Generation Of An Amplifiable Biosensor Based On Conjugation For Detection Of Salmonella In Food Samples

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Abstract

Foodborne illness caused by ingestion of pathogens represents a significant, increasingly complex global health challenge. The gold-standard of food pathogen detection is becoming outdated, representing a reasonable target for reducing foodborne illness. The work in this thesis aims to develop and characterize an amplifiable biosensor system based on conjugation from a non-pathogenic E. coli to Salmonella for detection in food samples. The assay functions via conjugation of a plasmid pDETECT to Salmonella, inducing production of luxI and homoserine lactones, which induce fluorescent readout via pLux-driven transcription of thermal green protein (TGP) in an E. coli sensor. With optimized conditions for conjugation and fluorescent readout, the estimated current limit of detection of the assay is $10^4$-$10^5$ CFU/mL in spiked food samples, with a total assay time ranging between 11-13 hours. The conjugative biosensor used in this project is a novel approach to food pathogen detection that may potentially be refined through future research.

Keywords

Foodborne illness, Pathogen testing, Synthetic biology, Microbial biosensor, Salmonella, Campylobacter, Gastroenteritis
Summary for Lay Audience

Foodborne illness (FI), commonly known as ‘food poisoning’, is caused by consuming contaminated food. Contamination can be caused by several factors, but most cases are caused by bacteria. Your food’s journey from the farm to your kitchen table is a complex one, involving imports and exports from all over the globe, handling by many different people and equipment, and distribution networks with many moving parts. All these factors provide ample opportunity for bacterial contamination of food products and/or equipment used in food production. Food producers perform tests to evaluate whether contamination has occurred, but they are limited in their capacity to provide fast, reliable results. The work in this thesis aims to develop a test that can address these limitations. The potential design uses non-harmful (NH) *E. coli* to detect harmful bacteria in food samples. This thesis investigates the potential utility of this test using *Salmonella*, a widespread bacterium and one of the most common causes of FI. The proposed design relies on a DNA communication system between NH *E. coli* and *Salmonella*. *E. coli* delivers a piece of DNA to *Salmonella* in the sample, which leads to the production of messengers in *Salmonella*. These messengers relay a signal back to *E. coli*, triggering a cascade of signals that ultimately results in production of thermal green protein (TGP) that can be measured to estimate levels of contamination. This research begins by assessing TGP production by the DNA communication system, and determining optimal conditions to produce a fast, strong signal. Next, the transfer of the DNA signal between *E. coli* and *Salmonella* is optimized. Finally, the system was put to the test by artificially spiking leafy greens with *Salmonella* – and established initial proof of principle that the assay design could detect concentrations >10,000 cells/mL with a total assay time 11-13 hours. Future work will focus on making this system specific for *Salmonella* and trying to improve assay sensitivity.
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# Table of Contents

Abstract ......................................................................................................................................................... ii  
Summary for Lay Audience ........................................................................................................................... iii  
Acknowledgments .......................................................................................................................................... iv  
Table of Contents .......................................................................................................................................... vi  
List of Tables .................................................................................................................................................. ix  
List of Figures ................................................................................................................................................ x  
List of Appendices ......................................................................................................................................... xii  
Chapter 1 .................................................................................................................................................... 1  
  1 Introduction .............................................................................................................................................. 1  
    1.1 Foodborne Illness ................................................................................................................................. 1  
      1.1.1 Sources of Foodborne Illness ........................................................................................................ 2  
      1.1.2 The food industry as a complex network ...................................................................................... 5  
      1.1.3 Foodborne illness on the rise ......................................................................................................... 6  
      1.1.4 Challenges to management of foodborne illness ........................................................................... 7  
    1.2 Food pathogen detection as a leverage point for prevention of foodborne illness ......................... 9  
    1.3 *Salmonella* spp. .................................................................................................................................. 11  
      1.3.1 Background ..................................................................................................................................... 11  
      1.3.2 Clinical presentation ...................................................................................................................... 12  
      1.3.3 Epidemiology ................................................................................................................................... 13  
      1.3.4 Transmission and Reservoirs ......................................................................................................... 15  
    1.4 Biosensors as an alternative to traditional food pathogen detection ................................................. 18  
      1.4.1 Applying AHL-induced quorum sensing to food pathogen detection ........................................... 20  
Chapter 2 .................................................................................................................................................... 23  
  2 Rationale, Hypothesis, and Objectives ..................................................................................................... 23
2.1 Rationale ......................................................................................................................... 23
2.2 Hypothesis ......................................................................................................................... 23
2.3 Objectives ......................................................................................................................... 23

