate therapeutic agents, respectively, lengthening the drug's half-life by allowing its reabsorption into the bloodstream (*33*).

The ability of the gut microbiota to metabolise drugs depends on the drugs' exposure to the bacteria-rich distal gut (*32*). Figure 1-1 shows the potential interaction of therapeutic agents with the gut microbiota through the human digestive tract. Drugs with high solubility and permeability are quickly absorbed into the bloodstream in the upper gut with minimal microbial interaction, while drugs absorbed slowly are exposed to significantly higher concentrations of microbes (*32*). Additionally, variations in microbiome composition also affect an individual's ability to metabolise drugs (*34*).

Knowledge of these interactions have allowed for the development of prodrugs that are specifically metabolized to an active form by microbial enzymes

(*35*). However, microbial drug modification often leads to unintended effects, such as drug inactivation, undesired enterohepatic circulation, or the production of toxic products (*18*,*25*). Some of these interactions are described in the following sections.

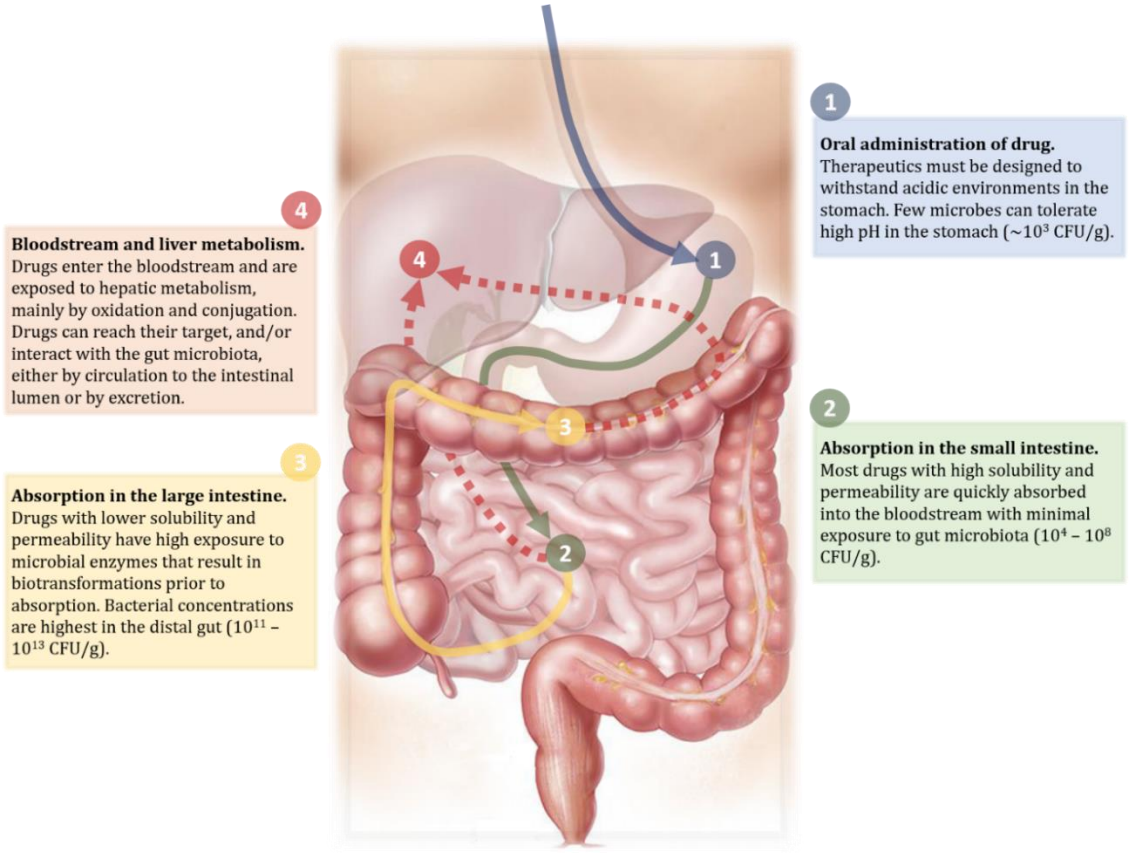
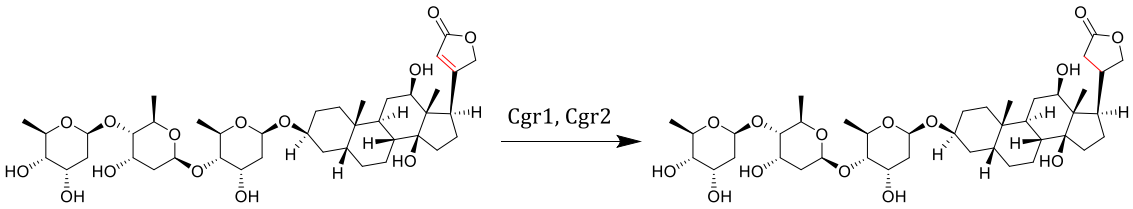


Figure 1-1: The biotransformation of therapeutic agents along the human digestive tract.

Orally administered drugs travel through the mouth, esophagus, (**1**) stomach, (**2**) small intestine, and (**3**) large intestine prior to absorption into (**4**) the bloodstream. To maintain bioactivity, these drugs must be designed to be soluble and stable in digestive fluids, and permeable to the intestinal lining. In addition, drug pharmacology must consider both liver and bacterial metabolism. Drugs administered intravenously may also have contact with liver and bacterial enzymes. The potential for microbiota-mediated drug modifications depends on the drug's exposure to bacteria, with highest levels present in the distal gut. Bacterial concentrations are noted in CFU/g (colony forming units per gram of contents), as reviewed by Sousa *et al* (*32*). Parts of this figure were adapted from "*Digestive appareil*" by www.cancer.gov and used under the Creative Commons Attribution-ShareAlike 4.0 International License (*36*).

1.2.1. The inactivation of digoxin by the gut bacterium *Eggerthella lenta*

The cardiac drug digoxin is among the best studied cases of microbiome-mediated drug inactivation. Digoxin is a drug with a narrow therapeutic range that was known to be ineffective in some patients; its inactivation by the gut microbiome was hypothesized as early as the 1960s (*37*). The bacterium responsible for this inactivation was first identified in 1983 as *Eubacterium lentum*, now referred to as *Eggerthella lenta* (*38*). Thirty years later, a study by Haiser *et al*. identified a two gene operon in *E. lenta* capable of reducing digoxin to its inactive form dihydrodigoxin, shown in Figure 1-2 (*37*,*39*). Through *in vivo* mouse model studies, the presence of this two gene operon in the gut microbiome was shown to significantly reduce the amount of active digoxin available to the mouse, and could explain digoxin inactivation in humans (*39*). Understanding the pharmacomicrobiomics of these therapeutic agents can thus provide a cause for varying patient outcomes, while helping to identify ways to improve treatment. For example, the same 2013 study also investigated the modulation of digoxin inactivation by diet, suggesting that higher arginine consumption could inhibit *E. lenta*-mediated drug reduction and potentially restore drug activity (*39*).



digoxin
(active)

dihydrodigoxin
(inactive)

Figure 1-2: Digoxin is inactivated by bacterial reductases in the gut microbiome.

The cardiac drug digoxin is reduced to dihydrodigoxin by Cgr1 and Cgr2 of the cardiac glycoside reductase operon in the gut bacterium *Eggerthella lenta*. Digoxin is rendered inactive by the saturation of a double bond in its lactone ring, highlighted in red. This mechanism was described by Haiser *et al*. in 2014 (*37*).

1.2.2. The reactivation of irinotecan by bacterial β-glucuronidases

Another example of undesired bacterial metabolism of a therapeutic agent is the reactivation of irinotecan, or CPT-11, a topoisomerase I inhibitor used as an anticancer drug (*40*). Irinotecan is administered as a prodrug converted to its active form SN-38 by mammalian carboxylesterases and is eliminated by hepatic glucuronidation, where liver glucuronosyltransferases conjugate glucuronic acid to SN-38 to produce the inactive SN-38G (*40*). However, Wallace *et al*. showed that gut bacteria expressing β-glucuronidases may utilize glucuronic acid as a carbon source, reactivating SN-38 and causing severe side effects including diarrhea, resulting in irinotecan's dose-limiting drug toxicity (*41*,*42*). The identification of this mechanism has allowed for the development of bacterial β-glucuronidase inhibitors that can be co-administered with irinotecan to overcome drug toxicity and manipulate host-microbiome interactions to improve chemotherapy treatments (*42*).

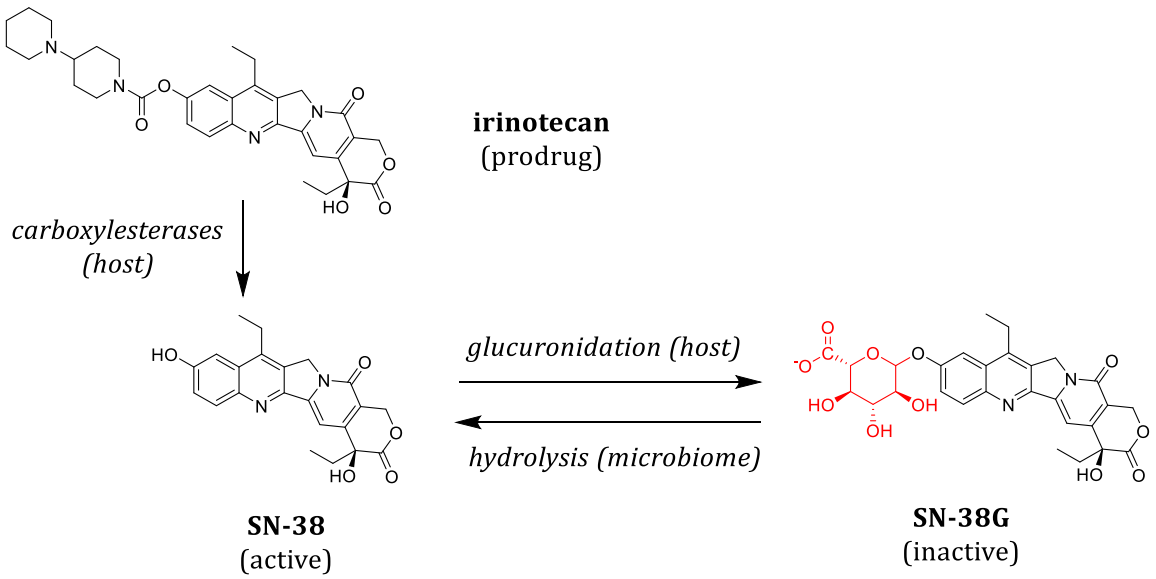


Figure 1-3: SN-38, derived from irinotecan, is reactivated by bacterial β-glucuronidases.

Irinotecan is administered as a prodrug and converted to its active form SN-38 by host carboxylesterases. Host liver enzymes inactivate the drug by the addition of glucuronic acid, shown in red. This reaction is catalyzed by glucuronosyltransferases that convert SN-38 to the inactive SN-38G for elimination. Bacterial β-glucuronidases can remove the added glucuronic acid by hydrolysis, resulting in the active SN-38 and causing dose-limiting side effects such as diarrhea (*42*).

1.3. The gut resistome is a source of drug inactivation genes

Unlike drugs like digoxin, antibiotics have the added effect of selecting against sensitive bacteria, thus potentially enriching the microbiota for inactivation mechanisms (*43*,*44*). Characterizing the gut resistome, the collection of antibiotic resistance genes found in the gut microbiome which includes drug-inactivating mechanisms, has been a key research aim in combating antibiotic resistance (*45*). The collection and prevalence of these drug resistance genes and their ability to be shared across the gut microbiota, aid in studying how drug-inactivating genes may influence pharmacomicrobiomics. A metagenomic study of 162 individuals identified 1093 antibiotic resistance genes by comparing gut microbiome genes to a known set of resistance genes from the Antibiotic Resistance Genes Database (*46*). Using these data, the prevalence of resistance genes can be compared across populations in different countries to identify region-specific genes responsible for antibiotic resistance and inactivation.

1.3.1. The inactivation of antibiotics

The challenges of widespread antibiotic resistance in the clinic has resulted in thorough investigations into mechanisms of antibiotic resistance (*47*). Among mechanisms including efflux, target protection, and target modification, drug modification is a significant contributor to antibiotic resistance (*48*). Drug inactivation is mediated by a wide range of bacterial enzymes that primarily inactivate antibiotics through hydrolysis, reduction, the transfer of chemical groups (i.e. acylation, phosphorylation, and glycosylation), or the activity of lyases (*48*). Many of these genes are disseminated across commensal bacteria and thus these gene products may be inactivating drugs in the gut (*49*). Understanding the gut resistome, mechanisms of drug inactivation, and the selection and transfer of antibiotic resistance elements will help in study of drug-microbiome interactions.

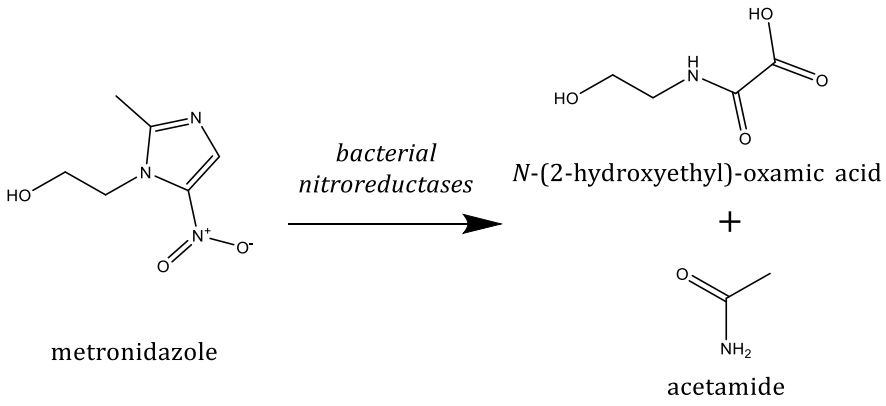


Figure 1-4: The antibiotic metronidazole is modified by bacterial enzymes.

Bacterial enzymes are capable of degrading antibacterial drugs. The antibiotic metronidazole is reduced by bacterial nitroreductases to an active form, before further reduction to *N*-(2-hydroxyethyl)-oxamic acid and acetamide, products only formed by bacterial inactivation (*50*).

The antibiotic metronidazole, for example, has been shown to be inactivated by the murine gut microbiota (*50*). While the drug is activated by bacterial nitroreductases to produce an intermediate highly reactive to DNA, the drug may be further broken down and inactivated by gut bacteria such as Enterococci and Clostridia to produce *N*-(2-Hydroxyethyl)-oxamic acid and acetamide, shown in Figure 1-4 (*50*,*51*). These experiments used mouse models to observe the presence of these metabolites in the feces and urine of microbiota-colonized mice that were absent in germ-free mice. These same metabolites have also been observed in human urine, indicating that a similar microbiome-mediated inactivation of metronidazole may occur in the human gut (*32*).

Most classes of antibiotics are reported to be inactivated by bacterial enzymes, including β-lactams, macrolides, aminoglycosides, rifamycins, tetracyclines, and streptogramins (*48*). Like metronidazole, some of the antibiotics may be inactivated by gut bacteria. Ampicillin, a β-lactam antibiotic, is inactivated by hydrolysis by β-lactamases (*52*). When mice were treated with ampicillin orally, there was an

increased prevalence of β-lactamase genes in the murine gut microbiome, suggesting that ampicillin is inactivated by gut bacteria (*53*).

Sommer *et al*. studied the gut resistome functionally, showing that fecal isolates cultured from two healthy individuals had widespread resistance to most antibiotics in a panel of thirteen drugs (*49*). Many of the genes responsible for resistance were antibiotic-inactivating enzymes, including β-lactamases, aminoglycoside-modifying enzymes, and chloramphenicol acetyltransferases. Approaches that study the microbiota by identifying resistance genes, instead of focussing on species-level interactions, recognize the effect of horizontal gene transfer that may also influence microbial drug inactivation (*54*). Yet, the impact of interactions between antibiotics and the gut microbiota *in vivo* is complex: while bacteria may be capable of inactivating antibacterial drugs, these drugs also place pressure on the gut microbiota by inhibiting bacterial growth (*18*). Though many studies have analyzed the effects of antibiotics on the gut microbiota, the influence of the gut microbiota on the efficacy of antibiotic treatments is not well characterized.

1.3.2. The activation and inactivation of antineoplastics

Microbial interactions with anticancer agents have been studied for the development of many cancer treatments; however, most of these interactions have focussed on activation of cancer prodrugs, such as anthocyanins, genistin, and naringin, which depend on bacterial β-glucuronidases to release the active drug (*25*). Compared to the undesired enterohepatic recycling of irinotecan described earlier, these drugs demonstrate that bacterial drug metabolism can result in both intended and unintended effects for drug pharmacokinetics.

Given that many antineoplastics have antibacterial activity due to similar targets in bacteria and rapidly dividing cancer cells, microbial metabolism similar to the mechanisms of antibiotic inactivation may also affect anticancer agents (*55*). The antineoplastic doxorubicin was shown to be inactivated by reduction, catalyzed by an

NADH (nicotinamide adenine dinucleotide) dehydrogenase of a soil-dwelling *Streptomyces* (*56*). This reaction is described in further detail in Section 3.2. The next step for these studies is to identify the degree of drug inactivation *in vivo* to characterize the pharmacomicrobiomics of antineoplastic drugs, helping to improve and personalize chemotherapy treatments.

1.4. The effect of antibiotics and antineoplastics on the gut microbiome

While microbial metabolism may affect various types of therapeutic agents, selective pressure is present when the drugs have antibacterial effects (*44*). These drugs thus have the potential to shape the microbiome, and selectively enrich for resistance mechanisms. Antibiotic therapies are known to cause dysbiosis, affecting microbiota populations by acting against certain groups of bacteria, reducing overall species diversity, and changing microbial metabolic phenotypes (*57*). An increased percentage of drug-resistant organisms during and after treatment have been reported for a wide range of antibiotics, including ciprofloxacin, tetracycline, ampicillin, and clindamycin (*43*,*53*,*58*). Some of these studies also demonstrate the long-term enrichment of resistance genes several years after antibiotic administration (*43*,*58*,*59*).

Antineoplastics also have a disruptive effect on the gut microbiota. Chemotherapy treatments may induce mucositis, which involves host-specific pathophysiology and the inflammation of the intestinal epithelium (*60*). Changes in microbiota composition are observed alongside intestinal inflammation, while antineoplastics may directly inhibit bacterial growth or alter microbial metabolic potential (*61*).

For instance, the antineoplastic chlorambucil, which inhibits tumour growth by cross-linking DNA as an alkylating agent, causes dysbiosis in the absence of antibiotic treatment and has been associated with *Clostridium difficile* infections,

traits similar to antibiotic therapy (*62*). Antineoplastics including 5-fluorouracil (5-FU), mitomycin, and etoposide have shown antibacterial effects at achievable plasma concentrations, and are also often used in combination with other anticancer agents and co-administered with antibiotics (*55*). Overall, the ability of these drugs to directly manipulate and apply selective pressure on the microbiome contribute to the complexity of elucidating drug-microbiome interactions, and should be considered when studying drug pharmacomicrobiomics, especially for chemotherapeutic agents.

1.5. Implications for health care and research

Pharmacomicrobiomics has exposed a web of interactions that appear increasingly complex, involving host genetics and epigenetics, metabolomics, and microbiomics (*24*). Even the role of the host-microbiota relationship is multilayered, involving microbiota-mediated effects that affect host gene expression, immune system response, and metabolite production. Two recent studies in *Science* have described the importance of the gut microbiota in promoting tumour response to cyclophosphamide treatment by manipulating the host immune system (*63*,*64*).

Patterson and Turnbaugh suggest that the microbiota plays a critical role in "biochemical individuality", with many implications for variations in treatment efficacy (*65*). The complexity of multiple host and microbial factors and the seeming unpredictability of drug pharmacology has led Nicholson and Wilson to describe therapeutic metabolism as 'Pachinko', or Japanese pinball, where probable outcomes and pathways steer the ball – the drug – through the host-microbiota system (*21*). In this comparison, factors including host metabolic pathways, bacterial enzymes, and transporters, each affected by single nucleotide polymorphisms and varied gene expression, all contribute to the drug's pharmacokinetics in a form of "probabilistic metabolism" (*21*). Despite these challenges, identifying drug-microbiome interactions help to determine these potential interactions that may increase drug toxicity or

decrease efficacy. Combined with a systems biology approach, researchers can map out the network of possible drug interactions (*21*,*26*).

Currently, aside from a few specific examples, the ability of the microbiota to reduce therapeutic efficacy has not been well characterized (*25*). For digoxin and metronidazole, *in vivo* models demonstrate that the presence of certain bacterial gut strains can reduce or eliminate drug efficacy. However, the gut microbiome has not been implicated in the pharmacology of most other drugs, even antibiotics like β-lactams with widespread inactivation resistance mechanisms. A study by Harmoinen *et al*. co-administered proteolysis-resistant recombinant β-lactamases with ampicillin to promote drug inactivation in the gut to reduce selective pressure on the microbiota (*66*). This approach, which successfully reduced antibiotic side effects such as dysbiosis and *C. difficile* infections, did not affect ampicillin concentrations in blood plasma. Thus, drug inactivation in the gut microbiome may have highly variable influence on drug pharmacology; identifying which interactions have an *in vivo* effect is critical for informing health care decisions and preventing unforeseen consequences.

An example of an unexpected drug interaction took place in 1993 with the antiviral drug sorivudine, which led to the deaths of eighteen patients in Japan before the drug was pulled from the market (*67*). The affected patients had taken both sorivudine and the previously mentioned cancer drug 5-fluorouracil (5-FU). Gut enterobacteria metabolised sorivudine to 5-(2-bromovinyl)uracil, an inhibitor of the host's 5-FU detoxification enzymes, causing death due to 5-FU accumulation (*67*,*68*).