Chapter 3 ................................................................................................................................. 25

3 Materials and Methods ......................................................................................................... 25

3.1 Bacterial strains and growth conditions ........................................................................... 25

3.2 Plasmids ........................................................................................................................... 26

3.2.1 Transformation of plasmid DNA into E.coli and Salmonella ....................................... 26

3.2.2 Plasmids of interest ....................................................................................................... 27

3.3 Fluorescent biosensor assay ............................................................................................. 29

3.3.1 Optimization of pDETECT for Salmonella ................................................................. 31

3.4 Optimizing conjugation of pDETECT from E.coli to Salmonella .................................... 33

3.4.1 Conjugation time .......................................................................................................... 33

3.4.2 Recipient:donor ratio .................................................................................................... 33

3.4.3 Optimizing removal of conjugation donor .................................................................... 34

3.4.4 Measuring fluorescent output following conjugation of pDETECT to Salmonella and outgrowth of E.coli donor ................................................................. 34

3.5 Fluorescent detection of Salmonella in artificially spiked leafy greens ......................... 37

3.5.1 Evaluating autofluorescence by spring mix samples .................................................... 38

Chapter 4 .................................................................................................................................. 39

4 Results .................................................................................................................................. 39

4.1 Characterizing TGP fluorescent output by E.coli + pREADOUT in response to Salmonella + pDETECT ........................................................................................................... 39

4.1.1 Evaluating media conditions for optimal fluorescent output by E.coli + pREADOUT when co-cultured with Salmonella + pDETECT ......................................................... 42

4.1.2 Estimating the minimum Salmonella + pDETECT concentration necessary to induce fluorescent readout by E.coli + pREADOUT ......................................................... 46
4.1.3 Evaluating two promoters for optimal induction of fluorescence by *Salmonella* + pDETECT ................................................................. 48

4.2 Optimization of conjugation of pDETECT from an *E.coli* (dapA-) donor to *Salmonella* ....................................................................................... 51

4.2.1 Conjugation time.............................................................................. 51

4.2.2 Recipient:donor ratios ..................................................................... 52

4.2.3 Outgrowth for sufficient removal of donor *E.coli* (dapA-).............. 54

4.2.4 pDETECT produces measurable fluorescent readout when conjugated to *Salmonella* from a donor *E.coli* (dapA-)........................................... 57

4.3 Demonstrating proof-of-principle for the fluorescent biosensor assay in detecting *Salmonella* in artificially spiked food samples ......................... 59

4.3.1 Optimizing outgrowth conditions for reduction of background fluorescence by unspiked spring mix samples........................................... 66

Chapter 5 ........................................................................................................ 77

5 Discussion ..................................................................................................... 77

5.1 *Salmonella* + pDETECT induces TGP fluorescence by *E.coli* + pREADOUT .. 77

5.2 Conjugation of pDETECT to *Salmonella* by an *E.coli* (dapA-) donor produces detectable transconjugants in the fluorescent biosensor assay ...................... 79

5.3 Proof-of-principle for detection of *Salmonella* in food samples using the fluorescent biosensor assay .......................................................... 81

5.4 Limitations .............................................................................................. 85

References ....................................................................................................... 89

Appendices ..................................................................................................... 111

Curriculum Vitae ............................................................................................. 126
List of Tables

Table 1: Public Health Agency of Canada-Reported *Salmonella* Outbreaks in Canada from 2014-2022. ............................................................... 14

Table 2. Summary of bacterial strains, characteristics, and antibiotic selection. ............... 25

Table 3. Primer sequences for Gibson Assembly of the rpsM promoter into pDETECT backbone. ............................................................... 32
List of Figures

Figure 1. The farm-to-fork pathway ................................................................. 3

Figure 2. Overview of proposed fluorescent biosensor assay design ......................... 21

Figure 3. Plasmid maps ..................................................................................... 28

Figure 4. Overview of fluorescent biosensor assay for detection of Salmonella ............. 30

Figure 5. Overview of conjugation, outgrowth, and fluorescent biosensor assay for detection of Salmonella ........................................................................................................ 36

Figure 6. Comparison of maximum fluorescent output by pREADOUT co-cultured with pDETECT in 0.2% L-arabinose and 0.2% glucose ................................................................. 40

Figure 7. Relative fluorescence and absorbance trends for E.coli + pREADOUT co-cultured with E.coli + pDETECT(1) or Salmonella + pDETECT(1) ........................................ 41

Figure 8. Evaluating various L-arabinose concentrations for optimal fluorescent output by E.coli + pREADOUT when co-cultured with Salmonella + pDETECT ..................................... 43

Figure 9. Absorbance patterns as an indicator of growth by co-cultures of E.coli + pREADOUT and Salmonella + pDETECT in various concentrations of L-arabinose .......... 45

Figure 10. Assessing minimum initial concentration of Salmonella + pDETECT to produce measurable TGP fluorescence by E.coli + pREADOUT in 0.02% arabinose ........................................ 47

Figure 11. Comparison of fluorescent readout of Salmonella + pDETECT(Anderson) and Salmonella + pDETECT(rpsM) co-cultured with E.coli + pREADOUT 0.2% arabinose .... 49

Figure 12. Comparison of fluorescent readout of Salmonella + pDETECT(Anderson) and Salmonella + pDETECT(rpsM) co-cultured with E.coli + pREADOUT 0.02% arabinose .... 50

Figure 13. Evaluating different conjugation times for optimal delivery of pDETECT(rpsM) from E.coli (dapA-) donor to Salmonella ................................................................. 52
Figure 14. Evaluating the ratio of *Salmonella* recipients to *E.coli* (dapA-) + pDETECT(rpsM) donors for optimal conjugation efficiency

Figure 15. Evaluating outgrowth conditions for sufficient removal of *E.coli* (dapA-) + pDETECT(rpsM) following conjugation with *Salmonella*

Figure 16. Relative fluorescence and absorbance of *Salmonella* + pDETECT(rpsM) transconjugants co-cultured with *E.coli* + pREADOUT following conjugation and outgrowth of a donor *E.coli* (dapA-)

Figure 17. Fluorescent detection of *Salmonella* + pDETECT(rpsM) transconjugants in artificially spiked spring mix samples

Figure 18. Unspiked spring mix samples spotted on antibiotic-free LSLB

Figure 19. Ratios of *Salmonella* transconjugants to un-conjugated *Salmonella* recipients pre- and post-outgrowth for removal of *E.coli* (dapA-) + pDETECT(rpsM)

Figure 20. Fluorescent biosensor assay for detection of *Salmonella* + pDETECT(rpsM) transconjugants from *Salmonella*-spiked spring mix

Figure 21. Fluorescent biosensor assay for detection of *Salmonella* + pDETECT(rpsM) transconjugants under various outgrowth conditions for removal of donor *E.coli* (dapA-)

Figure 22. Measuring pDETECT(rpsM) transconjugants in *Salmonella*-spiked spring following removal of *E.coli* (dapA-) under various outgrowth conditions

Figure 23. Comparison of outgrowth for removal of *E.coli* (dapA-) + pDETECT(rpsM) donors in antibiotic free or double antibiotic selection

Figure 24. Evaluating unspiked spring mix samples for autofluorescence in the fluorescent biosensor assay

Figure 25. Fluorescent biosensor assay in spring mix spiked with various concentrations of *Salmonella*

Figure 26. Overview of total fluorescent biosensor assay and time investment per step
List of Appendices

Appendix A: Summary of initial experiments with *Campylobacter jejuni*……………….113
Chapter 1

1 Introduction

Foodborne illness is a complex challenge for public health and the food industry to navigate. This chapter will begin with a general review of foodborne illness, how it arises during the journey from farm to fork, and the challenges of detecting foodborne pathogens. The discussion narrows toward the end of the chapter to the pathogen of interest, *Salmonella*, and its impact as a common foodborne pathogen.