Thus, developing a map for these microbial drug interactions is important for the pharmacokinetics research and its health care implications. Coupled with diagnostic tools to determine the presence of a gene or strain linked to drug inactivation, various responses can be used to address drug-microbiome interactions. First, alternative treatments can be considered, if available, to identify the optimal treatment for a patient given known pharmacokinetic interactions, similar to how

BRCA mutations that affect tumour sensitivity to certain therapies are used to guide treatments for ovarian cancer (*69*). Knowledge about possible drug inactivation and toxicology can also guide decisions for dosage to optimize efficiency and limit side-effects (*25*,*70*). Another solution is to develop inhibitors of bacterial metabolism, such as those identified by Wallace *et al*. to prevent irinotecan reactivation (*42*). Additionally, characterizing drug-microbiome interactions enables the development of treatments such as prodrugs that take advantage of bacterial metabolism, while prebiotic and probiotic therapies can be used to intentionally direct drug pharmacokinetics (*23*,*25*). Westman *et al*. also suggest the use of selectively inactivating or detoxifying drugs at non-target sites to eliminate side effects (*56*). In all, studying drug-microbiome interactions have many potential applications for health care and drug research and development.

1.6. Project objectives

This project has two main aims: to develop a platform for identifying drug-microbiome interactions and to use this platform to identify drug inactivation in the gut. A protocol was developed for identifying and comparing drug metabolism by the human gut microbiota, summarized in Figure 1-5. This project utilized gut strains isolated from healthy donor fecal samples prepared in collaboration with the Surette laboratory. These samples provided diverse populations of gut microbes, which were expected to have varying metabolic capabilities. Drugs of interest in this study included a panel of antibiotics and antineoplastics, to survey the gut microbiota's ability to harbour and utilize a wide range of genes capable of metabolising various drugs. This project identified general drug resistance patterns among the gut strains and characterized a specific drug-metabolism mechanism between doxorubicin and the gut bacterium *Raoultella planticola*.

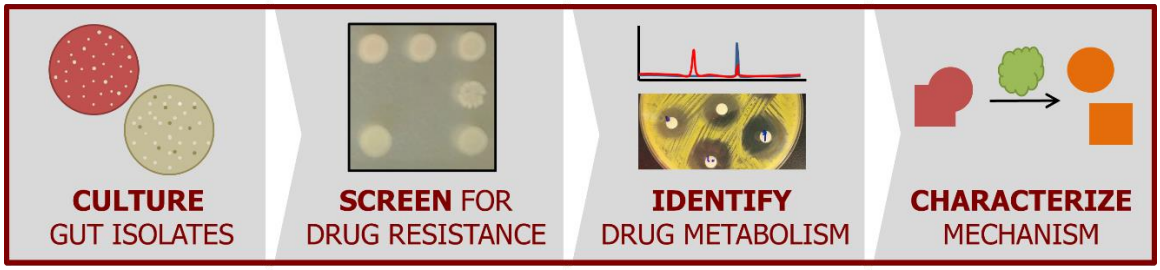


Figure 1-5: Developing a platform for identifying drug-microbiome interactions.

This project involved developing the protocols to identify drug-microbiome interactions. This platform involves four main steps. First, a collection of gut isolates is cultured from fecal samples. Then, this collection is screened for drug resistance on antibacterial agents to enrich for drug-inactivating strains. Next, these strains are then tested for their ability to metabolize or inactivate drugs to filter out strains with other resistance mechanisms. Lastly, strains known to inactivate drugs can be studied further to characterize the reaction mechanism.

Chapter 2 describes the rationale and construction of a gut isolate collection and the screen developed to identify potential gut-microbiome interactions. Chapter 3 discusses various hits from the screen and the identification of the Gram-negative strain, *Raoultella planticola*, capable of doxorubicin inactivation. Chapter 4 details the characterization of anaerobic doxorubicin inactivation by *R. planticola*, while Chapter 5 outlines the study of doxorubicin inactivation in other Enterobacteriaceae and the identification of a potential reaction mechanism involving molybdoenzymes. Lastly, Chapter 6 concludes this report with a final discussion, and outlines potential future steps for this project and the implications of drug-microbiome research.

Chapter 2.
Screening for drug-microbiome interactions

2.1. Key objectives

In order to identify potential therapeutic inactivation by the human gut microbiome, a protocol must be developed to both culture gut microbes and screen for drug interactions. This chapter describes the rationale used for the creation of a library of fecal isolates from a healthy donor and the screening methods used to identify resistant strains, enriching for isolates capable of modifying therapeutic agents. For this project, a total of 190 bacterial strains were isolated and screened on seven antineoplastic and antibiotic drugs. These protocols were designed to be scalable to enable the high-throughput identification and analysis of potential drug-microbiome interactions.

2.2. Culturing the gut microbiota

A significant problem for microbiologists is the bias against studying “unculturable" strains, due to the limitations of common laboratory culturing techniques (*71*). For instance, some bacterial strains may require complex media conditions, rare nutrients, or require metabolites produced by other bacteria (*72*). Given the diversity and complexity of the microbiome, a significant proportion of the microbiome is considered “unculturable”, with some estimates as high as 80% (*59*,*72*). These challenges are being addressed on many fronts, including the development of culture-independent tools and improvements to culturing techniques.

The development of metagenomic tools has provided greater insight into the diversity and composition of the microbiome while overcoming the limitations of culture-based techniques (*17*). Though many microbiome studies now use these sequencing methods exclusively, culture-based methods are still required for

"definitive links to bacterial physiology" (*73*). While metagenomic data can be used to identify putative inactivation genes, the context for these genes may be lost, such the identity of their source organism and the presence of regulatory elements (*74*). Additionally, given that sequence-based studies also rely on comparisons to known sequences, metagenomic studies are limited in their ability to find novel genes such as those with new "biocatalytic activities" (*75*). Therefore, for this project, culture-based methods are better suited for their ability to directly link interactions between gut strains and specific drugs, and potentially capture novel drug-inactivation mechanisms.

Various approaches to improve culturing of the microbiota involve the use of simulated environments, including host-associated elements and co-culturing techniques, all aiming to mimic the bacteria's natural environment and provide a diverse range of metabolites and nutrients that may be required for growth (*72*,*76*). Other techniques encourage the growth of specific bacterial classes that may be slow-growing or outcompeted by fast-growing bacteria, often by inhibiting the growth of common dominant strains (*76*).

At McMaster University, Jennifer Lau of the Surette laboratory has been developing protocols to effectively culture the human gut microbiota, obtaining significantly higher proportions of the bacterial community compared to other studies (personal communication). This diversity was obtained by growing mixed pools of bacterial strains on over thirty media types both aerobically and anaerobically, enabling co-culturing and enriching for underrepresented populations. If needed, bacterial strains could be isolated from these pools. These mixed populations and individual gut strains could then be studied and screened to identify potential drug resistance and inactivation by the human microbiome. Some of these resources and techniques were utilized for this project.

2.2.1. Using fecal samples as a representation of the gut microbiota

Stool samples are often used for the study and characterization of the gut microbiome, mainly due to their ease of collection (*77*). Generally, the composition of feces serves as a representation of microbial population along the large intestine, including both mucosal and luminal associated bacteria (*78*). Yet, different sites along the intestinal tract may host unique communities that are poorly represented by fecal samples. Eckburg *et al*. described the variability and differences between the bacteria isolated from mucosal tissue and stool samples, as well as distinct bacterial populations between different mucosal sites, suggesting "patchiness" or "microanatomic niches" (*77*). These niches have not been studied in the context of microbial drug metabolism.

Variations in different types of tissue and stool samples are important factors to consider when using fecal samples to represent the gut microbiome, particularly when comparing microbial populations across samples and individuals. However, for identifying drug-microbiome interactions mediated by specific bacteria, fecal samples should provide sufficient diversity of both luminal and shed mucosal bacteria for the identification of drug-inactivating strains. Future studies can be designed to enrich for adherent mucosal-specific strains and other species that may be underrepresented when using fecal samples.

2.2.2. Challenges using mixed bacterial populations to identify drug-microbiome interactions

The use of mixed bacterial populations for finding gut-microbiome interactions allows for interspecies interaction and enriches for the growth of strains that require co-culturing. The Surette laboratory has been using these techniques to enable the cultivation of many "unculturable" bacteria by pooling together strains grown on solid media using dozens of media types and incubating with and without oxygen. These pools are referred to as "plate pools", and are identified by their isolation media and incubation condition. In some cases, the Surette laboratory was

able to culture over 95% of the operational taxonomic units present from donor fecal samples, verified by comparing metagenomic sequencing of the source sample to the plate pools. Using these techniques, plate pools were collected by the Surette lab from both healthy donors and patients with irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD). Both conditions are associated with dysbiosis in the gut microbiota (*79*).

Initially, this project aimed to use these plate pools to identify the potential metabolism of drugs by mixed bacterial populations, and compare the metabolic potential of the microbiota between healthy donors and patients with IBS or IBD. A protocol was developed to culture these organisms in media supplemented with drug, obtain the conditioned media, and determine the presence or absence of drug through bioactivity assays and analytical tools like high-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC/MS). If drug inactivation had occurred, the conditioned media, also referred to as the spent media, would no longer contain the active drug but may contain the inactivated product.

Approximately four hundred plate pools from four healthy donors and four patients, including three patients with IBS and one with IBD, were selected for screening. However, using these plate pools to analyze drug metabolism yielded many challenges. Primarily, the plate pools were difficult to culture reproducibly. Biological replicates, where two different cultures were inoculated from the same glycerol stock for incubation, yielded varying results when the conditioned media was assayed for drug content. Technical replicates, where the same conditioned media sample was analyzed, had high reproducibility, indicating that variation was due to the culturing of mixed bacterial populations. Preparing simulated mixed cultures from known gut isolates yielded a similar result. Thus, these data were unreliable and another approach using individual bacterial strains was needed. Other challenges involved in using these mixed communities included variations in plate pool composition after

storage as frozen glycerol stocks, and the need for a high-throughput purification step prior to running samples of conditioned media on the mass spectrometer.

Being able to use plate pools still offers unique advantages for studying drug-microbiome interactions, as these techniques enrich for "unculturable" bacteria while a mixed population can better mimic the complex gut community compared to bacterial monocultures. Plate pools are also better tools for comparing metabolic potential between different patients, as culturing a comprehensive library of gut isolates for each individual is impractical. Improving and developing new strategies to utilize these plate pools and assay for drug metabolism is a continued interest but is not explored further in this project.

2.2.3. Constructing a library of bacterial isolates

Due to the challenges of culturing mixed bacterial populations, a library of gut isolates were prepared for screening purposes. The use of individual strains allowed for reproducible results and prevented certain strains from outcompeting others. Observed drug inactivation could then be directly linked to a specific strain, enabling direct follow-up studies and genomic sequencing if required. These attributes made the use of a library of bacterial isolates favourable. Initially, a previously prepared gut isolate library was used for this project, but problems with contamination in the library complicated follow-up studies. As a result, a new set of isolates were prepared as described in the methods below.

2.2.4. Limitations for *in vitro* strain-based studies

Despite interest in the interaction between host metabolism and the gut microbiome, *in vivo* studies are limited by the availability of reliable gut models. Animal studies, including those using gnotobiotic mice to control microbiome composition, have been essential tools in microbiota research (*10*,*39*). These studies are required to confirm potential drug-microbiome interactions, as the presence of a drug-inactivating gene may not necessary result in varied drug pharmacokinetics,

especially for therapeutic agents with limited exposure to microbes (*32*,*66*). Thus, in addition to finding drug-microbiome interactions, downstream *in vivo* studies are needed to analyze and contextualize these potential interactions in the map of pathways present in the complex host-microbiota system (*21*).

2.3. Materials and methods

2.3.1. Media types and antibiotic additions

Media	Supplier
bacteroides bile esculin	– *
bifidobacterium selection medium	Fluka
brain heart infusion, modified	BD (BBL™)
cooked meet broth	Fluka
de Man, Rogosa, Sharpe media	BD (BBL™)
MacConkey agar	BD (BBL™)

Antibiotics and supplements	Supplier
agar	Fisher
L-cysteine	Aldrich
actinomycin D	AG Scientific
ampicillin	BioShop
cefalexin	Sigma
doxorubicin	AK Scientific
rifampicin	Sigma
vancomycin	Sigma

Table 2-1: List of media, supplements, and antibiotics used in Chapter 2.

These reagents were used in the experiments described in Chapter 2. Their suppliers are listed above. *Bacteroides bile esculin media was prepared by the Surette laboratory using a recipe described by Atlas (*80*).

Various types of media and supplements were used for the growth of gut isolates, shown in Table 2-1. Anaerobic handling and incubation of samples took place in the Bactron IV 600 SHEL LAB Anaerobic Chamber at the Surette laboratory. Media

preparations were made according to suppliers' instructions, unless otherwise stated, and sterilized by autoclaving. Bacteroides bile esculin was made by the Surette lab by preparing 40.0 g/L trypticase soy agar, 20.0 g/L Oxgall, 1.0 g/L esculin, and 0.5 g/L ferric ammonium citrate in distilled water; after autoclaving, 2.5 ml of 5 mg/ml hemin, 2.5 ml of 0.04 mg/ml gentamicin, and 1.0 ml of 10 mg/ml vitamin K1 were sterilized and supplemented per litre of media (*80*). Media used for anaerobic culturing were pre-equilibrated in the anaerobe chamber for a minimum of three hours prior to use.

2.3.2. Isolation of a diverse gut strain collection from a stool sample

Fecal samples from a healthy donor (HV07-11) were received by Jennifer Lau on May 20, 2014 and plated anaerobically at various concentrations ranging from 10^{-3} to 10^{-11} times dilutions onto five types of solid media: MAC (MacConkey's agar), BSM (bifidobacterium selecting media), BEEF (a cooked meat broth), MRS (de Man, Rogosa, Sharpe media), and BDE (bacteroides bile esculin). These five media types were prepared with agar and supplemented with 0.05% L-cysteine (L-cys) to maintain a reducing environment in anaerobic conditions (*81*).

These plates were incubated at 37°C anaerobically for 48 hours. Plates with no colonies or too many colonies were discarded; the ideal dilutions are highlighted on Table 2-2 in Section 2.4. A total of 190 colonies representing the five plates were picked and inoculated in 1 ml BHI (brain heart infusion) broth containing 0.05% L-cysteine in deep 96-well plates, with one blank well on each plate serving as a negative control. These cultures were incubated for 48 hours anaerobically at 37°C, before the addition of sterile glycerol, to a final concentration of 20% glycerol. To prevent contamination of the original stock plates, 200 µl from each well on the deep-well plate was transferred to 96-well plates designated as source plates for replica pinning. Strains were labelled by their plate position, starting from P1A1 to P2H12. All plates were stored at -80°C.

2.3.3. Screening the gut isolate library for antibacterial resistance

Using a 96-well pin tool, cultures were stamped in anaerobic conditions from thawed source plates onto Nunc® OmniTray™ Single-Well plates (VWR) containing 30 ml of BHI agar + 0.05% L-cys, with and without drug. Pins were sterilized between each stamping with 70% ethanol. Plates were prepared with six drugs with antibacterial activity: doxorubicin (5, 20, 50, 150 μg/ml), vancomycin (5, 20, 50 μg/ml), rifampicin (5, 20, 50 μg/ml), actinomycin D (15 μg/ml), cefalexin (50 μg/ml), and ampicillin (50 μg/ml). Plates were incubated overnight at 37°C, anaerobically. A set of isolates were also pinned onto BHI agar + 0.05% L-cys without drug and incubated in aerobic conditions at 37°C to test for oxygen tolerance. Photos were taken after 24 hours, and the ability for each strain to grow at each condition was recorded.

2.4. Preparing a library of gut isolates

A collection of gut isolates were prepared from a stool sample from a healthy donor who was providing stool samples for fecal transplants. The use of a healthy individual's samples provided a profile of a healthy, balanced microbiota. While there are limitations in extrapolating an individual's microbiome's metabolic capacity to the general population, identifying strain-specific mechanisms or certain genetic elements enables the study of its prevalence in the human population, providing insight on the clinical implications of a drug-microbiome interaction.

Gut strains were cultured and isolated in an anaerobic chamber to enable the study of obligate anaerobes, which make up an estimated 99% of the gut microbiota (*82*). The use of five different media types aimed to capture a diverse range of bacterial strains, including Gram-positive and Gram-negative organisms and many dominant gut-associated bacterial genera: *Bifidobacterium*, *Bacteroides*, *Lactobacilli*, and *Escherichia* (*77*). These media are described in Table 2-2. In all, 190 strains were collected and used for downstream screening and study.

Media	Selection techniques	Ref.	Dilutions prepared	Colonies picked
MAC MacConkey	selection of coliform bacteria; contains bile salts	(*83*,*84*)	**10^{-1}**, 10^{-3}, 10^{-4}, 10^{-6}	24
BSM Bifidobacterium selection medium	selection for bifidobacteria; based on MRS media	(*85*)	10^{-4}, 10^{-5}, **10^{-6}**, 10^{-7}	44
BEEF Cooked meat broth	a rich non-selective medium, used for culturing anaerobic bacteria	(*86*)	10^{-4}, 10^{-5}, **10^{-6}**, 10^{-7}	39
MRS de Man, Rogosa, Sharpe	favours growth for *Lactobacilli*; contains Oxoid peptone, various sugars	(*87*)	10^{-3}, 10^{-4}, **10^{-5}**, 10^{-7}	42
BBE Bacteroides bile esculin	enriches for *Bacteroides spp*.; can identify *B. fragilis* strains by visualizing esculin hydrolysis	(*88*)	10^{-4}, 10^{-5}, **10^{-7}, 10^{-8}**	41

Table 2-2: Media types used for the selection of strains in the gut isolate collection.

These five media types were used to isolate a diverse range of strains from a healthy donor fecal sample. Several dilutions of each fecal sample were prepared. Dilutions that yielded distinct separate colonies on the various agar plates (bolded above) were used for the selection of colonies. The number of colonies picked from each media type is also listed above.

2.5. Screening for drug resistance to enrich for drug-inactivating strains

Methods developed in Section 2.2.2 for studying plate pools detected drug inactivation by testing the conditioned media for the presence or absence of drug using bioactivity assays or chemical analysis. However, this process can be time consuming and also requires high-throughput sample preparation. Given that drug inactivation is a form of bacterial resistance, strains that are able to grow in the presence of antimicrobial compounds may be able to degrade or modify these drugs (*48*). Thus, a screen was developed to enrich for the strains capable of inactivating various antibacterial compounds by first selecting for resistant bacteria. After filtering the gut strains by resistance, the mechanism of resistance could be further studied by bioactivity assays or chemical analysis to identify drug-inactivating strains.

This screen utilizes the gut resistome as a source of drug inactivation genes, though this approach is limited to drugs with antibacterial activity. The inactivation

of other therapeutic agents can still be performed by chemical analysis with the development of a high-throughput purification protocol and the use of HPLC or LC/MS.

2.5.1. Initial drug resistance screen

Six drugs with antibacterial activity representing various drug classes, including four antibiotic and two antineoplastics, were screened against the gut isolate collection by pinning strains onto BHI agar containing drug. BHI was chosen as a general purpose, rich-nutrient broth. Drugs were screened at 50 μg/ml, with exception to doxorubicin at 150 μg/ml and actinomycin D at 15 μg/ml, based on previously performed experiments. The properties and uses of these drugs are listed in Table 2-3, alongside known examples of drug inactivation. An example of the pinned plates is shown in Figure 2-1.

Drug name	Drug type (class)	Drug target / activity	Clinical use	Known inactivation	Ref.
actinomycin D	antineoplastic	binds DNA / inhibits DNA transcription	cancer treatments, Wilm's tumour	none observed	(*89*)
doxorubicin	antineoplastic (anthracycline)	intercalates DNA / inhibits topoisomerase	various cancer treatments	NuoEFG (bacterial NADH dehydrogenase)	(*56*)
ampicillin	antibiotic (β-lactam)	binds penicillin binding proteins / inhibits cell wall synthesis	bacterial infections, namely *Enterococci*	various β-lactamases	(*90*)
cefalexin	antibiotic (β-lactam)	binds penicillin binding proteins / inhibits cell wall synthesis	bacterial infections, pneumonia, urinary tract infections	various β-lactamases	(*90*)
rifampin	antibiotic (rifamycin)	binds bacterial RNA polymerase / prevents RNA synthesis	tuberculosis, leprosy treatment, in combination	glycosylation, ADP-ribosylation, phosphorylation, decomposition	(*91*)
vancomycin	antibiotic (glycopeptide)	binds bacterial cell wall lipid II / inhibits cell wall synthesis	bacterial infections, MRSA, *Clostridium difficile*	none observed	(*92*)

Table 2-3: Drugs screened against the gut isolate collection to identify resistant strains.

These six drugs were screened at various concentrations against the gut isolate collection to develop resistance profiles among the 190 gut strains. Resistance is a possible indication for drug inactivation.

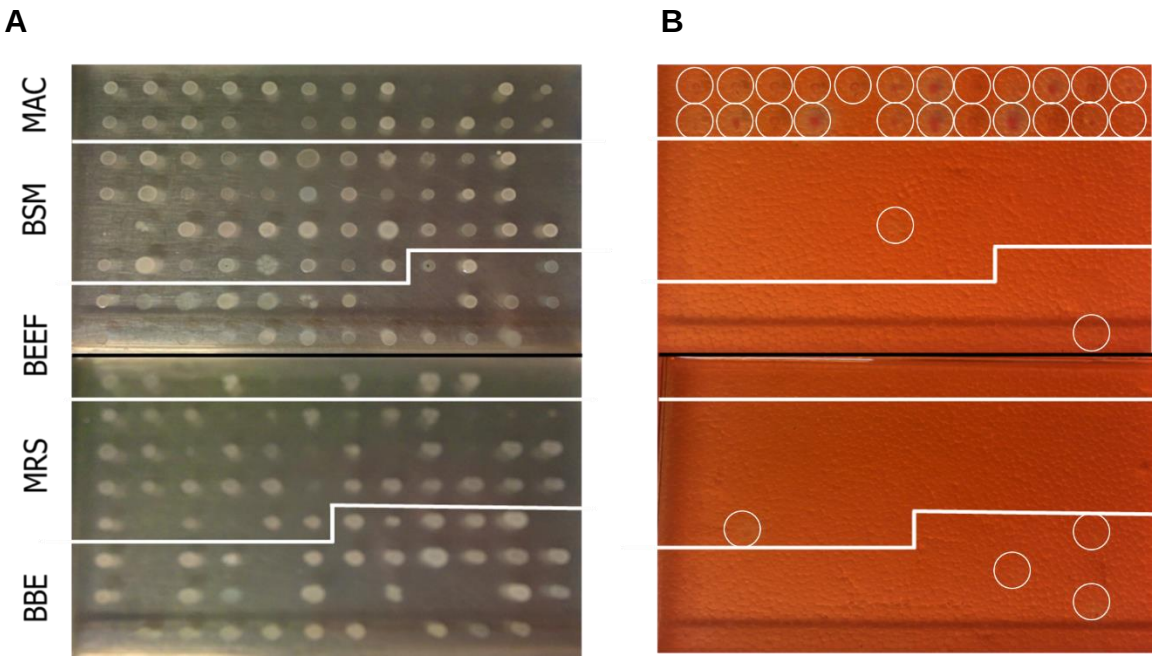


Figure 2-1: Gut isolate collection pinned onto BHI (A) with and (B) without doxorubicin.