1.1 Foodborne Illness

Foodborne illness (FI) represents a major public health problem impacting the social and economic welfare of nations across the globe (Newell, 2010). FI can be caused by the ingestion of bacteria, parasites, viruses, toxins, metals, and prions from contaminated sources (Thomas, 2013). Infected persons typically experience mild, self-limiting vomiting and diarrhea (Thomas, 2013). However, outcomes such as severe and life-threatening neurological conditions can occur in very young and/or elderly people, pregnant people, and people with weakened immune systems (Thomas, 2013; Scallan, 2011).

World Health Organization estimates attribute 420,000 deaths to foodborne illness annually – 125,000 of which occur in children under 5 (W.H.O., 2022). Canadian estimates from national, provincial, and territorial public health surveillance systems estimate 4 million Canadians affected annually by foodborne illness, leading to 11,600 hospitalizations and 238 deaths (Public Health Agency of Canada, 2015). However, estimating the incidence of foodborne illness remains a challenge considering most cases are mild and few people seek medical care, and even less are hospitalized (Thomas, 2013; Drudge, 2019). Due to under-reporting and under-diagnosis of cases, public health surveillance systems are likely to underestimate the true burden of foodborne illness (Thomas, 2013).
Salmonella, Campylobacter, and Escherichia coli have commanded attention from researchers and regulatory bodies within the food industry since the 1990s (Newell, 2010). These three pathogens, along with Listeria monocytogenes, constitute the greatest burden of foodborne illness in cases where etiology is known (Newell, 2010). In Canada, Norovirus, Listeria monocytogenes, Salmonella, Campylobacter, and E.coli 0:157 represent the top foodborne bacteria causing illness and hospitalization (Public Health Agency of Canada, 2015).

Cases of foodborne illness are expected to rise due to several interacting factors. Outbreaks have more reach due to modern mass food production and widespread distribution networks and are becoming more difficult to treat due to rising antimicrobial resistance (Hall, 2002). In the face of these challenges, we require modern solutions targeting modifiable risk factors along the ‘farm-to-fork’ pathway to ensure health of consumers.

1.1.1 Sources of Foodborne Illness

The journey from farm to fork is an increasingly complex system with opportunities for contamination at any stage from pre- to post-harvest (Baraketi, 2018). Contamination of food products occur due to errors along this ‘farm-to-fork’ pathway, where pathogens can be introduced, fail to be eliminated, or multiplied due to improper handling procedures (Lukacsovics, 2014). For a visual representation of the farm-to-fork pathway and entry points for contamination please refer to Figure 1.
Figure 1. Sources of contamination along the farm-to-fork pathway. The farm-to-fork pathway refers to the complex system by which food is prepared for consumption by consumers. This figure outlines the main sources of foodborne pathogen contamination.
for pre-harvest (production) and post-harvest (processing, distribution, retail, and handling by consumers) stages. Adapted from ‘How Food Gets Contaminated’ (CDC, 2022).

In the pre-harvest stage, there are many points of introduction and transmission of pathogens, including (but not limited to) water, soil, manure, air, vehicles, workers, wildlife, birds, insects, and rodents (Torrence, 2018). Additionally, most foodborne pathogens are zoonotic, which may be introduced through food producing animals (Torrence, 2018). Some examples of zoonotic foodborne pathogens are S. aureus, Salmonella, Campylobacter, L. monocytogenes, and E. coli (Abebe, 2020). Manure and contaminated irrigation represent the two most important sources of pathogen transmission to both humans and animals in the preharvest stage (Park, 2012). Pathogens in animal manure can contaminate the environment where animals are raised, fed, and kept awaiting slaughter (Bintsis, 2018). Additionally, manure and other waste products serve as cost-efficient nutrient sources for produce, but improperly treated or untreated waste can pose contamination risk to produce (Alegbeleye, 2018). Irrigation method and frequency is important to produce safety, and contamination can be reduced by reducing the exposure of plant leaves to irrigation water (Park, 2012). Further complicating this challenge is the presence of insects and small rodents, which are widespread and can aide in the persistence, transmission, and amplification of pathogens within an agricultural environment (Alegbeleye, 2018). Ultimately, identifying sources of preharvest contamination is inherently complex - risk factors vary by location as individual farms have distinct topography, land-use interactions, and climate that may influence the frequency and transmission of foodborne pathogens (Alegbeleye, 2018).