Under anaerobic conditions, the gut isolate collection was pinned onto BHI agar using a 96-pin tool. Photos of each pair of plates were aligned to show the 190 strains, separated by the black line. Strains were pinned onto (A) BHI agar or (B) BHI agar containing 150 µg/ml doxorubicin, an example of results obtained using the drugs in Table 2-3. Strains were identified for resistance, their ability to grow despite presence of drug. Doxorubicin-resistant strains are circled for clarity. Notably, many doxorubicin-inactivating strains were able to decolourize the red drug. The divisions above divide the 190 strains by their isolation media, listed on the left: MAC (MacConkey's agar), BSM (bifidobacterium selecting media), BEEF (a cooked meat broth), MRS (de Man, Rogosa, Sharpe media), and BDE (bacteroides bile esculin).

Figure 2-1A shows the gut isolate collection pinned onto BHI agar without drug, with many strains showing unique colony morphology, size, and colour, a reflection of the diversity of strains that were isolated. Yet, only 168 of the 190 strains were able to grow in the absence of antibiotic selection. This result may be due to some strains requiring co-culturing or an inability to grow well on BHI media. Figure 2-1B shows the same 190 strains pinned onto BHI agar containing 150 µg/ml doxorubicin, a bright red drug, where only 29 strains were able to grow. Across all six drugs, strains were profiled for their ability to grow visible colonies after 48 hours of incubation.

These results are summarized in Table 2-4. Many strains were inhibited with the presence of drug, and most resistant strains were isolated on MacConkey agar. For example, of the 29 strains resistant to 150 μg/ml doxorubicin, 23 were MacConkey isolates, as well as all observed vancomycin, ampicillin, and cefalexin resistant strains. These results are expected as many of these drugs target sites that are more accessible in Gram-positive organisms while MacConkey selects for Gram-negative coliform bacteria. Gram-negative bacteria also have a protective outer membrane and various multidrug efflux mechanisms that may also contribute to antibiotic resistance (*93*).

Media	# of strains	# of strains capable of growth							
		No drug	DOX-150	VAN-50	RIF-50	ActD-15	CEF-50	AMP-50	+ O_2
BBE	41	32	3	0	ND	0	0	0	0
BEEF	39	31	1	0	ND	0	0	0	0
BSM	44	42	1	0	1	0	0	0	1
MAC	24	24	23	11	0	14	8	8	9
MRS	42	39	1	0	ND	0	0	0	0
TOTAL	**190**	**168**	**29**	**11**	**ND**	**14**	**8**	**8**	**10**

Table 2-4: Summary of drug resistance among gut isolates.

This table breaks down the number of strains picked from each media type and the number of resistant bacteria in each category. Drugs include doxorubicin (DOX), vancomycin (VAN), rifampin (RIF), actinomycin D (ActD), cefalexin (CEF), and ampicillin (AMP). Concentrations are listed in μg/ml. All strains were inoculated and incubated anaerobically, with the exception of aerobic incubation in the column marked '+ O_2'. ND: no data, MAC: MacConkey's agar, BSM: bifidobacterium selecting media, BEEF: cooked meat broth; MRS: de Man, Rogosa, Sharpe media, BDE: bacteroides bile esculin.

2.5.2. Second drug resistance screen

After the first screen, a second screen was performed using lower drug concentrations. This screen would help increase the resolution of analyzing drug resistance in the gut isolate collection, as some resistance strains with potential drug inactivation mechanisms may be missed if only strains were only screened against a higher concentration. The screen was repeated with doxorubicin, vancomycin, and rifampin at lower concentrations. The results are shown in Table 2-5.

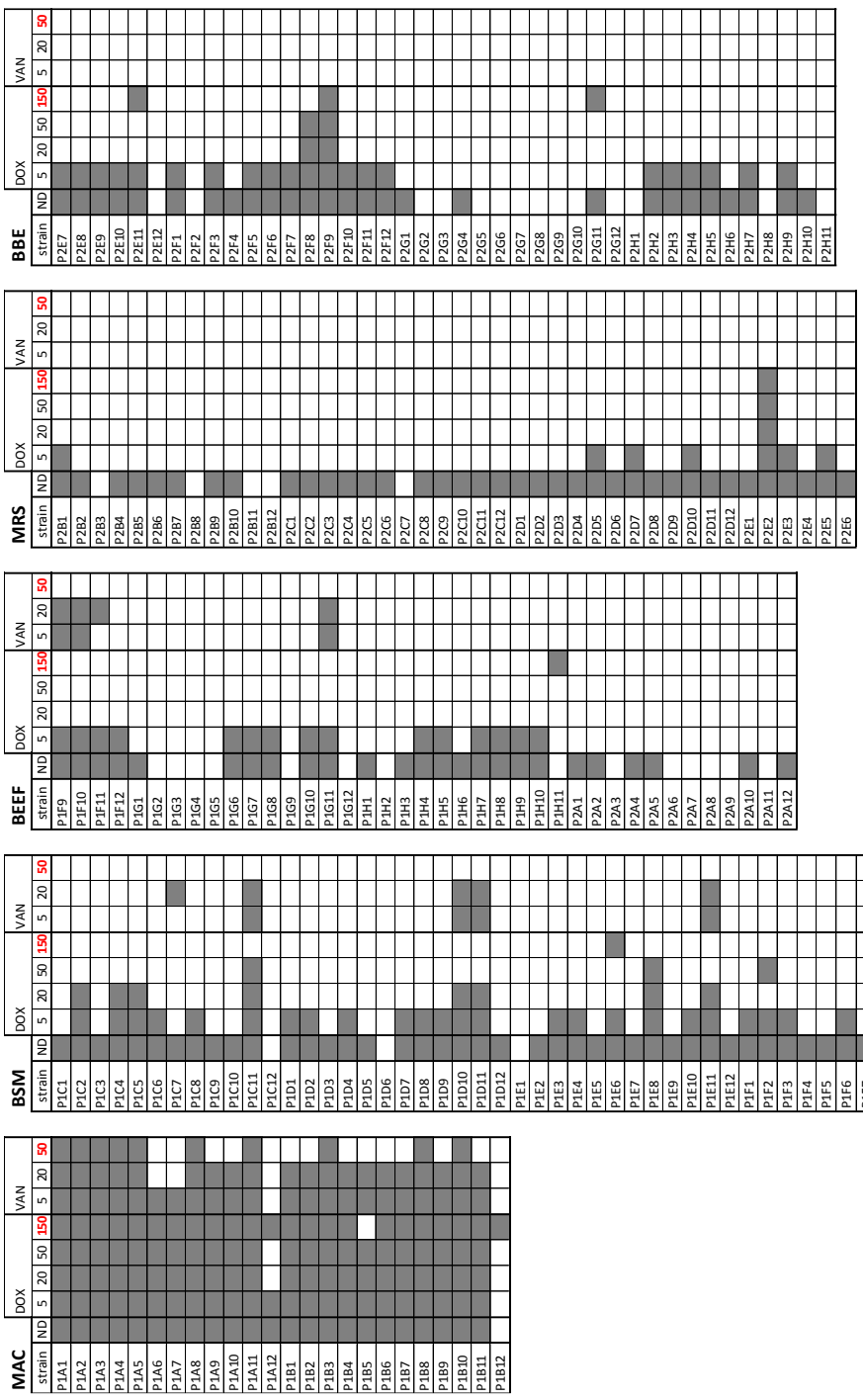


Table 2-5: Selected resistance profiles of the gut isolate collection.

The gut isolate collection was pinned onto BHI agar with and without drug. Vancomycin (VAN) was screened at 5, 20, and 50 µg/ml; doxorubicin (DOX) was screened at 5, 20, 50, and 150 µg/ml. Data with concentrations marked in red were taken from the initial screen, while all other concentrations including the no drug control (ND) were screened together. Shaded cells indicate growth of a visible colony after 48 hours of anaerobic incubation. MAC: MacConkey's agar, BSM: bifidobacterium selecting media, BEEF: cooked meat broth; MRS: de Man, Rogosa, Sharpe media, BDE: bacteroides bile esculin.

These results mainly confirm the resistance profiles from the initial screen, while identifying additional resistant strains that were able to grow at lower concentrations of drug. These identified strains, especially those outside of the MacConkey isolates likely to be capable of drug efflux, may be enriched in drug-inactivating mechanisms. Some strains, such as P2E11, appear to be resistant to doxorubicin at 5 and 150 µg/ml, but not at 20 or 50 µg/ml; because the data for 150 µg/ml was taken from the initial screen, while the lower concentrations were used in the second screen, strains may have been affected by an additional freeze-thaw cycle. Yet as a whole, most of the data is consistent between the two screens, providing each strain with a resistance profile useful for further analysis.

2.6. Future steps and applications

A previously undescribed phenomena was observed on the doxorubicin-containing BHI agar plates. Many pinned colonies showed a zone of decolourization, as seen among many of the MacConkey isolates in Figure 2-1B. This result was not described by Westman *et al*. in their study of doxorubicin inactivation by NADH dehydrogenase in *Streptomyces*, and was confirmed to be a previously unobserved phenomenon (personal communication). As a result, these "decolourizing" strains were studied further in the experiments described in Chapter 3, guiding this project's focus on doxorubicin inactivation.

As a result, most of the remaining screening data from this chapter have not been fully studied, providing other potential drug interactions that have yet to be identified. The resistant gut strains identified above can be screened for the inactivation of the other six antibacterial agents, using similar protocols described in the following chapters for characterizing doxorubicin inactivation. Furthermore, the gut isolate collection's 190 strains may be used to screen for the inactivation of other therapeutic agents of interest.

Additionally, these protocols may be used to culture different stool samples that represent a diverse range of patients or time points. While this study used a sample from a healthy donor, a patient's disturbed microbiota may yield strains with different metabolic activities, especially among patients taking various therapeutic agents. These platform can be used to further study whether antibiotic resistance genes are enriched in patients taking antibiotics, and whether this enrichment also selects for antibiotic inactivation genes.

Other future directions aim to optimize the collection of gut isolates to ensure that the strains collected are representative of the diversity in the gut microbiota, including developing protocols for the reproducible culturing of plate pools. The strain identities of the current gut isolate collection should also be characterized to determine how well these strains represent the human gut microbiota and guide any future changes to improve this platform for identifying drug-microbiome interactions.

Chapter 3.
Identification of doxorubicin-inactivating strains

3.1. Key objectives

After the screening of gut isolates in Chapter 2, several strains were identified for their resistance to the panel of antibacterial drugs. Notably, doxorubicin-resistant strains were able to decolourize the red drug (see Figure 2-1), a phenomenon that was previously unobserved. Chapters 3 to 5 describe the efforts to characterize these strains and identify the specific enzymes involved in this reaction.

This chapter documents the identification of several doxorubicin-inactivating strains. This activity was shown to be specific to anaerobic conditions. One of these isolates was characterized as *Raoultella planticola* through 16S rDNA sequencing and subsequent genome sequencing. This strain was used for downstream studies in Chapter 4.

3.2. Doxorubicin and its bacterial inactivation

Doxorubicin, or adriamycin, is an anthracycline antineoplastic agent produced by *Streptomyces peucetius* var. *casius* and is a frontline chemotherapy drug in the treatment of a wide range of cancers including acute leukemia, breast cancer, and non-Hodgkin lymphomas (*94*,*95*,*96*). All anthracyclines consist of a quinone-containing, rigid, planar, aromatic-ring structure with a glycosidic bond to a daunosamine aminosugar. Doxorubicin appears bright red, is photosensitive, and can be observed by spectrophotometry at an absorbance of 480 nm; its structure is shown in Figure 3-1 (*97*).

Anthracyclines induce many concentration-dependent effects that involve intercalating with DNA, inhibiting DNA synthesis, and targeting topoisomerase IIα

(*94*,*98*). These drugs may also act as electron acceptors and generate free-radicals, inducing DNA damage and forming other toxic radicals and peroxides (*98*,*99*). These interactions ultimately result in DNA damage and cell death. Doxorubicin's high affinity for membranes can also cause lipid peroxidation, resulting in significant cell damage even when the drug is bound to large, insoluble agarose beads that cannot penetrate the cell membrane (*96*,*100*).

Doxorubicin treatment is dose-limited due to its high cardiotoxicity, linked to high levels of free radical formation in the mitochondria-rich cardiac tissue lacking in antioxidant defenses (*95*). The drug is given intravenously due to its acid sensitivity, and is rapidly taken up by cells, with a distribution half-life of three to five minutes and a terminal half-life of 24-36 hours. Efforts to develop oral formations of doxorubicin are ongoing, utilizing carriers or lipid-based systems (*101*).

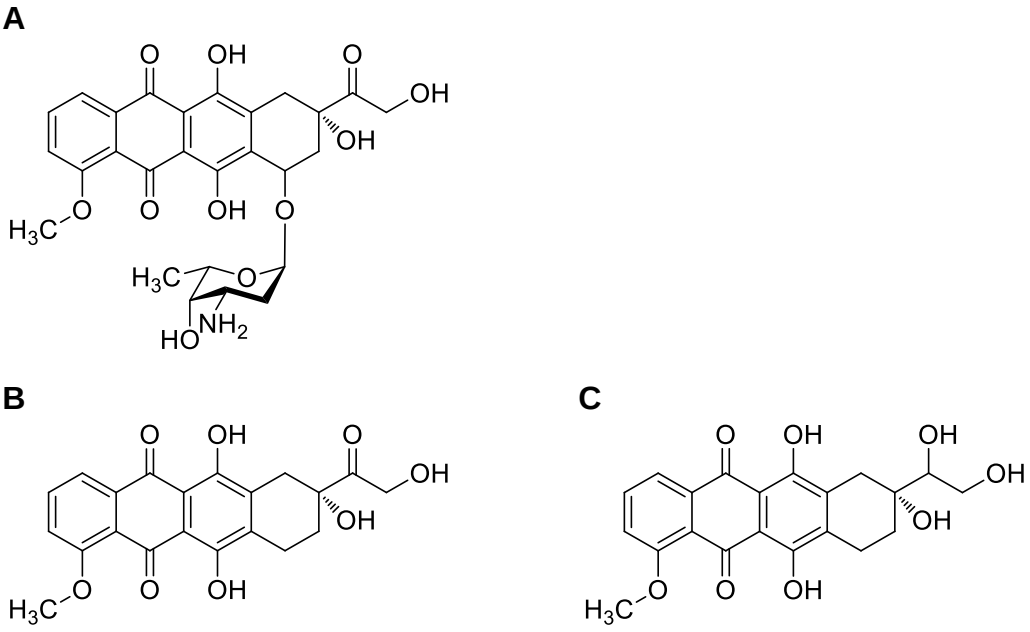


Figure 3-1: Structures of doxorubicin and its aglycone derivatives.

Key features of (A) doxorubicin include a quinone ring, a semiquinone ring, and a daunosamine sugar. A study by Westman *et al*. show the conversion of doxorubicin to two aglycone forms: (B) 7-deoxydoxorubicinone and (C) 7-deoxydoxorubicinonol.

Doxorubicin, though used as an antineoplastic agent, also has antibacterial activity. A report by Bodet III *et al*. tested the activity of doxorubicin and five other anticancer drugs against a panel of bacterial strains. Several strains (17 of 41) conferred high level resistance at >100 μg/ml (*55*). The authors suggested that the effect of anticancer drugs on the gut microbiome may be limited due to low achievable plasma concentrations of doxorubicin (0.5 μg/ml). However, studies must also account for the rapid uptake of drug and the localization of anthracycline drugs to cell membranes that may lead to high intracellular concentrations.

In 2012, Westman *et al.* identified doxorubicin inactivation by a bacterial NADH dehydrogenase in a strain of soil-dwelling *Streptomyces* (*56*). The NuoEFG complex promotes the formation of an unstable doxorubicin semiquinone leading to the loss of its daunosamine sugar. Structures of doxorubicin and two of its inactive aglycone products, 7-deoxydoxorubicinolone and 7-deoxydoxorubicinol, are shown in Figure 3-1.

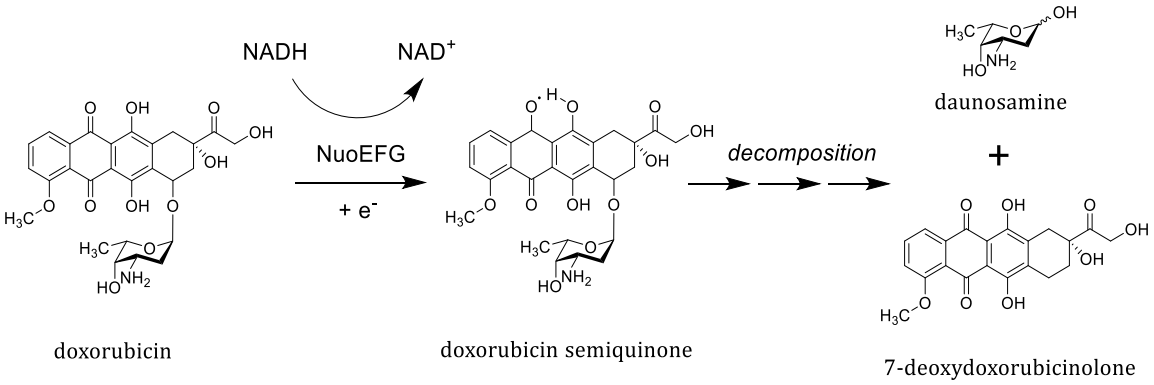


Figure 3-2: Simplified reaction mechanism of doxorubicin inactivation by the bacterial NADH dehydrogenase NuoEFG.

Doxorubicin is converted to a doxorubicin semiquinone by bacterial NuoEFG, a respiratory complex. The unstable doxorubicin semiquinone decomposes to daunosamine and 7-deoxydoxorubicinolone. While this reaction has been demonstrated in environmental bacteria and not by gut bacteria, the NuoEFG complex is found in all bacteria. For full reaction mechanism, see Westman *et al.* (*56*).

While doxorubicin is currently administered intravenously, potential for interactions in the gut microbiota due to the drug's antibacterial activity and the ongoing development of oral therapies warrant interest in drug-microbiome interactions. Identifying strains that may inactivate doxorubicin in the gut will aid in pharmacokinetic studies of oral treatments. Furthermore, doxorubicin, among other chemotherapies, is not selectively targeted towards tumour tissue. By identifying the enzymes responsible for drug inactivation, these enzymes can be utilized to selectively detoxify cancer drugs in non-tumour tissues, including in the intestinal tract as well as sites affected by doxorubicin-mediated side effects (*66*,*102*).

3.3. Materials and Methods

3.3.1. Media preparation

Gut strains in this chapter were grown in either BHI broth or M9 minimal media. Suppliers for media and supplements are listed in Table 3-1. Anaerobic handling and incubation of samples took place in the Bactron IV 600 SHEL LAB Anaerobic Chamber at the Surette laboratory.

L-cysteine was added to all liquid media used for anaerobic cell growth and to all solid media at a final concentration of 0.05% w/v to aid in maintaining a reducing environment in anaerobic conditions. M9 minimal media was made by diluting a stock of 5X M9 salts with sterile water to produce 1X M9 salts supplemented with final concentrations of 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 0.4% glucose. Doxorubicin was added at a concentration of 150 µg/ml when assayed.

Media and supplements	Supplier
brain heart infusion, modified	BD (BBL™)
5X M9 salts	Sigma
$MgSO_4$ (magnesium sulfate)	Fisher
$CaCl_2$ (calcium chloride)	Sigma
glucose	BioShop
L-cysteine	Aldrich
agar	Fisher

Table 3-1: List of media and supplements used in Chapter 3.

These reagents were used in the experiments described in Chapter 3. Their suppliers are listed above.

3.3.2. Growing isolates in the presence of doxorubicin

Thirteen strains from the gut isolate collection that were resistant to doxorubicin were selected and inoculated from their glycerol stock into 1 ml BHI + 0.05% L-cys. These starter cultures were incubated at 37°C, anaerobically, overnight.

Cultures were used to inoculate two media types: BHI broth and M9 media containing 0.4% glucose, each supplemented with 0.05% L-cys and prepared with and without 150 µg/ml doxorubicin. In 96-well plates, the two media types, with and without drug, were aliquoted in 200 µl volumes for each of the 14 strains. Each well was inoculated with the starter culture at a 1:100 ratio. After anaerobic incubation at 37°C for 48 hours, absorbance readings at 600 nm and 480 nm were read for culture growth and doxorubicin decolourization, respectively.

The starter cultures were also used to streak each strain onto BHI agar plates containing 0.05% L-cysteine, with and without 150 µg/ml doxorubicin. Plates were prepared anaerobically in duplicate: one replicate was incubated in the anaerobic chamber while one replicate was removed from the chamber and incubated aerobically. After 48 hours of incubation at 37°C, strains were observed for their ability to grow with and without oxygen, and decolourize doxorubicin in either condition.

3.3.3. BOX-PCR

BOX-PCR is a form of polymerase chain reaction (PCR) used to quickly fingerprint genomic DNA (gDNA) to compare if strains are identical or unique (*103*,*104*). DNA samples of each strain were extracted using the PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems). Strains were each inoculated in 1 ml BHI broth containing 0.05% L-cys and incubated overnight at 37°C, anaerobically. Cells were then removed from the anaerobic chamber and centrifuged at maximum speed (13 200 rpm) in an Eppendorf 5415 D centrifuge. Supernatants were discarded, and the pellets were resuspended in 50 µl of PrepMan® reagent, boiled for 15 minutes, and centrifuged for 5 minutes at maximum speed. The supernatant, containing the genomic DNA, was decanted and stored at -20°C.

BOX-PCR reactions were prepared in 25 µl volumes, containing: 2.5 Units of BioBasic Taq polymerase, 0.5 µl of the prepared DNA, and final concentrations of 1X BioBasic buffer, 0.8 µM BOXA1R primer, 0.2 µM dNTPs, and 2 mM $MgSO_4$ in sterile water. The primer sequence was 5' – CTA CGG CAA GGC GAC GCT GAC G – 3', and protocols were adapted from Versalovic *et al*. and Lanoot *et al*. (*104*,*105*). PCR was performed using the Eppendorf Mastercycler® gradient thermocycler, with the following parameters: initial denaturation (95°C, 7 min); thirty cycles of denaturation (95°C, 30 s), annealing (53°C, 1 min), and extension (65°C, 8 min), and final extension (65°C, 16 min). BOX-PCR bands were visualized on a 2% agarose gel stained with RedSafe dye (iNtRON Biotechnology), run at 120 V for 2 hours, and visualized using the Typhoon Trio+ (GE) scanner.

3.3.4. 16S rDNA sequencing

16S ribosomal DNA (rDNA) sequencing, also known as 16S rRNA sequencing, is used to classify an unknown bacterial strain based on its 16S ribosomal DNA sequence (*106*). This sequence is highly variable and can often be used to identify an organism at its genus, if not species, level. This protocol involves amplifying the 16S rDNA sequence from a preparation of a strain's genomic DNA (gDNA) using PCR.

DNA samples of each strain were extracted using the PrepMan Ultra Sample Preparation Reagent as described in Section 3.3.3. The primers used were: '16 BAC F', 5' – AGA GTT TGA TCM TGG CTC AG – 3'; and '16 BAC R', 5' – TAC GGY TAC CTT GTT ACG ACT T – 3'. PCR reactions contained 1 Unit of BioBasic Taq polymerase and 1 µl of the PrepMan DNA preparation in 50 µl reactions, with final concentrations of 1X BioBasic buffer, 0.4 µM forward primer, 0.4 µM reverse primer, 0.2 µM dNTPs, and 2 mM $MgSO_4$ in sterile water. PCR was performed using the Eppendorf Mastercycler® gradient thermocycler, with the following parameters: initial denaturation (94°C, 3:30 min); thirty cycles of denaturation (94°C, 30 s), annealing (47°C, 30 s), extension (72°C, 1:30 min), and final extension (72°C, 7 min). To confirm that the 16S rDNA was amplified, 5 µl of each reaction was visualized using agarose gel electrophoresis using a 1% agarose gel stained with RedSafe dye that was performed at 120 V for approximately one hour. Gels were visualized using the Typhoon Trio+ scanner to confirm the presence of a 1.5 kb band.

If the rDNA was successfully amplified, the remaining 45 µl reactions containing the 16S rDNA amplicons were purified using the GeneJET Gel Extraction Kit (Life Technologies) following manufacturer's protocols for PCR amplicon purification and eluted in the provided Tris-HCl Elution Buffer. The purified amplicons and 16S PCR primers were then sent to the MOBIX Lab at McMaster University for sequencing, which uses BigDye® terminator chemistry on a 3730 DNA Analyzer (Applied Biosystems). Results were then analyzed either using BLAST or GreenGenes to identify strain identity (*107*,*108*).

3.3.5. DNA preparation for genome sequencing

Genomic DNA (gDNA) was isolated using the Invitrogen PureLink™ Genomic DNA kit, following the protocol for preparing genomic DNA from a "Gram Negative Bacterial Cell Lysate" (*109*). *Raoultella planticola* cells were harvested from a 5 ml BHI overnight culture and lysed using PureLink™ Genomic Digestion Buffer, incubating at 55°C for four hours. An incubation time of 90 minutes had a low DNA yield.

DNA was eluted in 50 µl of water and quantified using the NanoDrop 2000 Spectrophotometer (Thermo Scientific). A polymerase chain reaction of the 16S rDNA was used to verify the identity and purity of *R. planticola* gDNA following the protocols described in Section 3.3.4. The purified amplicon was sent to MOBIX lab for sequencing, and the sequence was compared to the previously sequenced rDNA.

3.3.6. Genome sequencing and assembly

Genomic DNA was then diluted and sent for sequencing at the Farncombe Metagenomics facility on the Illumina platform with 2 x 250 bp paired-end reads. Genome assembly was performed with guidance from Nick Waglechner. Data files were trimmed at quality values of 30 using `skewer`, version 0.1.117, to remove adapters and low quality reads (*110*). Read pairs were merged using `FLASH`, "**f**ast **l**ength **a**djustment of **sh**ort reads", version 1.2.10, to merge paired reads to improve assembly (*111*). The genome was assembled using `SPAdes`, version 3.1.1, using default k-mers and the '`--careful`' flag, which aims to eliminate errors by reducing mismatches and short insertions/deletions (*112*).

3.3.7. MIC determination of *Raoultella planticola*

All these drugs were prepared in 1 mg/ml aqueous stock solutions, with the exception of rifampin which was dissolved in dimethyl sulfoxide (DMSO). An overnight starter culture of *R. planticola* in BHI broth was prepared, aerobically, and used to inoculate BHI broth containing drug at a 1:100 ratio (2 µl of culture in 198 µl of media). Drugs were prepared in 2-fold serial dilutions ranging from 64 µg/ml to 6.25 ng/ml. Cultures were incubated for 24 hours and observed for growth. The minimum inhibitory concentration (MIC) of each drug was recorded, the lowest concentration required to inhibit the growth of *R. planticola*.

Antibiotics	Supplier
ampicillin	BioShop
bacitracin	Sigma
ciprofloxacin	Sigma
kanamycin	BioShop
rifampin	Sigma
streptomycin	BioShop
vancomycin	Sigma

Table 3-2: List of antibiotics used for susceptibility determination in *R. planticola*.

These drugs were used for antibiotic susceptibility testing in *R. planticola* as described in Section 3.3.7. Their suppliers are listed above.

3.4. Characterization of doxorubicin resistant strains

Thirteen of the decolourizing strains were selected, seven originally isolated on MacConkey agar (P1A1, P1A6, P1A7, P1B2, P1B3, P1B6, P1B9) and six from the remaining media types (P1E6, P1H11, P2E2, P2E11, P2F9, P2G11). Though the latter six had comparably smaller zones of clearing compared to the MacConkey isolates, including a wider range of strains could help in finding different mechanisms of doxorubicin inactivation.

Table 3-3 shows the ability of these strains to decolourize doxorubicin. Three of the six strains grown on other media types had poor growth in BHI broth (OD_{600} < 0.1). Of the remaining ten, significant doxorubicin degradation was observed in six MacConkey isolates (P1A1, P1A6, P1A7, P1B2, P1B3, and P1B5). The other strains showed less decolourization and had limited growth in the presence of doxorubicin. This result confirms that the MacConkey isolates were significantly more capable of doxorubicin decolourization and are also able to confer resistance to 150 μg/ml doxorubicin in liquid BHI cultures.

	liquid media						*solid BHI agar*			
	BHI	**BHI+DOX**		**M9**	**M9+DOX**		**aerobic**		**anaerobic**	
Strain	**OD_{600}**	**OD_{600}**	**OD_{480}**	**OD_{600}**	**OD_{600}**	**OD_{480}**	**ND**	**DOX**	**ND**	**DOX**
P1A1	0.35	0.35	0.06	0.12	0.14	0.45	+	+	+	+D
P1A6	0.46	0.32	-0.11	0.13	0.30	0.57	+	+	+	+D
P1A7	0.44	0.11	-0.34	0.11	0.05	0.55	+	+	+	+D
P1B2	0.43	0.11	-0.32	0.12	0.14	0.28	-	-	+	+
P1B3	0.26	0.16	-0.11	0.13	0.17	0.5	+	+	+	+D
P1B5	0.17	0.04	-0.09	-0.01	0.01	0.69	+	+	+	+D
P1B9	0.25	0.77*	0.43*	0.28	0.28	0.14	+	+	+	+D
P1E6	0.33	0.06	0.27	-0.01	0.03	0.71	+	-	+	-
P1H11	0.01	0.06	0.67	-0.01	0.01	0.7	+	+	+	+D
P2E2	0.04	0.04	0.23	-0.01	0.00	0.68	+	+	+	+D
P2E11	0.3	0.05	0.3	0.00	0.03	0.72	–	-	+	-
P2F9	0.04	0.14	0.83	0.00	0.04	0.73	-	-	+	-
P2G11	0.13	0.03	0.01	-0.01	0.03	0.73	-	-	+	-
control	0	0.09	0.51	0.00	0.02	0.72				

	OD_{600}		OD_{480}
no growth	0.00	high decolourization	0.00
high growth	0.50	low decolourization	1.00

Table 3-3: Capability of doxorubicin decolourization among thirteen doxorubicin-resistant strains.

Thirteen doxorubicin resistant strains were inoculated in a rich, BHI broth media and a minimal M9 media containing glucose, with and without drug at 150 µg/ml. End point absorbance readings at 600 nm and 480 nm were taken to monitor anaerobic culture growth and doxorubicin degradation, respectively. All readings are normalized to a media-only control. OD_{480} values are also normalized to the OD_{480} of a no-drug control of the same culture. Absorbance values can be compared by the colour gradient shown by the legend below. Strains were also streaked out on BHI agar and incubated aerobically or anaerobically to characterize doxorubicin degradation capabilities. A '+' indicates growth, while a '–' indicates no growth. A '+D' indicates that in addition to resistance, decolourization was observed. The asterisk (*) indicates a well of P1B9 sample which had unexpectedly higher readings likely due to an air bubble.

The thirteen strains were also streaked onto BHI agar plates with and without doxorubicin, and incubated aerobically and anaerobically (Table 3-3). P2E11, P2F9, P2G11, isolated on bacteroides selecting media, as well as P1B2, a MacConkey isolate, were obligate anaerobes while the other nine strains were facultatively anaerobic. No strains were capable of decolourizing doxorubicin in aerobic conditions, though eight of the nine oxygen-tolerant strains were resistant. These eight strains were capable of decolourizing doxorubicin anaerobically, while P1B2 was resistant but did not decolourize.

BOX-PCR was run on seven of these strains: P1A1, P1A6, P1A7, P1B3, P2B5, P1B9, and P1H11. All but P1B9 had an identical pattern when visualized on an agarose gel. 16S rDNA sequencing of P1A1 and P1B3, and typed these strains as *Raoultella planticola*, while P1B9 was a *Propionbacterium* species. P1B3 was selected for downstream studies as a strain of *R. planticola* capable of doxorubicin inactivation.

3.5. Identification of *Raoultella planticola*

3.5.1. Characteristics of *Raoultella planticola*

Raoultella planticola, previously classified as *Klebsiella planticola,* is a Gram-negative bacterium first described in 1981 by Bagley *et al.* (*113*). While early strains were mainly associated with aquatic and soil environments and usually isolated from agricultural products, *R. planticola* was also found in human urine samples, human wound infections, and bovine mastitis. *R. planticola* has been studied as a histamine-producing bacteria commonly found in fish, and has colonized an estimated 9 to 18% of humans (*114*).

The genus *Raoultella* was created after a comparative analysis of 16S rDNA and *rpoB* genes among *Klebsiella* in 2001, and describe a group of capsulated rods that are non-motile, Gram-negative, oxidase-negative, and capable of growing at 10°C unlike other *Klebsiella* spp. (*115*). While *R. planticola* is usually a commensal organism, rare

cases of clinical infection have been described, often in immunocompromised or elderly patients, in wounds following trauma, or in the form of a secondary bacteremia (*114*,*116*). A 2014 report in the Journal of Infectious Disease and Medical Microbiology described a case of *R. planticola* bacteremia in a cancer patient, which may have been linked to seafood consumption (*117*).

R. planticola was originally characterized as being resistant to ampicillin but sensitive to most other drugs tested, with few reported cases of multidrug resistance compared to some pathogenic *Klebsiella* species (*113*). *R. planticola* bacteremia can be treated with conventional antibiotics including ciprofloxacin and ceftriaxone (*116*,*117*). However, given the close similarity of the two genera, the possibility of the emergence of multi-drug resistant *R. planticola* remains a possibility and should be monitored.

3.5.2. Genome sequencing and assembly of *Raoultella planticola*

One strain of *R. planticola* was selected for genome sequencing. Genomic DNA was isolated using the Invitrogen PureLink™ Genomic DNA kit. The gDNA was then diluted and sent for sequencing at the Farncombe Metagenomics Facility.

The *R. planticola* genome was assembled on 44 scaffolds with a total genome size of 5.85 Mbps, with the largest contig of 812663 bps and a N50 of 463914 bps. This sequence can be used to help identify elements associated with doxorubicin degradation and investigate antibiotic resistance.

3.5.3. Determining antimicrobial sensitivities of *Raoultella planticola*

The sensitivity of *Raoultella planticola* to various antibiotics and antimicrobial compounds was identified and utilized for the development of genetic tools and resistance markers in future experiments. The antibiotics tested were: ampicillin, bacitracin, ciprofloxacin, kanamycin, rifampin, streptomycin, and vancomycin. The MICs of the various antibiotics against *R. planticola* are shown in Table 3-4.

These data indicate that *R. planticola* is sensitive to ciprofloxacin, kanamycin, rifampin, and streptomycin. Resistance to vancomycin and bacitracin are expected due to the drug's main activity against Gram-positive organisms, while resistance to ampicillin in *R. planticola* was previously known and may be due to the β-lactamase resistance elements identified in its genome. Based on prior experiments, the MIC for doxorubicin was greater than 64 μg/ml, and was able to grow on solid BHI media containing up to 750 μg/ml doxorubicin.

Drug	MIC (μg/ml)
ampicillin	>64
bacitracin	>64
ciprofloxacin	<0.063
kanamycin	4
rifampin	8
streptomycin	4
vancomycin	>64

Table 3-4: Minimum inhibitory concentrations of various antibacterial compounds against *Raoultella planticola*.

Seven different antibiotics were tested against *R. planticola* to determine the strain's susceptibility to various antibacterial drugs. The minimum inhibitory concentration of each of these drugs was identified.

3.6. Future steps and directions

Chapter 4 continues with the study of *R. planticola* by confirming the inactivation of doxorubicin, testing the activity of cell lysates, and analyzing inactive doxorubicin products. These steps aim to identify the mechanism of doxorubicin inactivation and explain the decolourization observed in anaerobic conditions. The ability for anaerobic inactivation and the previously unobserved decolourization phenotype suggest that the method of inactivation by *R. planticola* is independent of the NuoEFG NADH dehydrogenase.

While this project focussed on *R. planticola*, identified from strain P1B3, the other strains described in this chapter can also be studied for their ability to inactivate doxorubicin. Other strains of *R. planticola* were identified in the screen, such as P1A1, that may represent different biotypes with varying metabolic and resistance capabilities (*118*). Even if the mechanism of inactivation was the same, different levels of gene regulation may help in the study and identification of the responsible enzymes.

Other strains were also found to be resistant to doxorubicin though with limited decolourization. These strains can also be further studied, and may have a different mode of interaction than *R. planticola*. Some preliminary experiments aimed to characterize and study these strains, including members of the genera *Sutterella*, *Coprococcus*, and *Ruminococcus*; the latter two were studied briefly in Section 5.2.2 in comparison to *R. planticola*.

Chapter 4.
Anaerobic doxorubicin inactivation by *Raoultella planticola*

4.1. Key objectives

This chapter describes the investigation of a mechanism of anaerobic doxorubicin inactivation by *Raoultella planticola*, a strain among the doxorubicin-decolourizing isolates studied in Chapter 3. While the previous chapters showed that *R. planticola* was resistant and could decolourize doxorubicin, the work described in this chapter confirms the inactivation of doxorubicin and the production of its aglycone derivative, 7-deoxydoxorubicinolone.

This chapter includes the use of whole cell and cell lysate assays coupled with chemical analysis to study the doxorubicin inactivation reaction. The material and methods describe the techniques used to study *R. planticola*, including anaerobic lysate preparation and drug inactivation assays. The clinical implications of drug-microbiome interaction studies can be better understood by characterizing the drug inactivation mechanism.

4.2. Materials and methods

4.2.1. Media and assay conditions

The following media conditions were used to culture *R. planticola* and test for its ability to inactivate doxorubicin. Anaerobic handling and incubation of samples took place in the Bactron IV 600 SHEL LAB Anaerobic Chamber at the Surette laboratory. L-cysteine was added to all liquid media, solid media, and assay buffers at a final concentration of 0.05% w/v, but was not added in HPLC and LC/MS buffers and the media used for the aerobic culturing of *Micrococcus luteus*, an obligate aerobe.

Media, assay buffers, and supplements	Supplier
brain heart infusion, modified	BD (BBL™)
trypticase soy broth	BD (BBL™)
lysogeny broth	BioShop
5X M9 salts	Sigma
$MgSO_4$ (magnesium sulfate)	Fisher
$CaCl_2$ (calcium chloride)	Sigma
glucose	BioShop
L-cysteine	Aldrich
EDTA (ethylenediaminetetraacetic acid)	Fisher
doxorubicin	AK Scientific
agar	Fisher
methanol	Caledon
NaCl (sodium chloride)	Fisher
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	BioShop
glycerol	Caledon
NADH (nicotinamide adenine dinucleotide)	Sigma
NADPH(nicotinamide adenine dinucleotide phosphate)	Sigma
acetonitrile	Caledon
formic acid	Sigma-Aldrich

Table 4-1: List of media, buffers, and supplements used in Chapter 4.

These reagents were used in the experiments described in Chapter 4. Their suppliers are listed above.

4.2.2. *R. planticola* culture doxorubicin inactivation

R. planticola was inoculated anaerobically in BHI broth and M9 media, supplemented with 0.05% L-cysteine, with and without doxorubicin. Cultures were aliquoted into 1 ml volumes and incubated in 1.5 ml micro-centrifuge tubes at 37°C, anaerobically. A set of cultures were removed every 8 hours over 80 hours, and monitored for growth and drug decolourization. Absorbance values were measured using the SpectraMax Plus 384 (Molecular Devices) by transferring 200 µl of the suspended culture and the conditioned media, obtained by centrifuging the cultures, into a 96-well plate. By measuring the absorbance of the culture at 600 nm and the conditioned media at 480 nm, the growth of *R. planticola* and decolourization of doxorubicin in the media could be monitored over time, respectively. Doxorubicin

concentrations were measured using the conditioned media instead of the cultures because cells can also absorb light at 480 nm.

4.2.3. HPLC analysis of cell culture supernatants

The conditioned media, also referred to as culture supernatants, were analyzed to confirm the inactivation of doxorubicin and to identify potential reaction products. Samples were analyzed by reverse phase HPLC (Waters e2695) using an Xselect CSH C18 semi-preparative column (Waters, 5 μm, 10x100 mm). The mobile phases used were water (A) and acetonitrile (B), both containing 0.05% formic acid. Samples were run at 25°C using the following nine minute method with a flow rate of 1.0 ml/minute: 0 to 1 min, 95% A; 1 to 7 min, a linear gradient increasing B from 5% to 97%; 7 to 8 min, 97% B; 8 to 8.5 min, a linear gradient increasing A from 3% to 95%; 8.5 to 9 min, 95% A. Absorbance readings from 210 to 550 nm at a 6 nm resolution were observed using the Waters 2998 photodiode array detector. Chromatograms were extracted at 480 nm to observe doxorubicin and other red inactivation products.

4.2.4. Preparation of cell lysates in anaerobic and aerobic conditions

R. planticola lysates were prepared by harvesting 1 L cultures of BHI broth containing 0.05% L-cys. All media were first equilibrated in the anaerobic chamber overnight to ensure anaerobic culturing conditions. A starter culture of 1-5% of the final culture's volume was inoculated with a colony of *R. planticola* on a BHI + 0.05% L-cys agar plate, and incubated overnight at 37°C, anaerobically, without shaking. The starter culture was then used to inoculate the 1 L flask(s) of BHI L-cys and incubated at the same conditions for six to eight hours. The culture was then poured into a 1 L centrifuge bottle, which were also pre-equilibrated anaerobically. The bottle was sealed by closing the lid with an O-ring tightly, and removed from the anaerobic chamber. Cells were then pelleted at 8000 x g, 4°C, for 20 minutes. The centrifuge bottle was returned to the anaerobic chamber prior to opening the bottle and

decanting the spent media to reserve the cell pellet. If stored, the pellet was sealed in the closed centrifuged bottle and frozen at -20°C.

In the anaerobic chamber, cells were then resuspended in 10-20 ml of HEPES assay buffer (50 mM HEPES, pH 7; 100 mM NaCl; 0.05% L-cys; anaerobic) and transferred to the 'Small Chamber' of the BioShop BeadBeater with approximately 10 ml of 0.1 mm glass beads. The Small Chamber was sealed, transferred outside of the anaerobic chamber, and placed in an ice-water enclosure. Cells were lysed by the BeadBeater in 30 second on-off intervals, for a total of four minutes of beating over eight minutes. The small chamber was opened in the anaerobic chamber and the cell lysate was decanted into 35 ml centrifuge tubes. The tubes were tightly closed and centrifuged for 20 minutes at 20 000 g, 4°C. In the chamber, the lysate was removed with a pipette and passed through a 0.45 μm filter to remove any remaining whole cells or cell debris. These lysates were then used for *in vitro* assays to determine doxorubicin inactivation activity.

Aerobically-prepared lysates were also made by following the above protocol with all steps taking place in aerobic conditions. Cultures were inoculated and incubated aerobically, and aerated by shaking at 250 rpm. L-cysteine was kept in the media to minimize variation between aerobically and anaerobically prepared lysates.

4.2.5. Protein quantification of cell lysates using the Bradford Assay

Ten microlitre dilutions of bovine serum albumin (BSA) standards and lysate samples were set up in duplicate and mixed with 200 μl of 1X Bradford reagent (BioRad) in a 96-well plate. Reactions were incubated for five to thirty minutes at room temperature. The absorbance at 595 nm of each well was determined with the SpectraMax Plus 384, and a standard protein concentration curve was generated using known concentrations of BSA to predict the concentration of proteins in the cell lysate samples.

4.2.6. Doxorubicin inactivation disc diffusion assay

An assay for doxorubicin inactivation using drug-soaked paper discs was modified from Mehta *et al.* (*119*). Six millimetre paper discs (BD) were submerged in 150 µg/ml doxorubicin in methanol for one minute, then air-dried at room temperature and stored at 4°C in the dark. To assay for *R. planticola* lysate activity, individual paper discs were placed in wells of a 96-well plate, and incubated with 200 µl of prepared lysates at 37°C for 24 hours, anaerobically. A no-lysate control using HEPES assay buffer, and a no-drug control using plain paper discs, were also prepared. After incubation, these discs were air-dried and assayed against the doxorubicin-sensitive organism *Micrococcus luteus* using a disc-diffusion assay.