Contamination is more commonly related to post-harvest procedures including handling, processing, transport, and preparation of foods (Baraketi, 2018). The post-harvest phase is at the forefront of regulations and establishing standards for pathogen control, such as the Hazard Analysis and Critical Control Point (HACCP), and other interventions (Torrence, 2018). HACCP is an internationally recognized food safety system based on preventing, eliminating, and reducing hazards through seven main principles: 1) conducting hazard analysis, 2) determining critical control points, 3) establishing critical
limits, 4) establishing monitoring procedures, 5) establishing corrective actions, 6) establishing verification procedures and 7) establishing record-keeping and documentation procedures (Center for Food Safety and Applied Nutrition, 2022). At farms and food processing plants, cross-contamination between food products and/or on surfaces are the most common sources of pathogens (Nyachuba, 2010). In meat production, inadequate hygiene and handling in slaughterhouses represents a major contamination point (Bintsis, 2018). Feces present on hides or fleece of livestock is recognized as a major source of foodborne pathogens, which may spread to the carcass, to the slaughterhouse personnel, or equipment (Sheridan, 1998). Food processing environments have many surfaces where bacteria may adhere and form biofilms – clusters of micro-organisms that adhere to non-biological surfaces (Hall-Stoodley, 2004). Biofilms are dynamic systems that allow for a protected growth environment which allows cells to survive in hostile environments (Hall-Stoodley, 2004). Modern food processing supports formation of biofilms due to mass production of products, lengthy production cycles, and vast surface areas for biofilm development (Lindsay, 2006). Formation of bacterial biofilms in food production lines can lead to direct or indirect contamination of food products via direct contact, spread by personnel and or pests, as well as air flow and cleaning systems (Holah, 1992).

### 1.1.2 The food industry as a complex network

The way food is produced and distributed has drastically changed over the past 100 years. Developments in rail and sea transport during the Industrial Revolution along with food preservation technologies such as canning and refrigeration have made long-distance food trade the norm (Ilbery, 1997; Naylor, 2000). The increasing size of our global population in parallel with increased consumer demand for a wider variety of foods has established a longer and more complex food chain (Fukuda, 2015). While consumers have the benefit of more options and convenient ready-to-eat meals, they rely on a system with many working parts.

As the food market has become increasingly globalized with imports and exports from all over the world, more potential points of contamination have also been introduced (Nyachuba, 2010; Newell, 2010). As Canadians, imports make up a significant portion of
our food market. In 2020 alone, Canada’s agricultural sector imported 37.5 billion USD of product (Agricultural Sector of Canada, 2022). Our reliance on imports is perhaps best highlighted through the fresh fruit market, as 80% is supplied through imports (Agricultural Sector of Canada, 2022). Although food safety is regulated domestically, imports may come from other countries with different microbiological safety procedures that may not meet our domestic regulations (Nyachuba, 2010).

Even when food products are sourced domestically, industrialization of the food market and shifts towards large-scale production and distribution of food has introduced extra challenges (Nyachuba, 2010; Newell, 2010). Pooling of raw materials together along with handling by many different people and equipment provides more opportunities for pathogens to spread (Nyachuba, 2010). Additionally, increased globalization has led to concentration of commodities with a smaller number of producers representing a growing proportion of the food supply chain (Rotz, 2015). This concentration of production and supply demands rigorous, complex regulations for food safety, but this is often out of reach for small-scale producers and further places food production in the hands of more industrialized producers (Rotz, 2015).