M. luteus, which forms bright yellow colonies on agar plates, was inoculated in a 5 ml culture of trypticase soy broth (TSB), incubated at 37°C while shaking at 250 rpm, and grown to an OD_{600} of 0.1. Using a sterile cotton swab, the *M. luteus* culture was spread onto a TSB agar plate. The air-dried discs were then placed on top of the agar. Zones of inhibition on the lawn of *M. luteus* were measured after 36 hours of incubation at 30°C. The presence of these zones of inhibition would indicate the presence of doxorubicin, while the absence of these zones would suggest doxorubicin inactivation.

4.2.7. Doxorubicin inactivation liquid buffer assays

The ability of *R. planticola* lysates to inactivate doxorubicin was also shown in a liquid assay. Lysates were diluted by HEPES assay buffer as necessary, and added in a 1:1 ratio to HEPES assay buffer containing 150 µg/ml. Final concentrations of doxorubicin were thus 75 µg/ml.

This assay could be set up in many different conditions and formats. For the downstream chemical analysis of intermediates and products, reactions were set up with volumes between 400 µl to 1.0 ml in micro-centrifuge tubes. Serial dilutions of lysates were also prepared to study crude lysate activity and to identify the potential

need for a cofactor. Dilutions of the crude lysate could result in these cofactors becoming a limiting reagent in this reaction. These assays were set up in 96-well plates at 200 µl volumes. Additionally, cofactors NADH and NADPH could also be added to the reaction at a final concentration of 1 mM. Reactions were incubated in aerobic or anaerobic conditions at 37°C without shaking. The ability to inactivate doxorubicin was easily discernable by eye; assays could be photographed to demonstrate that the reaction had occurred.

4.2.8. HPLC and LC/MS analysis of doxorubicin inactivation products

Reaction products were visualized by HPLC analysis. Samples including doxorubicin-only and lysate-only controls, reaction supernatants, and reaction precipitates dissolved in methanol, were analyzed on the HPLC following the nine minute method described in 4.2.3. The identity of the precipitate was identified using liquid chromatography–mass spectrometry (LC/MS).

LC/MS analysis was performed using an Agilent 1100 Series HPLC system combined with an Applied Biosystems QTRAP LC/MS/MS. Samples were run on a SunFire™ analytical C18 column (5 µM, 4.6 x 50 mm) using a nine minute method similar to the method described above.

4.2.9. Preparation of a *R. planticola* transposon mutant library

Protocols were developed and optimized for the generation of a transposon mutant library in *R. planticola*. A strain of *E. coli* SM10 containing a *himar1* mariner transposon was kindly supplied by Dr. Brian Coombes at McMaster University. The transposon was located on a pFD1 plasmid, conferring both ampicillin and kanamycin resistance at 50 µg/ml and 25 µg/ml, respectively. The plasmid was purified from *E. coli* SM10 using the PureLink™ MiniPrep kit, following manufacturer's directions. DNA concentrations were quantified using the NanoDrop 2000 Spectrophotometer.

Electrocompetent cells were prepared by first growing a culture of *R. planticola* in BHI broth supplemented with 0.7 mM ethylenediaminetetraacetic acid

(EDTA). Cells were incubated aerobically at 37°C, shaking at 250 rpm, and harvested at an OD_{600} reading of 0.2 by centrifugation at 5000 g for 5 minutes at 4°C. Cells were then washed by resuspension in ice-cold 10% glycerol at the same volume of the original culture and centrifugation at the same conditions. Cells were washed three more times, before re-suspension in a final volume of one-hundredth of the original culture volume. Cells were aliquoted into pre-chilled, sterile micro-centrifuge tubes in 50 µl volumes and stored at -80°C.

Frozen aliquots of electrocompetent cells were thawed on ice, before the addition of purified pFD1 plasmid. Quantities of 0.1 to 1 µg of plasmid DNA per 50 µl aliquot were ideal. Sterile water or 10% glycerol was used as a negative control. Cells were then transferred to 1 mm gap, electroporation cuvettes (Fisherbrand®) and pulsed using the BIO-RAD MicroPulser™ using the Ec1 preprogrammed settings for a 1.8 kV pulse over 5.0 milliseconds. One millilitre of BHI broth, pre-warmed at 37°C, was immediately added to the cuvette. Cells were resuspended and transferred to a 1.5 ml micro-centrifuge tube and recovered at 37°C, shaking at 225 rpm for 1-3 hours.

After recovery, cells were pelleted at 5000 g for 2 minutes, and 800 µl of the supernatant was removed, leaving approximately 250 µl. The pellet was resuspended and 120 µl of cells were plated onto LB-agar plates containing 25 µg/ml kanamycin (Kan^{25}) using glass beads. Further dilutions and controls were also prepared. Plates were incubated at 37°C overnight, aerobically; colonies were counted after 24 hours of growth.

4.3. Anaerobic doxorubicin decolourization by *R. planticola* on solid media

Raoultella planticola, like other strains in the gut isolate collection described in Section 3.4, was able to decolourize doxorubicin when pinned onto a BHI agar plate. This activity was limited to anaerobic conditions, and can be observed clearly when streaked onto a BHI plate containing 150 µg/ml of doxorubicin and incubated aerobically and anaerobically (Figure 4-1).

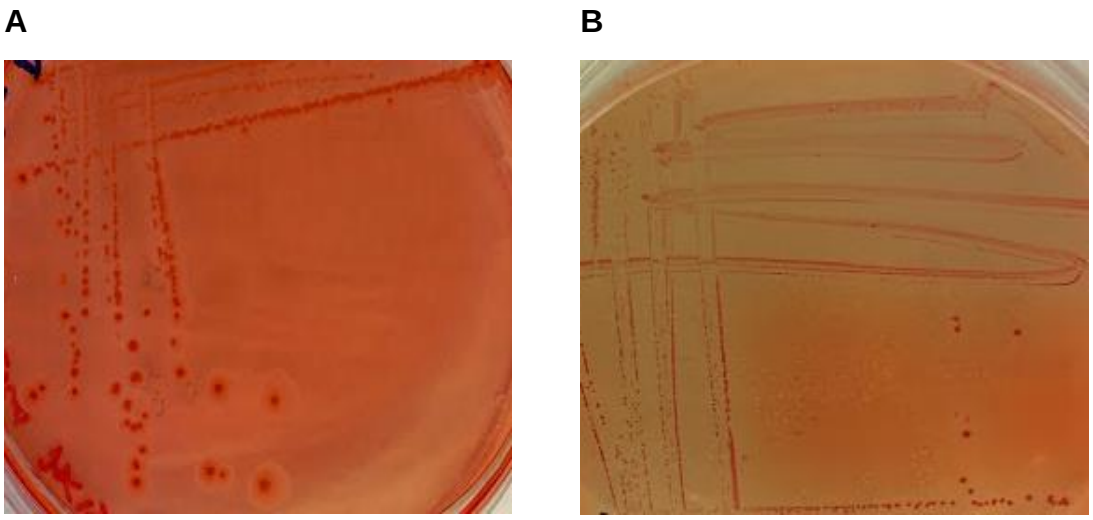


Figure 4-1: *R. planticola* grown on BHI agar containing doxorubicin in (A) aerobic and (B) anaerobic conditions.

R. planticola was streaked onto two BHI agar plates containing 0.05% L-cysteine and 150 µg/ml of doxorubicin. Both plates were incubated for 48 hours at 37°C, either in (A) aerobic or (B) anaerobic conditions. *R. planticola* colonies show a distinct polysaccharide capsule surrounding a dark red colony centre. The plate incubated anaerobically shows a distinct decolourization of the red drug around colonies that is not observed on aerobically-incubated plates.

In both cases, a red compound, likely to be either doxorubicin or a derivative, was localized to the centre of the *R. planticola* colonies. This interaction may be related to the high affinity of doxorubicin, a cationic anthracycline, to the anionic phospholipids in bacterial cell membranes (*99*). The dark red colonies on both plates suggest that *R. planticola* is able to sequester doxorubicin.

Yet, doxorubicin decolourization was specific to anaerobic conditions. No similar effect was seen in aerobically-incubated plates, even after incubation times of up to one week. These observations suggest doxorubicin may be metabolised or modified, though a process specific to anaerobic conditions. The ability for *R. planticola* to sequester doxorubicin may enable colonies to decolourize nearby doxorubicin. Furthermore, a plate of *R. planticola*, after 24 hours of aerobic incubation, did not show signs of decolourization when switched to anaerobic incubation

conditions, indicating that certain metabolic pathways active during cell growth may be necessary for doxorubicin inactivation. To enable further study of doxorubicin decolourization, this activity was studied in liquid culture, as described in Section 4.4.

4.4. Anaerobic doxorubicin decolourization by *R. planticola* cultures

To identify the degradation of doxorubicin over time, *R. planticola* was grown in liquid culture. Two types of media, the nutrient-rich BHI broth and the nutrient-poor M9 minimal media, were used to compare the strain's ability to inactivate doxorubicin in varying media types.

Figure 4-2 shows *R. planticola* growth curves and the degradation of doxorubicin over 80 hours in 1.5 ml micro-centrifuge tubes. Both media types showed cell growth in the first 8-16 hours that stayed constant for the remainder of the experiment. A higher final OD_{600} was observed in the BHI culture, possibly due to limited nutrients in M9 media. The increased absorbance at 480 nm in the first 16 hours was attributed to the *R. planticola* cells and eliminated by monitoring absorbance in the culture supernatant. Decolourization was observed in the BHI culture starting at 24 hours, with a steady decrease in absorbance at 480 nm from 1.3 to 0.3, resulting in light pink cultures after 72 hours. No significant decolourization was observed in M9 media.

These results can also be seen in photos of the cultures taken at 0 hours and 72 hours, shown in Figure 4-2C and D. These results suggest that a rich-nutrient media promotes anaerobic doxorubicin decolourization. Furthermore, cell pellets were red, indicating the affinity of doxorubicin and its potential metabolites for the cell membrane as demonstrated on solid media. Though the reaction was slow, the use of micro-centrifuge tubes and the tendency for *R. planticola* cells to tend to collect at the bottom of the tube may have limited exposure of the drug to the cells.

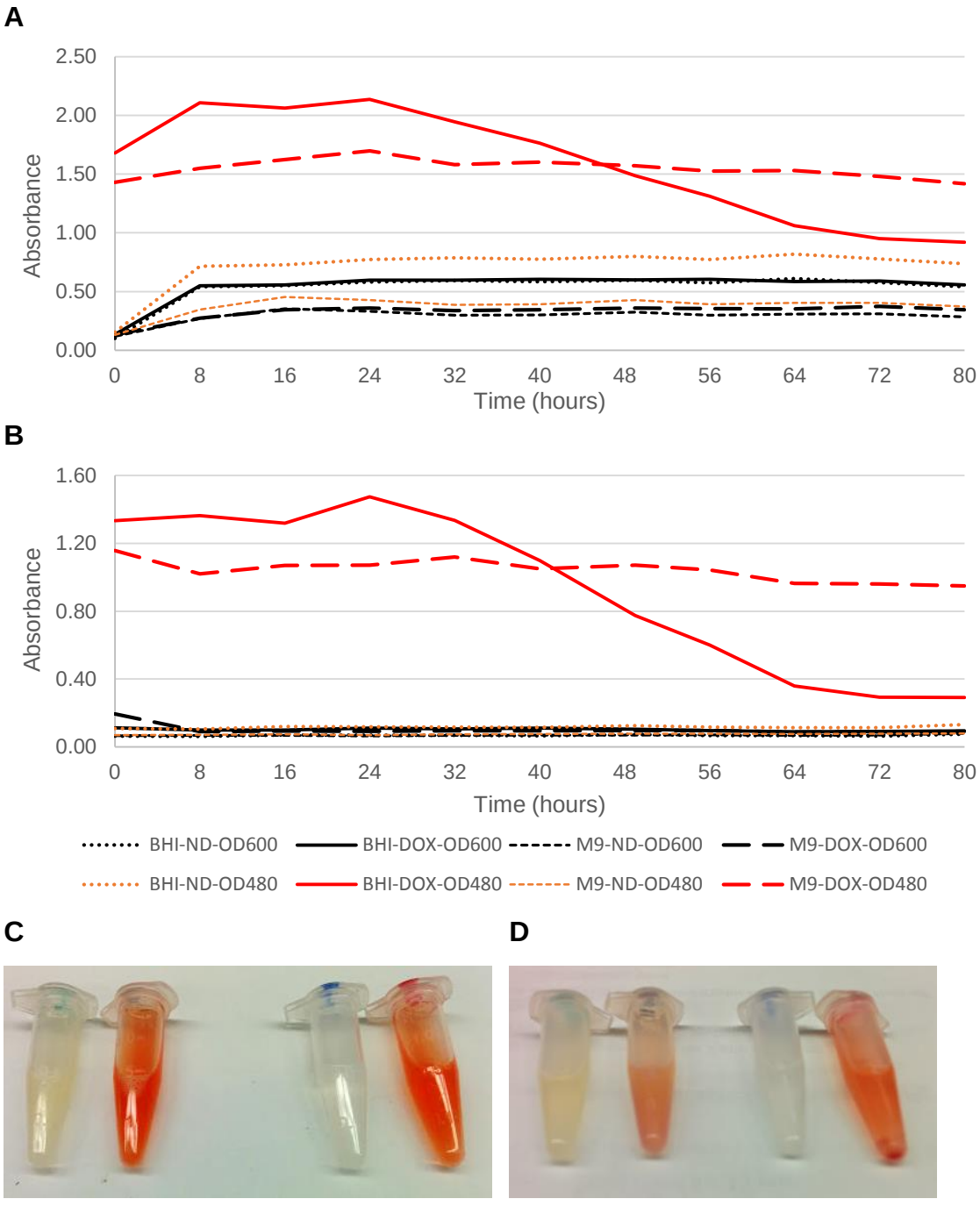


Figure 4-2: Doxorubicin degradation by *R. planticola* in BHI and M9 minimal media with glucose.

(A) Cell growth (absorbance observed at 600 nm, shown in black) and doxorubicin degradation (480 nm, red) in 1 ml *R. planticola* cultures in micro-centrifuge tubes over 80 hours in anaerobic conditions. (B) The same absorbance measurements were performed on the culture supernatants. Tubes were photographed at (C) 0 hours and (D) 72 hours; from left to right: BHI culture, BHI culture with doxorubicin, M9 culture, M9 culture with doxorubicin.

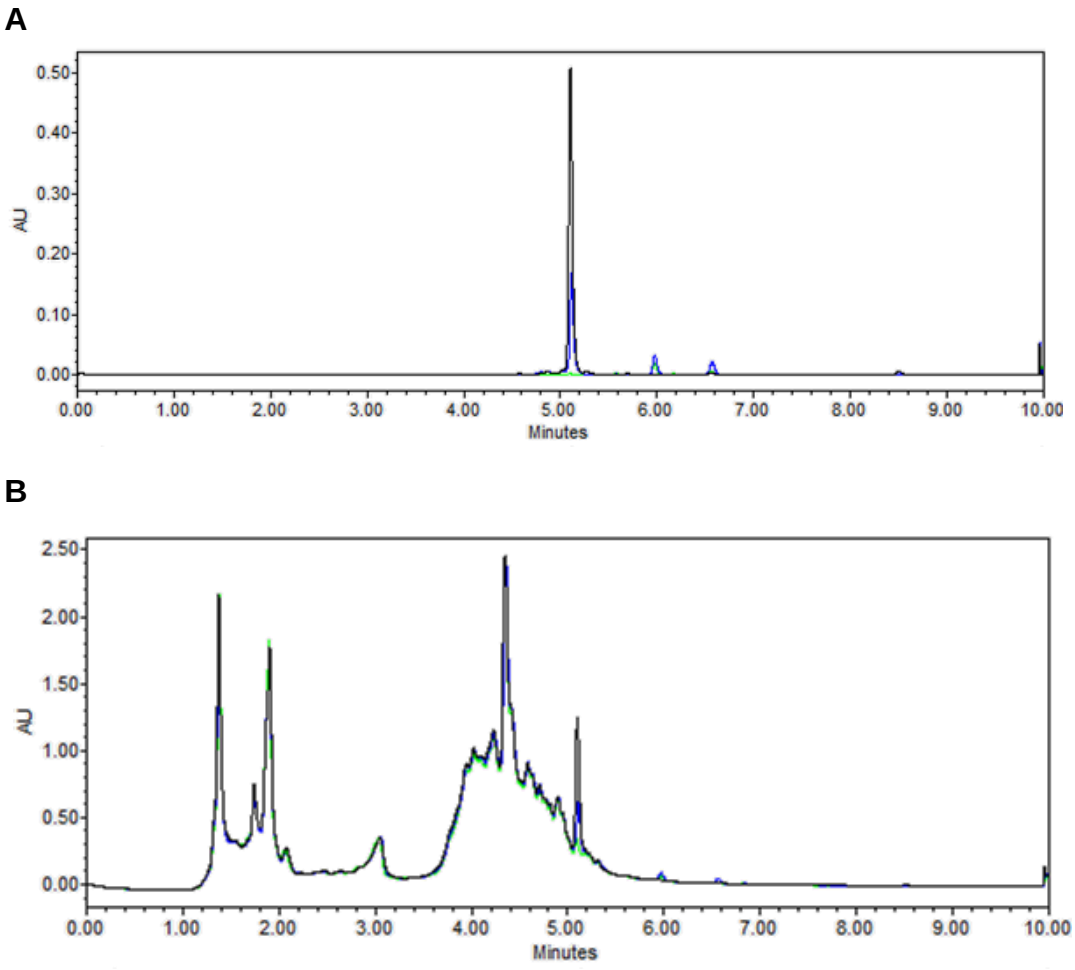


Figure 4-3: HPLC chromatograms showing doxorubicin inactivation by *R. planticola* conditioned media in BHI broth at (A) 480 nm and (B) 220 nm.

Supernatants of cultures taken at 0 hours (black), 48 hours (dark blue), and 80 hours (light green) were run on an Xselect CSH C18 column. (A) Chromatograms were extracted at 480 nm to observe doxorubicin and other possible derivatives. Doxorubicin was observed shortly after 5 min, while two products were observed at 6 min and 6.6 min after a 48 hour incubation, possibly representing 7-deoxydoxorubicinonol and 7-deoxydoxorubicinolone, respectively. (B) Chromatograms at 220 nm, among other wavelengths, were also extracted to identify possible further degradation products, but the complex media may be obscuring the observation of other possible peaks. AU: absorbance units.

Supernatants were analyzed by HPLC to identify possible degradation products, shown in Figure 4-3. Chromatograms extracted at 480 nm showed doxorubicin, which elutes at 5 minutes, being converted to compounds represented by peaks at 6 and 6.6 minutes, possibly representing 7-deoxydoxorubicinone and 7-

deoxydoxorubicinolone due to similarities in HPLC traces performed by Westman *et al.* (*56*). However, these peaks were significantly smaller than the doxorubicin peak in the no-lysate control. Doxorubicin and possible metabolites may have been sequestered by the *R. planticola* cells, converted to an insoluble form, thus precipitating with the cell pellet, or completed degraded and not identifiable on the HPLC trace.

The complexity of the rich BHI broth made identifying other potential degradation products difficult, as shown in Figure 4-3B. Various wavelengths were scanned for new peaks in the conditioned media, including at 220 nm, but no specific new peak was observed, possibly because the complex media could obscure the presence of smaller peaks. While doxorubicin inactivation was reproduced in other nutrient-rich media like lysogeny broth (LB) and trypticase soy broth (TSB), this activity was not observed in other nutrient-pool media including Bennett's media and M9 media supplemented with 0.4% glucose, acetate, or glycerol as carbon sources, that may have enabled the identification of other inactivation products. Instead, doxorubicin inactivation by cell lysates was used to reduce the HPLC footprint of the media and also test if this reaction could be replicated *in vitro*.

4.5. Anaerobic doxorubicin inactivation by *R. planticola* cell lysates

Cell lysates were prepared in anaerobic conditions by harvesting *R. planticola* cultures in BHI media. Cells were lysed by glass bead beating to maintain anaerobic conditions, in a HEPES assay buffer (50 mM HEPES, pH 7; 100 mM NaCl; 0.05% L-cys; anaerobic) to maintain a constant pH and ionic strength and support a reducing environment. Lysates were then studied for its ability to inactivate doxorubicin using a disc diffusion assay and a liquid solution-based assay.

4.5.1. Demonstration of cell lysate activity using a disc-diffusion assay

When incubated with cell lysates, doxorubicin-soaked discs changed from a red colour to a lighter, orange colour, as seen in Figure 4-4A between the middle and top rows. When these discs were plated on a lawn of *M. luteus*, the doxorubicin-containing disc incubated with lysate had no zone of inhibition, while the no-lysate control had a zone of 9 mm, suggesting doxorubicin inactivation. The no-drug disc incubated with lysate had no zone of inhibition, indicating that the crude lysate on its own had no significant inhibitory activity against *M. luteus*. This experiment demonstrates that the *R. planticola* lysate is capable of inactivating doxorubicin, potentially to a product with an orange-red colour.

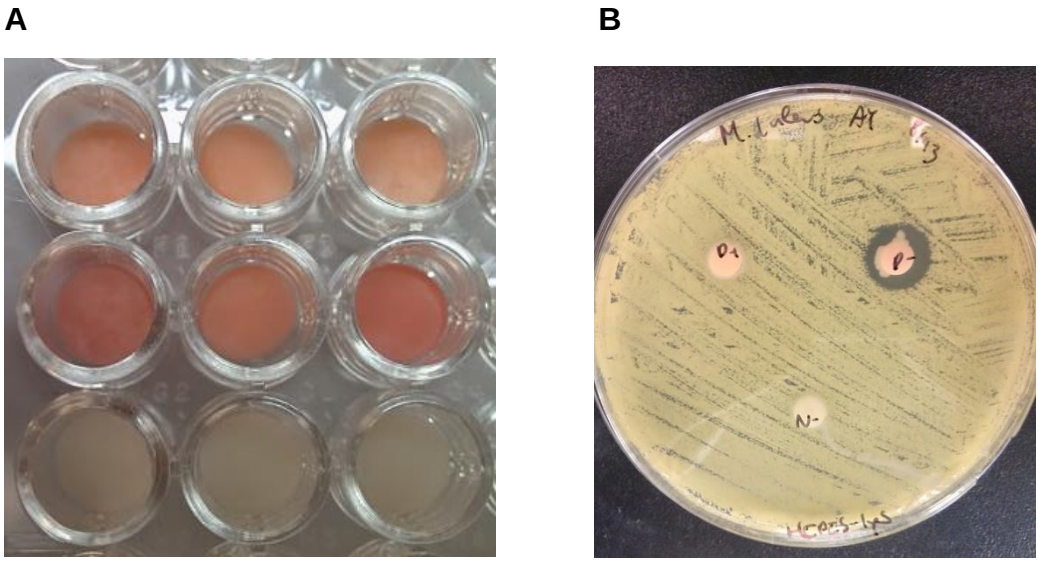


Figure 4-4: *R. planticola* crude lysate inactivates doxorubicin on paper discs.

Cell lysates of *R. planticola* were prepared anaerobically and assayed for activity using doxorubicin-containing filter discs. Discs were soaked in 150 µg/ml DOX in methanol for one minute, dried, and (A) incubated with *R. planticola* lysates in triplicate. The top row of DOX-containing discs were incubated with lysate; the middle row of DOX-containing discs were incubated with HEPES assay buffer; the bottom row of discs with no drug were incubated with lysate. (B) A set of these discs were placed onto a TSB-agar plate streaked with doxorubicin-sensitive *M. luteus*, with the doxorubicin + lysate disc in the top left, no-lysate control in the top right, and the no-drug control on the bottom. The plate was incubated at 30°C for 48 hours, aerobically.

4.5.2. Demonstration of cell lysate activity using a liquid assay

Lysates were added to assay buffer with and without doxorubicin, incubated at 37°C anaerobically and analyzed by high-pressure liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC/MS). Figure 4-5 shows doxorubicin inactivation in reactions containing 50% or 10% lysate, or 0.14 mg/ml and 0.028 mg/ml of protein, respectively.

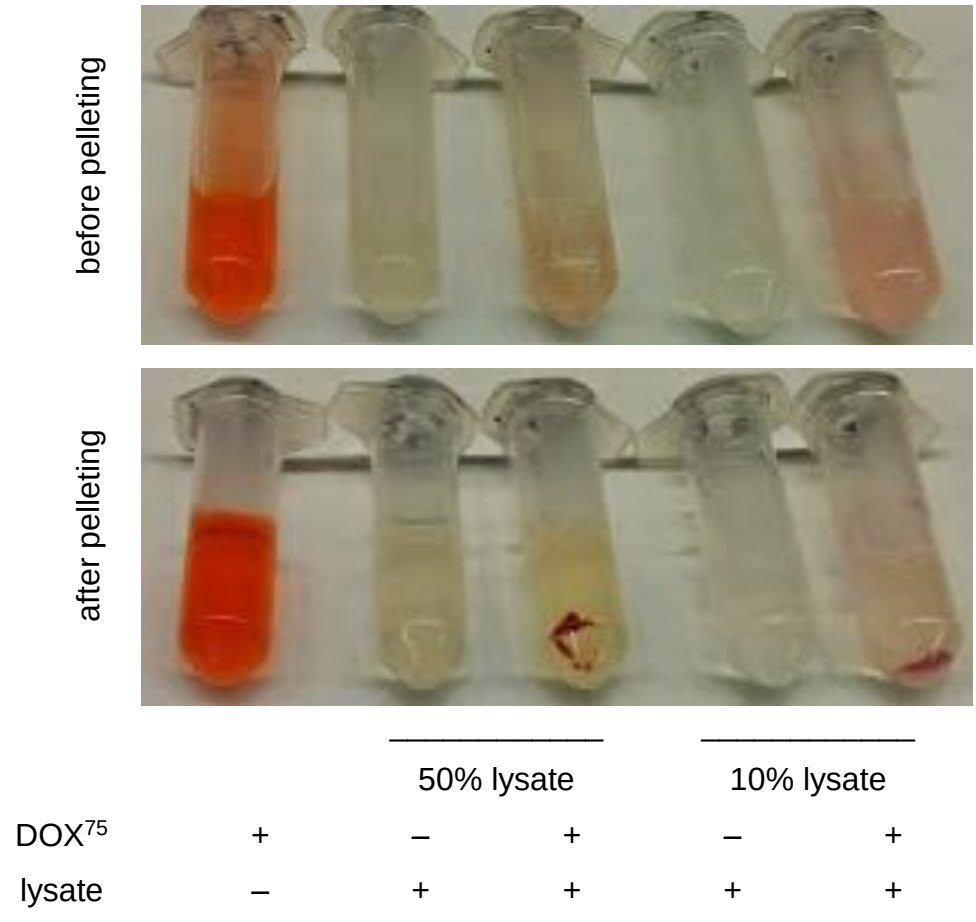


Figure 4-5: *R. planticola* crude lysate inactivates doxorubicin in a liquid assay.

Cell lysates of *R. planticola* were prepared anaerobically and added to HEPES assay buffer at either 10% or 50% of the final volume. Doxorubicin was added at a final concentration of 75 μg/ml. Drug-only and lysate-only controls were also prepared, and all reactions were incubated anaerobically at 37°C for 24 hours. Pictures were taken before and after spinning down the precipitate.

While the left tube representing a no-lysate doxorubicin control remained bright red, doxorubicin was converted to an insoluble precipitate by both lysate reactions, appearing cloudy before centrifugation and forming a dark red pellet afterwards. These pellets could be dissolved in methanol, resulting a dark orange-red solution, and were analyzed on the HPLC along with the supernatant and controls with no-drug or no-lysate, as described in Section 4.5.5.

4.5.3. Doxorubicin inactivation requires anaerobic lysate preparations and assay conditions

Section 4.5.2 describes the activity of the cell lysate when *R. planticola* was grown and lysed anaerobically, and incubated with doxorubicin anaerobically. The cell lysate assay was repeated with lysates prepared aerobically and anaerobically, with both aerobic and anaerobic incubation conditions. Assays in anaerobic conditions were unable to inactivating doxorubicin, suggesting that the reaction cannot occur in the presence of oxygen. This result could be due to oxygen-sensitive enzymes, cofactors, or intermediates that prevent the reaction mechanism from converting doxorubicin to its aglycone form.

Lysates prepared aerobically were also inactive, even after over two weeks of incubation with doxorubicin. Thus, a doxorubicin inactivation assay with an aerobically prepared lysate cannot be 'rescued' by anaerobic incubation. This result suggests that anaerobic growth conditions may induce metabolic changes in *R. planticola*, such as variations in gene expression, that allow the anaerobically prepared cell lysates to be capable of doxorubicin inactivation. Coupled with knowledge that *R. planticola* inactivates doxorubicin most effectively in rich media, anaerobic metabolic genes are hypothesized to be responsible for this mechanism.

4.5.4. Anaerobic doxorubicin inactivation by cell lysates may involve oxygen-sensitive compounds

While supernatants in these reactions were usually colourless, supernatants with high lysate concentration tended to yield a yellow colour that was not present in the lysate-only control, as seen above between the second and third tubes in Figure 4-5. Further experimentation showed that this observation was specific to anaerobic conditions, as the supernatant would turn pink within an hour after exposure to oxygen. This phenomenon is shown in Figure 4-6 and further suggests that this reaction may involve oxygen-sensitive intermediates or components that explain the need for anaerobic conditions for doxorubicin inactivation. This effect was also observed in another liquid assay that was photographed anaerobically (Figure 4-9), which used serial dilutions of crude lysates; the supernatant is more yellow in reactions with high lysate concentrations.

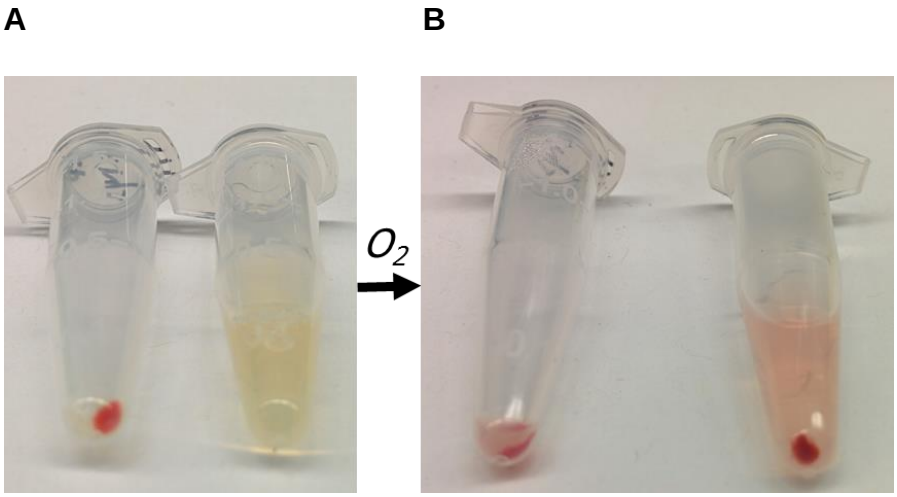


Figure 4-6: Anaerobic doxorubicin inactivation by high concentrations of *R. planticola* lysate yields an oxygen-sensitive yellow reaction supernatant.

Doxorubicin was incubated with *R. planticola* crude lysates in HEPES assay buffer (1:1 buffer to lysate ratio) with a final protein concentration of 0.14 mg/ml. (A) The reaction yielded a dark red precipitate and a yellow supernatant which was separated by centrifugation and decanting. (B) This supernatant turned pink when exposed to oxygen and resulted in further precipitation of a dark red pellet, shown above after centrifugation.

4.5.5. HPLC analysis of reaction products

Doxorubicin inactivation products from the cell lysate assays were analyzed by HPLC, yielding similar results as the cell culture supernatants in Figure 4-3 but with much clearer peaks of doxorubicin and its inactivation products. These results are shown in Figure 4-7, which compares a no-lysate doxorubicin control with its main peak at 5 minutes and the reaction precipitate, with peaks at 6 and 6.6 minutes, corresponding to 7-deoxydoxorubicinol and 7-deoxydoxorubicinolone as described earlier. The cell lysate and a clear reaction supernatant were also analyzed with no clear peaks observed at 480 nm. Other wavelengths were scanned for the production of a new peak, but no such peak was observed.

Of the two main peaks in the reaction precipitate, the latter peak was much larger, indicating the predominant conversion of doxorubicin to 7-deoxydoxorubicinolone. This product was confirmed with the use of mass spectrometry.

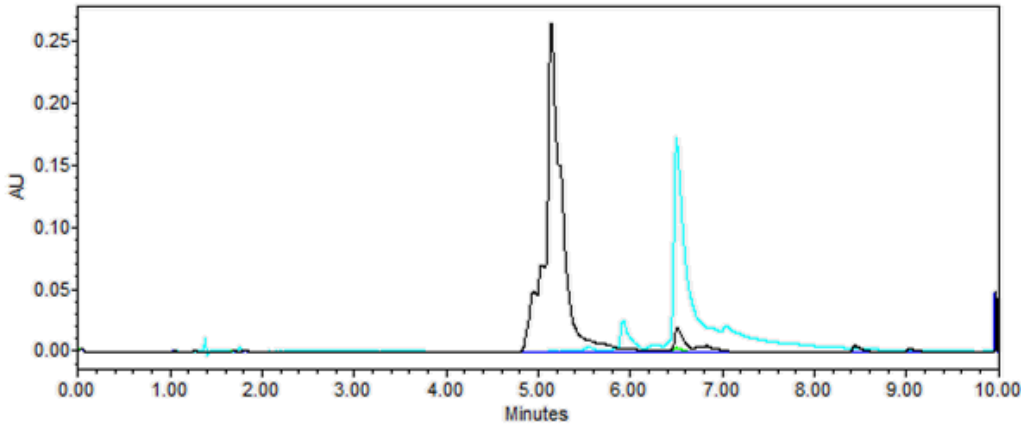


Figure 4-7: HPLC analysis of doxorubicin inactivation by *R. planticola* lysates.

Doxorubicin was incubated with cell lysate in assay buffer, yielding a supernatant and red precipitate, which was then dissolved in methanol. 4 samples were run on a CSH C18 column: a no-lysate doxorubicin control (black); a lysate-containing, no-drug control (dark blue); the clear reaction supernatant (light green); and the precipitate dissolved in methanol (light blue). Chromatograms were extracted at 480 nm to observe doxorubicin and other possible derivatives. AU: absorbance units.

4.5.6. LC/MS analysis of reaction products

Liquid chromatography–mass spectrometry was also used to confirm the identity of the doxorubicin aglycone product. The precipitate, dissolved in methanol, was applied on the Applied Biosystems QTRAP LC/MS/MS. The mass of the suspected 7-deoxydoxorubicinolone peak was 399.42 m/z in positive ion mode $[M+H]^+$ and 397.44 m/z in negative ion mode $[M-H]^-$.

These results fit the expected mass of 7-deoxydoxorubiciolone of 398.4 g/mol, and suggest that this compound is the primary product of anaerobic doxorubicin inactivation by *Raoultella planticola*, similar to the NuoEFG-mediated reaction described by Westman *et al*. under aerobic conditions (*56*). To ensure that this reaction was not caused by the same NADH dehydrogenase, the following section studies the effects of NADH on anaerobic doxorubicin inactivation by *R. planticola*.

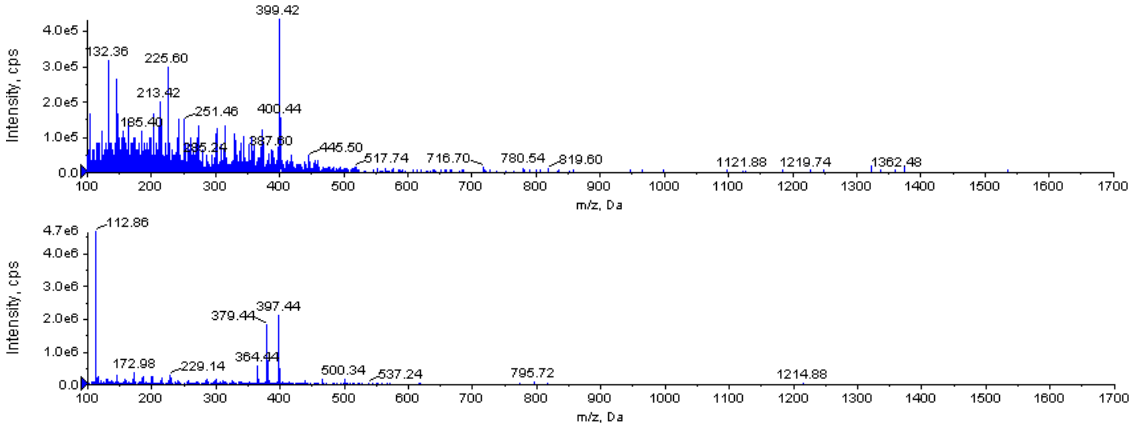


Figure 4-8: Liquid chromatography–mass spectrometry analysis of 7-deoxydoxorubicinolone.

The precipitate from anaerobic doxorubicin inactivation by *R. planticola* cell lysates was dissolved in methanol and run on the Applied Biosystems QTRAP LC/MS/MS system. The ion fragments of the main peak are shown above, and match the mass of 7-deoxydoxorubicinolone, 398.4. The graph on top shows the mass fragments in positive ion mode; the bottom shows negative ion mode.

4.6. Cell lysate activity is independent of NADH or NADPH

The *R. planticola* cell lysate doxorubicin inactivation assay set up in Section 4.5.2 used a drug concentration of 75 μg/ml, equivalent to 138 μM of doxorubicin. In other Gram-negative bacteria, such as *E. coli* and *Salmonella*, the concentration of intracellular NADH is around 20 μM (*120*). Wimpenny and Firth measured levels of NADH in both anaerobic and aerobic conditions, suggesting that almost all of the bacteria screened had 1.4 to 1.9 μmol of NADH per gram of dry cell mass, including in strains of *Klebsiella* (*121*). Either of these estimates indicate that the final NADH concentration in the cell lysate should be significantly less than 20 μM. As a result, given that the assays further dilute the cell lysates, the doxorubicin concentration should exceed the NADH concentration by at least tenfold even with undiluted cell lysates. The observation that the reactions can inactivate all the doxorubicin present in the assay buffer, suggest that this reaction is NADH-independent.

Additionally, the doxorubicin inactivation assay was performed in the presence and absence of added NADH and the related cofactor NADPH (nicotinamide adenine dinucleotide phosphate). Reactions with a final doxorubicin concentration of 75 μg/ml in HEPES assay buffer with a serial dilution of cell lysates were prepared, from 2X dilution to 1024X dilution. One set of reactions was set up without cofactor while two other sets were supplemented with a final concentration of 1 mM NADH or NADPH. Results are shown in Figure 4-9.

If the reaction was dependent on NADH from the crude lysates, dilutions would force NADH to become the limiting reagent, and doxorubicin inactivation would no longer be observed when NADH concentrations were too low. Supplemented cofactor would thus restore doxorubicin inactivation at low lysate concentrations. Yet, added NADH or NADPH was unable to restore decolourization, suggesting that anaerobic doxorubicin occurs independently of NADH dehydrogenase. This experiment was repeated alongside lysates prepared from other strains in Section 5.3 to differentiate *R. planticola* doxorubicin inactivation from other cofactor-dependent reactions.

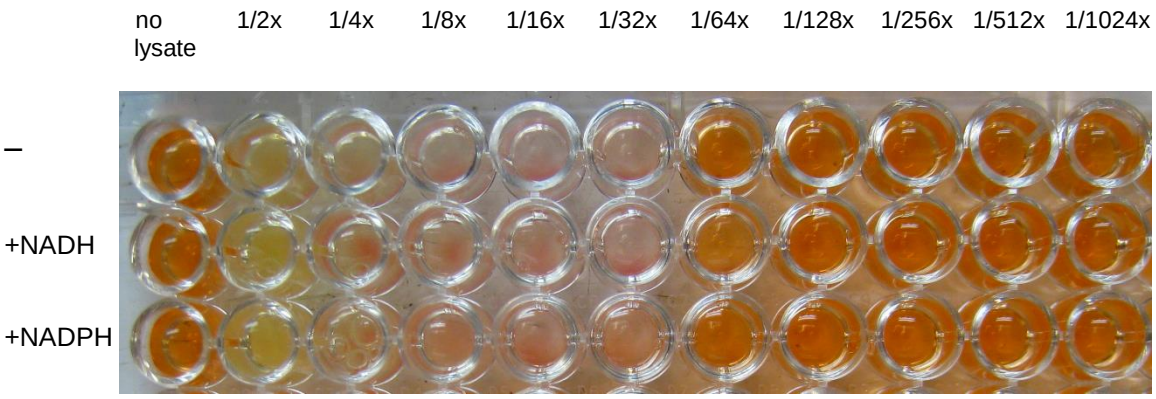


Figure 4-9: Doxorubicin inactivation by *R. planticola* lysates is independent of NADH and NADPH.

R. planticola lysates were anaerobically prepared and diluted serially from 1X to 1/512X in HEPES assay buffer. 100 µl of lysate was added to 100 µl of HEPES assay buffer containing 150 µg/ml, for a final concentration of 75 µg/ml with lysate ranging from 1/2x to 1/1024 dilutions. Reactions were also set up with NADH or NADPH cofactor supplemented at 1 mM. The plate was photographed after incubation at 37°C for 48 hours, anaerobically. This figure also demonstrates the production of the yellow, oxygen-sensitive compound in reactions with high lysate concentrations.

4.7. Next steps: finding the enzyme responsible for doxorubicin inactivation

In this chapter, the ability for *Raoultella planticola* to inactivate doxorubicin was observed and characterized *in vivo* and *in vitro*. The reaction primarily converted doxorubicin to its aglycone form, 7-deoxydoxorubicinolone, and required anaerobic conditions, but did not need NADH or NADPH cofactors. Doxorubicin inactivation *in vivo* also required a rich-nutrient medium. These observations suggest that NuoEFG is not involved in anaerobic doxorubicin inactivation, and that the reaction mechanism may be related to anaerobic metabolism and involve oxygen-sensitive cofactors, enzymes, or intermediates.

However, the specific enzyme(s) required for this reaction remains to be elucidated. The next steps for this project aimed to identify this enzyme using three different approaches: activity-guided protein purification and the analysis of reaction products; the generation of a transposon mutant library in *R. planticola*; and

observing doxorubicin inactivation in other strains, including related Enterobacteriaceae. The first two aims are summarized below, while the third approach was most successful and described in detail in Sections 5.4 and 5.5.

4.7.1. Further characterization of anaerobic doxorubicin inactivation in *R. planticola*

The use of activity-guided purification and further chemical analysis of inactivation products are often used to identify enzymes involved in a reaction. This process involves using purification techniques to fractionate proteins, coupled with assays to identify fractions able to inactivate doxorubicin; these steps would be repeated until the enzyme responsible for inactivation was pure enough for determination by techniques such as protein fingerprinting by mass spectrometry. Activity-guided purification was used to identify NuoEFG as the enzyme complex responsible for bacterial doxorubicin inactivation in *Streptomyces* (*56*).

However, these protocols were difficult to follow because the reaction required a strict anaerobic environment. Crude lysates that were exposed to oxygen for an hour were unable to inactivate doxorubicin, thus requiring all purification steps to take place in the anaerobic chamber, while many robust purification techniques involve chromatography protocols that were impractical to set up anaerobically. Preliminary experiments using gravity flow columns such as desalting columns were performed but did not produce consistent results. These protocols may still be used in the future to purify the active enzyme, but using other tools to first identify a shortlist of potential enzymes was more practical.

The identification of reaction intermediates in addition to the aglycone products would also provide information that could narrow down possible reaction mechanisms. One possible intermediate was the compound responsible for the oxygen-sensitive yellow colour described in Section 4.5.4. Reaction supernatant samples were sealed anaerobically in glass vials or exposed to oxygen, and run on the

Bruker micrOTOF II mass spectrometer, but oxygen present in the buffer system may have interfered with results. A peak with the mass of 325.3 g/mol appeared to be more prominent in the anaerobic sample, but was still present in the aerobic sample at lower concentrations. Furthermore, the presence of this compound only in reactions with high lysate concentrations suggested that this intermediate may be a non-specific interaction with other lysate components and not specific to doxorubicin inactivation. Thus, these experiments were not pursued further for this project, but the use of these tools will be essential in elucidating a reaction mechanism once a potential doxorubicin-inactivating enzyme is identified.