1.1.3 Foodborne illness on the rise

While modern food production has made identifying and preventing foodborne illness trends more difficult, it is hypothesized that it will be further challenged by climate change and climate variability in the near future. Climate change and variability are among multiple factors that can alter the nature and occurrence of food safety hazard, which have direct impacts on food production and food security (Tirado, 2010; Parry, 2004). It is predicted that climate change may interact with food distribution, production, and consumer behaviors to affect future foodborne illness trends (Hall, 2002). Climate related factors may impact food safety through changes in temperature and precipitation patterns, increased frequency and intensity of extreme weather events, ocean warming and acidification, and changes in the transport pathways of complex contaminants (Tirado, 2010). Sanitation of food sources following extreme weather events is a concern, as they may lead to contamination of land, water, food, and animal feed with pathogens, chemicals, or other hazards (Tirado, 2010). Ocean warming and acidification can affect
water’s microflora and the distribution of organisms, including pathogens and harmful algal blooms (Tirado, 2010). In Canada, annual average air, water, and precipitation temperatures are expected to rise across the country with regional and seasonal variations (Natural Resources Canada, 2014). These factors can affect the abundance, growth, range, and survival of pathogens in crops, livestock, and the environment (Smith, 2019). Possibly the most important consequence of climate change is the potential for emergence or re-emergence of pathogens (Lake, 2018).

Associations between ambient temperature and foodborne illness have been established in previous research. Kovats was able to show a linear association between temperature and reported cases of Salmonellosis in 10 European populations (Kovats, 2004). In a Canadian study, Fleury et al. estimated the potential impact of increasing temperatures on enteric diseases by retroactively comparing weekly notifiable disease laboratory counts of *Salmonella*, *E.coli*, and *Campylobacter* to average weekly temperatures between 1992-2000 (Fleury, 2006). Weekly counts of enteric bacterial disease increased with weekly temperatures after controlling for seasonal, regional, and long-term trends (Fleury, 2006). The potential impacts of climate change upon human health may depend on the capability of the food system and public health to respond to existing foodborne pathogens and their adaptation potential against climate change (Lake, 2018).

1.1.4 Challenges to management of foodborne illness

A key interacting challenge in public health management of FI is antimicrobial resistance. Foodborne pathogens already represent a serious human health concern, and the emergence of antibiotic-resistant food pathogens has further compounded this problem (Lekshmi, 2017). Antimicrobial resistance (AMR) is a growing public health concern and food safety issue (FAO and WHO, 2022).

Excessive use and misuse of antimicrobials are recognized as two major drivers for acquired AMR (Singer, 2003). Antimicrobials have been used prophylactically at subtherapeutic doses to prevent infection, as well as growth promotion to increase animal growth rate and productivity (FAO, 2015; Marshall, 2011; MacEwan, 2002). Excessive use of antimicrobials can exert selective pressure on bacterial communities over time,
resulting in emergence of AMR serotypes (Witte, 1998). An estimated 63,200 tons of antibiotics were administered to livestock in 2010, a number that is projected to increase 37% by 2030 (Lekshmi, 2017; Van Boekel, 2015). The regular administration of low concentration of antibiotics results in 75-90% of the antimicrobial being lost to the environment via urine or feces of farm animals (Gelband, 2015). This may provide an opportunity for pre-harvest contamination by AMR pathogens.