Lastly, the further degradation of 7-deoxydoxorubicinolone may be possible. While no other peaks were observed at an absorbance of 480 nm, the aglycone product could be further broken down to products not observed at the scanned wavelengths. Quantifying the reaction products is a key step to determining if any further metabolism occurs, and could help to explain the observed decolourization phenomenon. This reaction might use a different mechanism and enzyme than the first, doxorubicin inactivation reaction, and illustrates the complexity of host-microbial metabolism.

4.7.2. Generating a transposon mutant library in *R. planticola*

Another method that could help identify the doxorubicin inactivating enzyme(s) involved the preparation of a transposon mutant library. The rationale of these experiments was to insert random mutations into *R. planticola* genome using the *himar1* mariner transposon system (*122*). A library of gene knockout strains would be produced and screened for their ability to decolourize doxorubicin anaerobically. Strains unable to demonstrate this activity would lack the genes essential for this reaction. Identifying these genes would help elucidate the reaction mechanism. The protocols used to develop these mutants using electroporation are described in Section 4.2.9.

No techniques for transformation by electroporation in *Raoultella* were previously described. Thus, electroporation techniques were adapted from the BIO-RAD MicroPulser™ manufacturer protocols and other publications that had optimized techniques for *E. coli* and other *Klebsiella* species (*123*). While initial experiments yielded no transformants, the addition of 0.7 mM EDTA to the culture media was found to be essential for successful electroporation. Adding EDTA is believed to inhibit the production of exopolysaccharides that cause low transformation efficiency (*124*). Furthermore, various optimizations showed that harvest cells at an OD_{600} reading of 0.2 yielded more efficient electrocompetent cells compared to cells harvested at an OD_{600} of 0.6 or 1.0, while adding 0.1 to 1 µg of plasmid DNA was ideal for transformation of about 80 transformants per 50 µl reaction. Further optimization could be identified to increase efficiency for the preparation of a transposon library.

After identifying anaerobic doxorubicin inactivation by *E. coli* BW25113 as described in Section 5.2.3, the KEIO knockout collection was used as a better characterized tool for the identification of a doxorubicin-inactivating enzyme. Yet, these techniques can be used for any future genetic studies in *Raoultella planticola* that may require preparation of electrocompetent cells or a transposon mutant library.

4.7.3. Applications of characterizing doxorubicin inactivation

Overall, the protocols described in this chapter demonstrate the many methods that can be used to verify drug inactivation and identify a drug inactivation mechanism, and thus can be broadly applied to study other drug-microbiome interactions. While identification of inactivation requires only a relatively quick assay, whether through a disc assay or HPLC analysis, characterizing a reaction mechanism requires various protocols that need to be adapted to each reaction and is the rate-limiting step in this platform. Yet, the growing knowledge base on the antimicrobial resistome and other mechanisms of microbial drug inactivation contribute to elucidating potential reactions that may be occurring in the gut microbiota.

Studying doxorubicin inactivation by *R. planticola* has enabled the identification of various conditions that are essential for this reaction. The requirement for anaerobic conditions and the need for a rich nutrient environment indicate that the intestinal tract may provide appropriate conditions for this interaction to occur in the gut. In future studies, these experiments need to be repeated in an *in vivo* system to study the role of this drug-microbiome interaction on doxorubicin pharmacology in the complex host-microbe system.

Chapter 5.
Studying doxorubicin inactivation in Enterobacteriaceae

5.1. Key objectives

The previous three chapters described the development of a library of gut isolates, a screen for drug-resistance and inactivation, and the identification of *R. planticola* and its ability to convert doxorubicin to inactive, aglycone forms. Because not much is known about *R. planticola*, other well-characterized strains can be used as resources for identifying the mechanism of doxorubicin inactivation. Strains also able to inactivate doxorubicin anaerobically may share the same reaction mechanism, while strains that are unable to show this same activity provide clues as to which genetic components and strain characteristics are essential for doxorubicin.

Three main approaches using other strains were taken to help elucidate the mechanism for anaerobic doxorubicin inactivation. First, *Raoultella planticola* lysates were compared with the lysates of other organisms in unrelated families that were able to inactivate doxorubicin, to observe if the same inactivation activity was widespread (Section 5.3). The ability of *R. planticola* to inactivate doxorubicin in the absence of added NADH or NADPH cofactors was a key component in distinguishing the anaerobic inactivation mechanism from other reactions likely mediated by the previously described NADH dehydrogenase.

The second approach observed the ability for other strains in the family Enterobacteriaceae, which includes *Raoultella*, *Klebsiella*, and *Escherichia*, to inactivate doxorubicin (Section 5.4). Similar to the first approach, these studies would help identify genotypic or phenotypic traits that may be important for this reaction mechanism. Through these experiments, *E. coli* BW25113, the strain used to generate the KEIO single-gene knockout collection, was found to inactivate doxorubicin. Thus, a third approach utilized the KEIO knockout collection to identify genes responsible for doxorubicin inactivation (Section 5.5).

These studies provided clues and insight into possible inactivation mechanisms, and have enabled the identification of a putative set of molybdopterin-dependent enzymes that may be responsible for anaerobic doxorubicin inactivation in *Raoultella planticola*.

5.2. Material and methods

5.2.1. Media and assay conditions

The following media conditions were used to culture *R. planticola* and other strains to test for their ability to inactivate doxorubicin. Anaerobic handling and incubation of samples took place in the Bactron IV 600 SHEL LAB Anaerobic Chamber at the Surette laboratory. L-cysteine was added to all liquid media, solid media, and assay buffers at a final concentration of 0.05% w/v.

Media, assay buffers, and supplements	Supplier
brain heart infusion, modified	BD (BBL™)
L-cysteine	Aldrich
doxorubicin	AK Scientific
agar	Fisher
methanol	Caledon
NaCl (sodium chloride)	Fisher
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	BioShop
glycerol	Caledon

Table 5-1: List of media and supplements used in Chapter 5.

These reagents were used in the experiments described in Chapter 5. Their suppliers are listed above.

5.2.2. Comparing doxorubicin inactivation by different cell lysates

To compare the activity of *R. planticola* doxorubicin inactivation to other organisms, two other strains resistant to 50 μg/ml doxorubicin were selected from

the gut isolate screen and identified by 16S rDNA sequencing to be *Ruminococcus* and *Coprococcus* species using the protocol described in Section 3.3.4. Cell lysates were prepared following the anaerobic lysate protocol described in Section 4.2.4. The strains were each harvested from 1 L of BHI + 0.05% L-cys and lysed by glass bead beating. Future experiments could use desalting purification procedures to remove any cofactors that could be involved in downstream reactions.

The doxorubicin-inactivating activity of *Raoultella*, *Ruminococcus*, and *Coprococcus* cell lysates were compared. All lysates had been prepared within two weeks of this assay and stored at -20°C. Concentration determination by the Bradford assay indicated that all three strains had comparable protein levels in the prepared crude lysates. In a 96-well plate, various serial dilutions of each cell lysate were prepared, diluted to 4X, 16X, 64X, and 256X in HEPES assay buffer (50 mM HEPES, 100 mM NaCl, 0.05% L-cys). A no-lysate control was also prepared. By diluting the lysate, any cofactors that may be involved in the reaction mechanism would be diluted and become the limiting reagent. Thus, reduced doxorubicin inactivation would be expected with each serial dilution of cell lysate. If the reaction mechanism required NADH or NADPH, doxorubicin inactivation activity would be restored with the supplemented cofactors.

At each lysate concentration, four reactions were set up: lysate with DOX^{75} (75 µg/ml); lysate with DOX^{75} and 1 mM NADH; lysate with DOX^{75} and 1 mM NADPH; and lysate only, without cofactors or doxorubicin. The plate was incubated at 37°C for 48 hours. The resulting plate was photographed to visually observe doxorubicin inactivation.

5.2.3. Comparing doxorubicin inactivation in other Enterobacteriaceae

Three other strains in addition to *Raoultella planticola* were streaked from glycerol stocks onto BHI agar containing 0.05% L-cysteine: *Klebsiella pneumoniae* subsp*. pneumoniae* (ATCC® 22495™), *Escherichia coli* BW25113, and *Escherichia coli*

BL21(DE3). Each strain was incubated aerobically at 37°C for 24 hours. These plates were then transferred to the anaerobic chamber and streaked onto BHI-DOX^{150} agar plates with 0.05% L-cysteine and incubated anaerobically at 37°C. Another set of plates were also prepared for aerobic incubation. Plates were observed at 24 hours and then at 48 hours, when the plates were removed from the anaerobic chamber and photographed.

Cell lysates were also prepared from *E. coli* BW25113 to confirm *in vitro* doxorubicin inactivation following the anaerobic lysate protocol described in Section 4.2.4. Lysates were incubated with doxorubicin in HEPES buffer, and reaction products were visualized on the HPLC to confirm that its reaction mechanism was similar to that of *Raoultella planticola*.

5.2.4. Screening the KEIO collection for doxorubicin inactivation

The *E. coli* KEIO knockout collection was plated at 1536 pin density by Jean-Philippe Cote from Dr. Eric Brown's laboratory. BHI DOX^{200} agar were poured into Singer PlusPlates™ at 25 ml/plate, cooled at room temperature, and air-dried in a sterile flow-hood for 30 minutes. The ROTOR™ HDA (Singer Instruments) was used to replicate the three KEIO knockout collection source plates onto the BHI DOX^{200} agar plates in triplicate. These plates were then immediately transferred into the anaerobic chamber and incubated at 37°C. A set of plates was removed at 9 hours, 12 hours, and 16 hours and scanned for analysis. Strains that were unable to decolourize doxorubicin were considered to be strains of interest.

5.3. Doxorubicin inactivation in *R. planticola* is unique from NADH-dependent mechanisms in other gut strains

The ability of *R. planticola* crude lysates to inactivate doxorubicin were compared to the cell lysates derived from other resistant gut bacteria. These strains were cultured and identified as *Coprococcus* and *Ruminococcus* species by 16S rDNA

sequencing. Lysates were prepared from 1 L cultures and assayed for doxorubicin inactivation in a lysate dilution experiment with the addition of both NADH and NADPH, the former cofactor involved in the bacterial doxorubicin inactivation described by Westman *et al.* (*56*). Figure 5-1 shows the differences between *R. planticola* and these two Gram-positive strains. First, despite similar protein concentrations, the *R. planticola* lysates were significantly more active than the other two lysates, shown by decolourization at 1/64X dilutions, while only the 1/4X dilution in the Gram-positive organisms had noticeable decolourization. Furthermore, *R. planticola* activity was not boosted by the presence or absence of NADH or NADPH. Comparatively, at 4X and 16X dilutions, both *Coprococcus* and *Ruminococcus* appear to have increased activity when cofactors were supplemented.

In all, this result suggests that *Coprococcus* and *Ruminococcus* strains utilized an NADH or NADPH-dependent mechanism to degrade doxorubicin, much like the observed bacterial inactivation in *Streptomyces*. On the other hand, *R. planticola* inactivated doxorubicin in an NADH or NADPH independent manner, as suggested in Section 4.6 and thus involves a different inactivation mechanism. This experiment also showed that this mechanism is specific to *Raoultella* and not widespread among the gut isolate collection, though the ability for other similar strains to inactivate doxorubicin may be possible and is described in the following section.

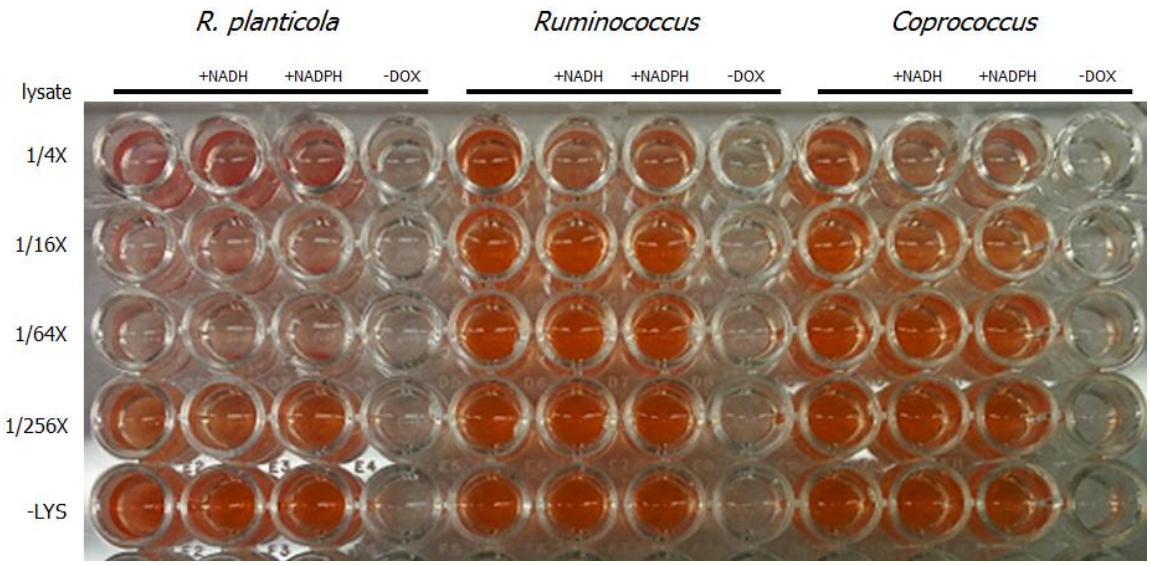


Figure 5-1: Comparison of anaerobic doxorubicin decolourization by *Raoultella*, *Ruminococcus*, and *Coprococcus* lysates.

Crude lysates were prepared by harvesting cells, glass bead beating, and centrifugation to remove cell debris. Lysates were diluted at 4X, 16X, 64X, and 256X, with the preparation of a no lysate control in HEPES assay buffer. For each dilution, four reactions were set up: lysate with DOX^{75} (doxorubicin at 75 µg/ml); lysate with DOX^{75} and 1 mM NADH; lysate with DOX^{75} and 1 mM NADPH; and lysate only (no cofactors or doxorubicin). The plate was incubated at 37°C for 48 hours and photographed.

5.4. Doxorubicin inactivation in other Enterobacteriaceae

Other members of the family Enterobacteriaceae, *Klebsiella pneumoniae* and various strains of *Escherichia coli*, were also grown on BHI-DOX^{150} agar plates. *Klebsiella* and *Raoultella* species share many similar characteristics (*115*). Figure 5-2 shows *R. planticola* and *K. pneumoniae* when streaked onto BHI-DOX^{150} agar and incubated in anaerobic conditions over 48 hours. As expected *R. planticola* showed its characteristic zone of doxorubicin decolourization around its colonies. Yet, *Klebsiella pneumoniae* showed zones of clearing that were even more distinct, clearly visible at 24 hours (not shown) and even larger at 48 hours.

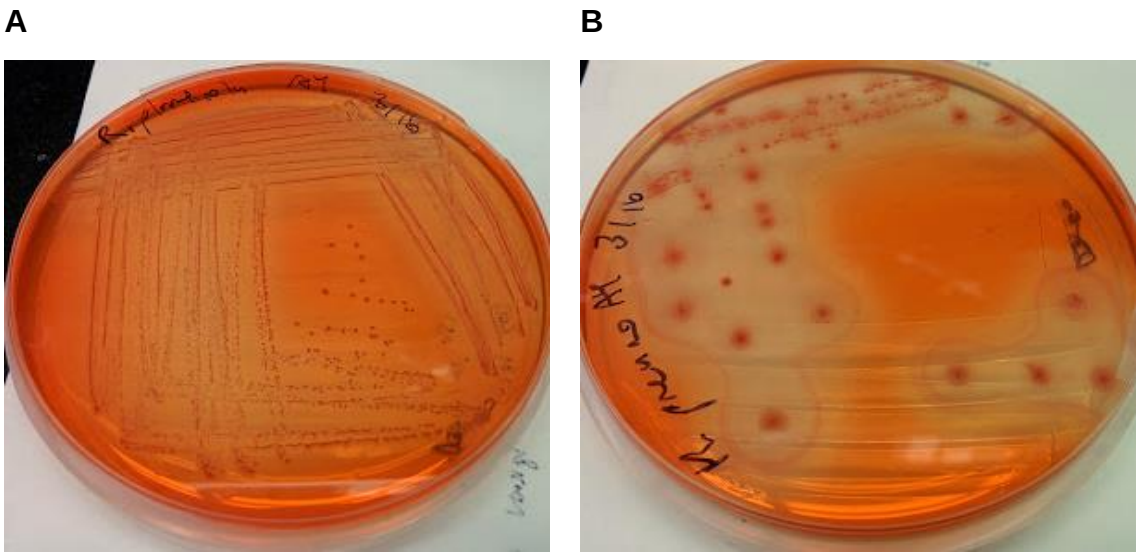


Figure 5-2: Comparison of anaerobic doxorubicin decolourization by (A) *R. planticola* and (B) *K. pneumoniae* on BHI agar.

(A) *R. planticola* and (B) *K. pneumoniae* were streaked onto BHI agar + 0.05% L-cysteine, incubated anaerobically for 48 hours, and photographed.

This result confirms that doxorubicin inactivation is not specific to *Raoultella planticola*, but may be shared in related strains. This same activity was then tested with two strains of *E. coli*: BL21(DE3), a B-type strain commonly used for expressing recombinant proteins and BW25113, a K-12 strain that has been well-characterised genetically. Unexpectedly, while both strains were able to grow well in the presence of 150 µg/ml doxorubicin, only the BW25113 strain was capable of displaying a zone of decolourization, as shown in Figure 5-3. Cell lysates of *E. coli* BW25113 were shown to inactivate doxorubicin in liquid assays and the HPLC analysis matched doxorubicin inactivation in *R. planticola*. This observation indicates that differences between these two strains of *E. coli* were sufficient to phenotypically affect anaerobic doxorubicin inactivation. However, many genotypic and functional differences are present between both strains of *E. coli*, including both cell metabolism and physiology (*125*). Thus, this result was insufficient in pointing to a specific gene or set of genes required for anaerobic doxorubicin inactivation.

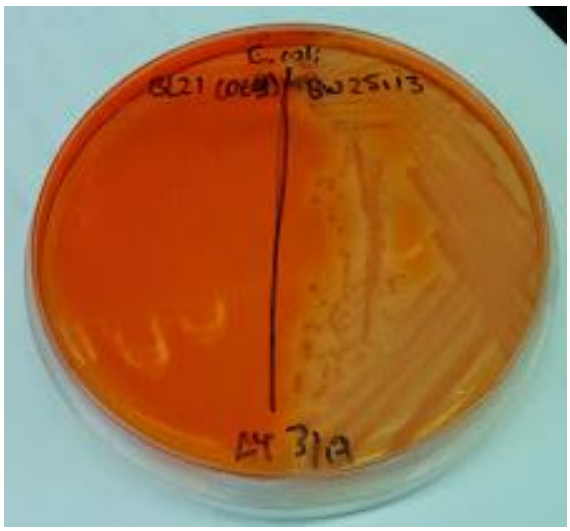


Figure 5-3: Comparison of anaerobic doxorubicin decolourization by *Escherichia coli* BL21(DE3) and BW25113 on BHI agar.

Two strains of *E. coli*, BL21(DE3), pictured on the left, and BW25113, right, were streaked onto BHI agar + 0.05% L-cysteine, incubated anaerobically for 48 hours, and photographed. Both strains were able to grow in these conditions, but only BW25113 was able to decolourize doxorubicin.

5.5. Studying DOX-inactivation in the KEIO knockout collection

Doxorubicin inactivation was observed in *E. coli* BW25113, the background strain of the KEIO collection. The inability for *E. coli* BL21(DE3) to inactivate doxorubicin suggests that the gene responsible may be unique to BW25113 and may also be nonessential. This result enabled the use of a doxorubicin decolourizing screen of the KEIO knockout collection, a set of single-gene deletions in the *E. coli* BW25113 (*126*). Identifying knockouts that were deficient in their ability to decolourize doxorubicin on doxorubicin-containing solid media would implicate these genes the doxorubicin inactivation mechanism. Strains with gene knockouts that increased the capability of doxorubicin inactivation may also provide further insight into how doxorubicin may be interacting with the bacterial cells.

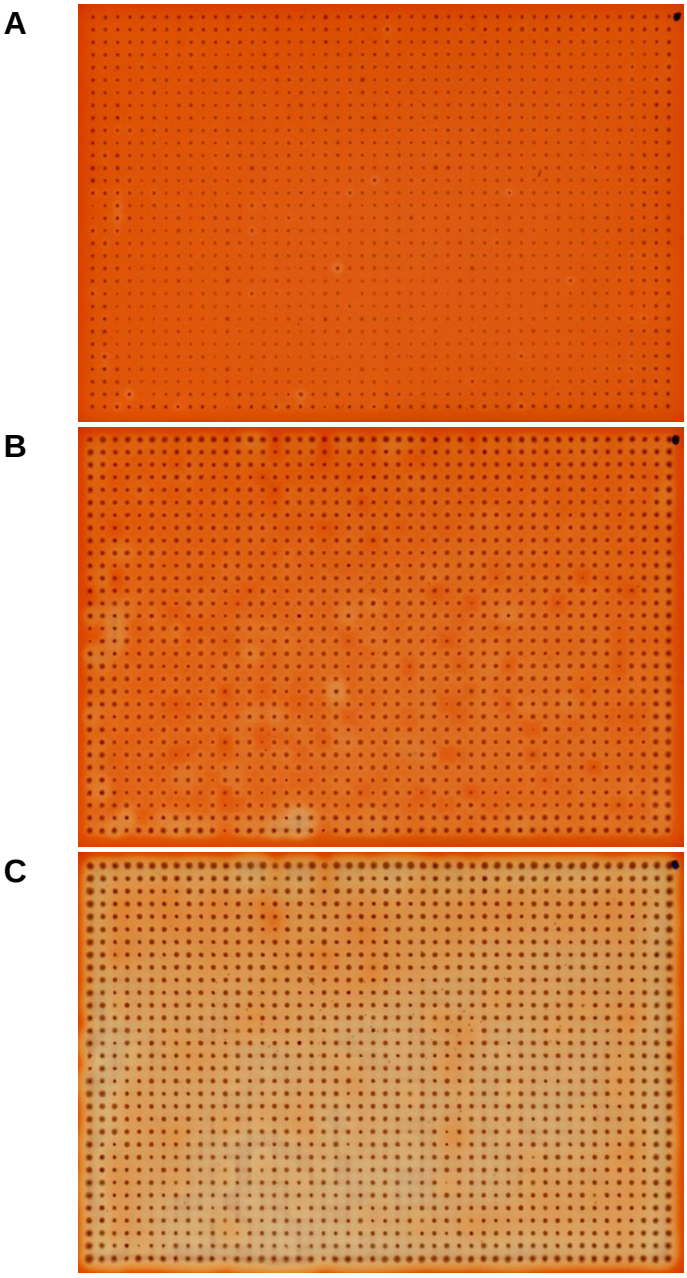


Figure 5-4: The KEIO knockout collection pinned onto BHI-DOX^{200} agar.

The KEIO knockout collection was pinned onto BHI agar containing 200 µg/ml doxorubicin and 0.05% L-cysteine in triplicate, and incubated anaerobically at 37°C. Strains were pinned at a 1536 density. Only Plate 2, of three sets of plates, is shown above. A replicate was removed at (A) 9 hours, (B) 12 hours and (C) 16 hours to observe three different time points for each plate. The degree of doxorubicin decolourization can be observed to identify strains that are particularly effective or ineffective at clearing doxorubicin from the plate.

5.5.1. NuoEFG is not a factor in anaerobic doxorubicin inactivation

To further confirm that bacterial NADH dehydrogenase does not play a role in anaerobic doxorubicin inactivation, the NuoEFG knockout strains were analyzed for their ability to decolourize doxorubicin in the KEIO knockout collection screening on BHI-DOX^{200} agar. As shown in Figure 5-5 at time points (A) 12 hours and (B) 16 hours, *nuoE*, *nuoF*, and *nuoG* knockouts have zones of decolourization like most other strains in the screen, without a decreased activity to inactivate doxorubicin. These data confirm that *nuoEFG* does not play a significant role in anaerobic doxorubicin inactivation.

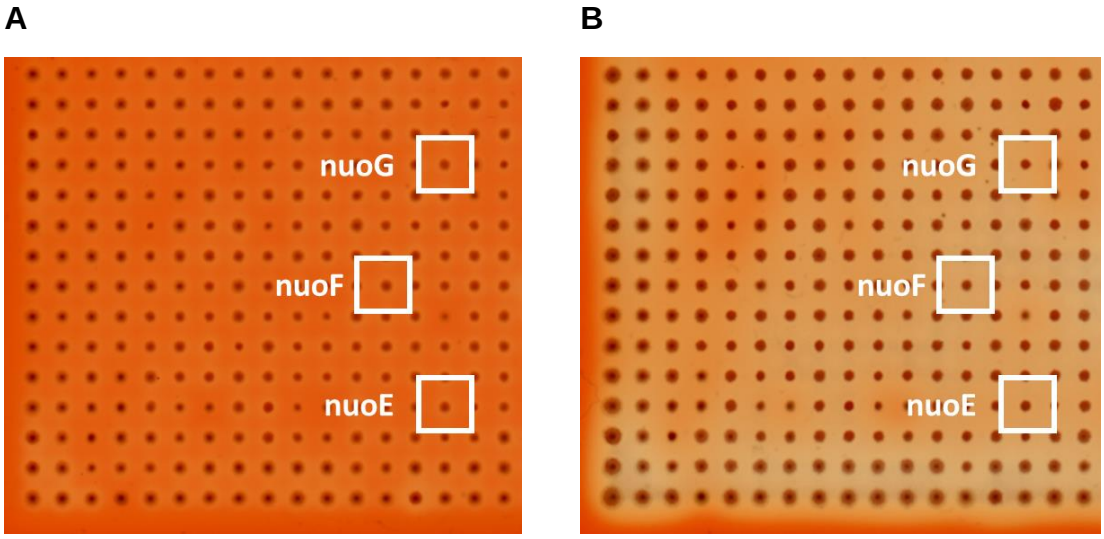


Figure 5-5: KEIO collection *nuoEFG* knockouts are capable of decolourizing doxorubicin.

The KEIO collection was pinned onto BHI agar containing 200 µg/ml doxorubicin and 0.05% L-cysteine in triplicate, and incubated anaerobically at 37°C. Knockouts of *nuoE* (strain 1-1C15), *nuoF* (1-0Y13), and *nuoG* (1-0U15), labelled above, were able to decolourize doxorubicin. (A) shows incubation at 12 hours, where small zones of inhibition can be observed. (B) shows incubation at 16 hours, where significant decolourization can be observed.

5.5.2. Gene knockout strains correlated with a decreased ability to decolourize doxorubicin

Strains were identified for their lack of doxorubicin decolourization, identified by eye for having no zone of decolourization at 12 hours incubation and either no zone or a very small zone of decolourization at 16 hours of anaerobic incubation. A list of these strains is shown in Table 5-2. The strain at position 3-0I19, deficient in production of the unknown protein JW5029 was excluded from this chart. Many of these genes were associated with molybdopterin biosynthesis, or other machinery involved in electron-transfer and specific metabolic pathways (*127*).

Position	Gene	Gene product	Deletion Phenotype	Ref
1-0W29	*moaA*	molybdopterin biosynthesis protein A	increased sensitivity to heat shock; loss of function in all molybdenum enzymes; unable to respire acetate, L-serine	(*128*)
2-0E16	*moeA*	molybdopterin molybdenumtransferase	chlorate resistant, lack active nitrate reductase	(*129*)
2-0F23	*fdx*	ferredoxin	significantly increased doubling time	(*130*)
2-0H20	*nifU/ icsU*	iron-sulfur cluster assembly scaffold	growth defect, low activity in succinate dehydrogenase, nitrate reductase	(*130*)
2-0M29	*yqiJ*	predicted inner membrane protein	unknown	(*131*)
2-0M44	*csgA*	curlin, major subunit	loss of curlin	(*132*)
2-0Y12	*moaD*	molybdopterin synthase small subunit	loss of function in all molybdenum enzymes	(*128*)
3-0D05	*fhlA*	FhlA transcriptional activator	regulatory of formate hydrogen lyase, induced in anaerobic conditions	(*133*)
3-0G09	*menD*	menaquinone biosynthesis	loss of selenate reductase activity	(*134*)
3-0L31	*potG*	putrescine ABC transporter	unknown	(*131*)
3-0V21	*sufD*	iron-sulfur cluster assembly	increased sensitivity to superoxide generating agents	(*135*)
3-1A30	*yehP*	putative conserved protein	defective swarming motility	(*131*)

Table 5-2: List of gene knockouts that reduce doxorubicin decolourization in *E. coli* BW25113.

The KEIO collection was grown on BHI-DOX^{200} and scanned at 9, 12, and 16 h. Strains with gene knockouts that were unable to decolourize doxorubicin were identified and listed above. Highlighted genes are directly related to molybdopterin biosynthesis.

5.5.3. Molybdoenzymes may be responsible for inactivating doxorubicin

The genes shown in Table 5-2 suggest that molybdopterin biosynthesis may be involved in anaerobic doxorubicin inactivation. On further inquiry, this observation corroborates with previous results, pointing towards a molybdenum cofactor dependent enzyme, or molybdoenzyme, that is responsible for doxorubicin inactivation.

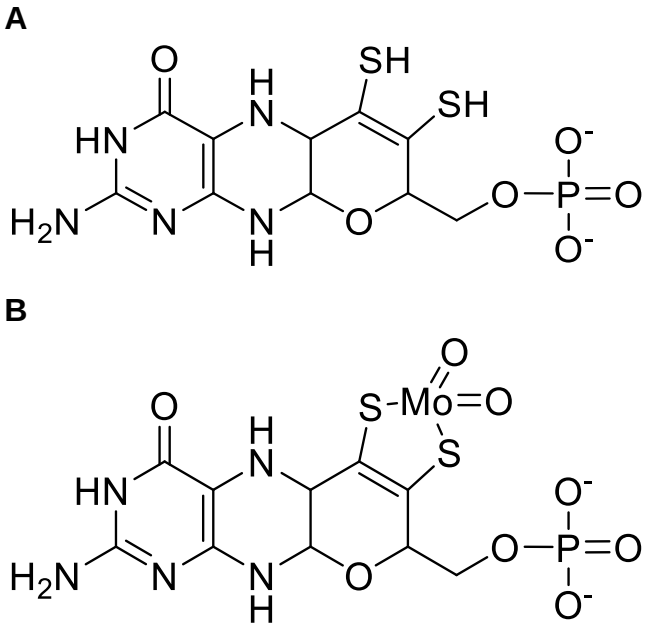


Figure 5-6: Structures of (A) molybdopterin and (B) molybdenum cofactor.

Structures of (A) molybdopterin, referred to as (MPT) and (B) molybdenum cofactor (Moco). MPT is synthesized from GTP by MoaABCDE. Molybdate is inserted by MoeAB to form Moco.

Molybdenum is a redox-active transition metal used as a catalytic site for various molybdoenzymes identified in bacteria, plants, and animals, including humans (*127*,*136*). Molybdenum is highly water soluble as its biologically active oxyanion molybdate and is found in seawater concentrations as high as iron at about 10^{-7} moles per litre (*127*). In *E. coli*, molybdate is taken up by the ModABC transporter, while ModE upregulates transcription of the molybdenum cofactor biosynthesis operon *moaABCDE* and other molybdenum-dependent enzymes (*127*). MoaA is

involved in the conversion of guanosine triphosphate (GTP) to cyclic pyranopterin monophosphate (cPMP), while MoaD helps to catalyze the conversion of cPMP to molybdopterin (MPT), which still lacks molybdenum (*137*). MoeA is involved in the insertion of molybdate into molybdopterin, resulting in the molybdenum cofactor, frequently referred to as Moco, which may be further modified depending on the molybdoenzyme (*127*).

E. coli strains lacking *modE*, along with *moaA* and *moaD*, were identified for their inability to decolourize doxorubicin. Each of the genes are involved in the Moco biosynthesis pathway described above. Additionally, IcsU is implicated in the sulfur-transfer activation of MoaD, while MoaA is dependent on oxygen-sensitive iron-sulfur clusters that are assembled by machinery such as the *suf* cluster (*127*). Furthermore, *E. coli* BL21(DE3), which was unable to decolourize doxorubicin, is deficient of molybdenum transport machinery, with deletions of *modABC*, *modE*, and *modF* (*138*). These deletions strengthen the link between molybdoenzymes and doxorubicin inactivation, explaining why BW25113 but not BL21(DE3) can explain the specificity of doxorubicin inactivation between the two strains.

Additionally, all known bacterial molybdoenzymes are involved in anaerobic metabolic reactions, consistent with observations of doxorubicin inactivation. A list of known molyboenzymes in *E. coli* is shown in Table 5-3. Gene sequences of these molybdoenzymes were downloaded from the well-annotated genome of *E. coli* K-12 substrain MG1655 (*131*). BLAST was used to identify homologues in *R. planticola* using the genome assembled in Section 3.5.2, providing a shortlist for possible molybdoenzymes that may be involved in anaerobic doxorubicin inactivation in Enterobacteriaceae (*107*).

These results strongly suggest that molybdenum biosynthesis plays a key role in doxorubicin inactivation, and that anaerobically upregulated molybdoenzymes may be responsible for drug inactivation. This reaction would be independent of NuoEFG and does not require NADH or NADPH cofactors. The next steps for this

project are to characterize the specific enzyme responsible for doxorubicin inactivation, and determining the prevalence of this inactivation mechanism across the microbiome.

***E. coli* molybdoenzyme**	**Gene (cluster)**	**Presence in *R. planticola* genome**
Nitrate reductase A	*narGHI*	*narG* 86% | *narH* 85% | *narI* 85%
Nitrate reductase Z	*narZYV*	*narZ* 81% | *narY* 83% | *narV* 77%
Periplasmic nitrate reductase	*napABCGH*	-
TMAO reductase A	*torAC*	-
TMAO reductase Z	*torZY*	*torZ* 67%
DMSO reductase	*dmsABC*	*dmsA* 80%, 70% | *dmsB* 83% | *dmsC* 75%
Formate dehydrogenase N	*fdnGHI*	*fdnG* 84%, 76% | *fdnH* 82%, 77% | *fdnI* 84%
Formate dehydrogenase O	*fdoGHI*	*fdoG* 86%, 75% | *fdoH* 85%, 74% | *fdoI* 90%
Formate dehydrogenase H	*fdhF*	*fdhF* 85%, 67%
Biotin sulfoxide reductase	*bisC*	*bisC* 76%
Selenate reductase	*ynfEFGH*	*ynfE* 77% | *ynfF* 69%, 67% | *ynfG* 81% | *ynfH* 66%
Aldehyde oxidoreductase	*paoABC*	-
Xanthine dehydrogenase	*xdhABC*	-
Sulfite oxidase	*yedYZ*	*yedY* 77% | *yedZ* 73%
Function unknown	*ydhYVWXUT*	-
Function unknown	*ydeP*	-
Function unknown	*xdhD, ygfM*	-
Function unknown	*ycbX*	*ycbX* 73%
Function unknown	*yiiM*	*yiiM* 73%

Table 5-3: Known molybdoenzymes in *Escherichia coli* and the presence of homologous genes in *Raoultella planticola*.

A list of known molybdoenzymes was taken from a review by Iobbi-Nivol and Leimkühler (*127*). Gene sequences from *E. coli* MG1655 were used to identify homologous genes in *R. planticola* using BLAST. Homologous genes are listed with their percent identity to corresponding genes in *E. coli*. A few genes had multiple homologous genes in the *R. planticola* genome. The '-' indicates that no homologous genes were identified in *R. planticola*.

5.6. Future steps

Observations of anaerobic doxorubicin inactivation in other strains have led to the conclusion that molybdoenzymes are the likely cause of anaerobic doxorubicin inactivation in Enterobacteriaceae. Ongoing and future steps will be designed to

identify the specific enzyme responsible for anaerobic doxorubicin inactivation in *R. planticola*. Through these studies, a reaction mechanism can be proposed to explain how doxorubicin is inactivated by *R. planticola* and how these interactions may manifest *in vivo*.

To identify the role of molybdoenzymes in doxorubicin inactivation, assays can be designed to monitor the effect of supplemented molybdate. For example, the culture-based assay in Section 4.4 could be repeated with molybdate added to the nutrient-poor M9 media to restore the activity observed in nutrient-rich media which may contain more trace molybdenum. Restoring doxorubicin inactivation would help confirm that the reaction is catalyzed by molybdenum. Using a molybdate transport deficient mutant may provide further evidence, as trace molybdenum is difficult to remove from the water sources used in media or buffers (*139*). Additionally, molybdate supplementation may be insufficient without the presence of required electron acceptors such as TMAO, DMSO, formate, and nitrate; thus, doxorubicin inactivation with the presence or absence of these compounds should be also be assayed.

Results from the above experiments should confirm that the reaction is catalyzed by a molybdoenzyme and suggest its function based on the electron acceptors required. Homologous genes from *E. coli* can be found in the *R. planticola* genome to be cloned and expressed to better characterize doxorubicin inactivation using purified protein assays as opposed to crude lysates. These studies can be linked with other protocols to fully characterize the reaction mechanism and explain why certain knockouts in the KEIO collection are deficient in doxorubicin inactivation. Further studies into strains that decolourize well may also elucidate how doxorubicin interacts with bacterial cells. Once a gene can be linked to doxorubicin inactivation, metagenomic studies can be used to determine its prevalence across the microbiota and other Enterobacteriaceae, in addition to inactivation by *Escherichia*, *Raoultella*, and *Klebsiella* species.

Chapter 6.
Future directions for studying drug-microbiome interactions

6.1. Project summary

A platform was developed to culture a representative population of the gut microbiota from a human stool sample, identify resistance profiles of each strain against a panel of antibiotics and antineoplastics, select strains with notable drug-modifying phenotypes, and elucidate the mechanism of drug inactivation. This platform was able to identify doxorubicin inactivation by the gut bacterium *R. planticola*, and can be used in the future to study other gut-microbiome interactions.

For this project, a collection of 190 strains was prepared using five different media types to prepare a diverse library. The resistance profiles of these strains were identified. Thirteen strains were then studied for their ability to inactivate doxorubicin (Section 3.4), and a strain of *Raoultella planticola* was identified as a potent modifier of doxorubicin in anaerobic conditions. The genome of this organism was sequenced.

Through cell culture studies, *R. planticola* was found to require anaerobic conditions and a rich-nutrient media in order to inactivate doxorubicin. Cell lysates were also able to inactivate doxorubicin anaerobically, but only when lysates were prepared and assayed in anaerobic conditions. HPLC and LC/MS were used to show the conversion of doxorubicin to 7-deoxydoxorubicinolone, while many assays confirmed that was NADH-independent. Experiments involving activity-guided protein purification, transposon-mutant studies, and chemical analysis of unstable intermediates were set up to help elucidate the mechanism of action.

Additionally, the ability to inactivate doxorubicin anaerobically was studied in other organisms. Notably, *E. coli* BW25113, but not *E. coli* BL21(DE3) was able to inactivate doxorubicin. The KEIO collection, which uses *E. coli* BW25113 as a

background strain for single-gene knockouts, was thus used to identify genes that may be responsible for doxorubicin inactivation. This approach found that strains with mutations in molybdopterin biosynthesis genes were unable to decolourize doxorubicin, suggesting that a molybdoenzyme was responsible for anaerobic doxorubicin inactivation.

Future experiments aim to identify the enzyme from a shortlist of molybdoenzymes present in *R. planticola* and characterize the mechanism of anaerobic doxorubicin inactivation. Other drug-microbiome interactions may also be identified using the data from the screens performed, while the overall platform may be optimized and repeated with different fecal samples from various patients. Specific experiments and directions for each step in the platform are described at the end of each respective chapter.

6.2. Challenges in identifying drug-microbiome interactions

Many stages in this project presented its own challenges. One significant limitation in studying gut-microbiome interactions remains the challenge of the "unculturable" microbiome. While efforts at the start this project aimed to use mixed plate pools to capture a greater source of diversity, the results were not reproducible. The use of multiple media types to capture a diverse set of strains may have helped to enrich for some difficult to culture strains, but some of these strains were also unable to grow on BHI agar and thus were not represented in the screen. Thus, challenges in culturing the gut microbiome will continue to result in some strains being underrepresented. Increased diversity can be obtained by using more media types and picking many more colonies for a larger library, and by screening these isolates on their original isolation media; these decisions must be optimized to balance greater strain diversity and limited resources for the scalability of efficient high-throughput identification of drug-microbiome interactions.

A second challenge, reflecting the microbiome's diverse metabolic capability, was that many unique conditions might be required to observe specific metabolic reactions. For example, the inactivation of doxorubicin identified in *Raoultella planticola* required both rich-nutrient media and anaerobic conditions. Screening aerobically or on different media types may have missed this inactivation activity. For clinical applications, screening contextually in an environment that best mimics the human intestinal tract would be most appropriate, but this complex ecosystem is difficult to model. Screening using many different types of conditions may prove to be necessary to capture more microbial-drug interactions.

Another significant challenge for studying drug-microbiome interactions include the difficulty of rapidly characterizing an unknown enzyme function. Of the four steps in the platform, this step is by far the most labour intensive and time consuming, as finding an unknown enzyme in an uncharacterized strain may require many approaches before elucidating a mechanism. This project used activity-guided purification, genetic techniques, and chemical analysis before closing in on a shortlist of molybdoenzymes. However, a set of experiments can be organized based on observed drug metabolism patterns to streamline the process of characterizing these methods.

Lastly, a limitation for this platform is that only drugs with antibacterial activity can be used when screening for resistance. While these pinning assays provide a simple screen, protocols to identify the metabolism of non-antibacterial drugs can also be prepared using HPLC or LC/MS tools. These experiments would have to be developed on a high-throughput scale.

In all, this platform has room for optimization, and the identification of doxorubicin inactivation by *R. planticola* provides validation that these overall steps can be used to find mechanisms of microbial drug metabolism. Using drugs with known interactions with the gut microbiota or unexpected pharmacokinetics may be

ideal targets for future screens and guide the optimization of the platform for the discovery of novel drug-microbiome interactions.

6.3. Clinical need and implications: from drug-microbiome interactions to personalized medicine

Currently, the microbiome has only been implicated in the pharmacology of just a few therapeutic agents, and most of these mechanisms of bacterial drug metabolism have not yet been identified. However, the microbiota has many implications of drug pharmacology, and recent developments in the field have enabled the study of drug pharmacomicrobiomics. These interactions should be essential in pharmacokinetic analysis to ensure drug efficacy, explain variations in treatment, and prevent undesirable and potential lethal side effects. In the case for digoxin and irinotecan, knowledge of these interactions have enabled possible remedies such as dietary changes or co-administration of inactivation inhibitors to restore therapeutic efficacy.

Combining a platform to identify mechanisms of microbial drug metabolism and study these drug-microbiome interactions using *in vivo* models provide a robust contribution to the study of pharmacokinetics. In translating this research for the clinic, alternative therapies need to be proposed to utilize, prevent, or circumvent drug-microbiome interactions

The doxorubicin inactivation reaction shown in this project required an anaerobic environment with rich-nutrient media, conditions that are representative of the gut intestinal tract. *In vivo* models, including mouse studies, can better represent the complex host-microbial environment are required to study how this interaction may affect doxorubicin pharmacology. Undesired drug inactivation, if identified, could then be prevented by the development of inhibitors for the drug inactivating enzyme(s), such as a small molecule that would limit molybdate

transport. In other cases, changing the dosage can also lead to more efficient treatment. Furthermore, known drug inactivation enzymes may also be used to selectively detoxify active drugs in non-target sites, though applications for this mechanism of doxorubicin inactivation are limited due to the toxicity of 7-deoxydoxorubicinolone for types of mammalian tissue (*56*).

In addition to providing alternative treatments, personalized medicine requires the development of robust diagnostic tools. To predict drug-microbiome interactions, health care professionals must be able to characterize patients' microbiota. Identifying the presence of certain strains or genes related to these drug-microbiome interactions are essential for the implementation of personalized medicine, and many initiatives into developing these technologies are underway, including the use of biomarkers and improvements to microbiome metagenomic sequencing (*70*). Thus, in building a comprehensive pharmacokinetic map for therapeutic agents, including interactions with the microbiome, these tools and resources can be used to advance personalized medicine and develop more effective drug treatments.

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