The most relevant example is the rise of antimicrobial resistance to fluoroquinolones. Fluoroquinolones are highly effective broad-spectrum antibiotics that are used in human and veterinary medicine, with relatively low minimum inhibitory concentrations (MIC) (Mitchell, 2006). These antibiotics function by interrupting DNA synthesis by targeting topoisomerase II and IV, and DNA gyrase – leading to rapid cell death (Fabrega, 2009; Mitchell, 2006). Fluoroquinolones are poorly metabolized and absorbed in vivo, and large concentrations can leach into the environment via manure (Riaz, 2018). They are of particular concern due to poor degradability and strong binding potential to soil and sediments, allowing them to persist in the environment even when therapeutic administration ceases (Pikkemaat, 2016). Like most foodborne infections, Salmonella is not typically treated with antibiotics. However, in the case of invasive Salmonella infection (see section 1.6.2), fluoroquinolone drugs are often the first line of defense (CDC, 2019). However, the utility of fluoroquinolones as an effective treatment is threatened by the emergence of fluoroquinolone-resistant isolates. In 2017, the WHO listed fluoroquinolone-resistant Salmonella as high priority for the development of new treatments (Tacconelli, 2018). Quinolone resistance in veterinary and clinical Salmonella isolates is associated with mutations in genes for DNA gyrase, gyrA and gyrB, targets of quinolone drugs (Griggs, 1997). Resistance in Salmonella enterica serovar typhimurium has also been linked to expression levels of the AcrAB efflux pump, overexpression of which has been corelated with quinolone resistant serotypes (Nikaido, 1998; Giraud, 2000). Fluoroquinolones were widely used in agriculture to reduce chicken and turkey mortality because of bacterial infections. However, concerns over fluoroquinolone resistance in poultry and the transmission of fluoroquinolone-resistant bacteria to humans lead to its withdrawal by the U.S. Food and Drug Administration (FDA) (FDA, 2005). A study by Gu and colleagues found that in-vitro long-term exposure to sub-inhibitory
concentrations of enrofloxacin resulted in a versatile adaptive response of *S.* enteriditis, including mutations in *gyrA*, downregulation of outer membrane protein-related genes, and upregulation of transcriptional activators of the AcrAB efflux pump (Gu, 2021).

Though essential for modern food production and food security, use of antimicrobials is a double-edged sword, as AMR threatens to reverse decades of improvements in human healthcare outcomes (FAO, 2015). The rate at which AMR is developing far outstrips the rate at which new antimicrobials are being developed, thus we must focus on other leverage points among the food chain that lower the risk of illness in consumers (FAO, 2015).

1.2 Food pathogen detection as a leverage point for prevention of foodborne illness

As cases of foodborne illness are expected to rise, and we face more pressure from threats such as climate change and AMR, it becomes crucial to identify modifiable risk factors across the food change. The detection and enumeration of pathogens in food, as well as surfaces that encounter food, represents one such factor. The current methods of detection of foodborne pathogens are limited and becoming increasingly outdated as food demand and complexity of food production increases.

Conventional methods of food pathogen detection rely on microbiological and biochemical identification (Velusamy, 2010). Culture-based methods are considered the ‘gold standard’ for detection given their cost effectiveness, sensitivity, ability to confirm cell viability, and ease of standardization (Dwivedi, 2011). These rely on culturing on agar, followed by colony counts and subsequent serological and biochemical identification (Mahmoud, 2012). Prior to culturing, samples must undergo enrichment. Enrichment is often divided into two steps – pre-enrichment to resuscitate injured cells and/or increase levels of the target bacteria, and selective enrichment to select for growth of the target organism (Dwivedi, 2011). Thus, culture-based methods are time-consuming – preliminary identification can take 48-72 hours, and confirmation of the pathogen species can take >1 week (Baraketi, 2017). This method is also limited as bacteria can enter a dormancy state where they become viable, but non-culturable (VBNC), which
may lead to underestimates of pathogen numbers and/or failure to isolate pathogens altogether (Velusamy, 2010). Additionally, inter-laboratory inconsistencies in testing protocols may give rise to variable results (Mahmoud, 2012). This method of food pathogen detection is advantageous in that it is generally inexpensive, sensitive, and provides both qualitative and quantitative information about the microorganisms (Velusamy, 2010). However, this is greatly restricted by the requirement for initial enrichment, as pathogens typically occur in low numbers in food (Velusamy, 2010).

Rapid methods of pathogen testing have gained increasing interest in the food industry (Nugen, 2008). The two main rapid methods are immunological detection and nucleic acid-based detection. Immunological detection based on antigen-antibody binding to detect foodborne pathogens is widely used in the food industry (Velusamy, 2010). The typical immunological methods used are enzyme-linked immunosorbent assays (ELISA) and lateral flow immunoassay (Law, 2015). The main advantages to immunological detection include rapid assay time compared to standard culture, and high specificity as it is possible to develop monoclonal antibodies that only react with specific pathogens (Velusamy, 2010). Despite these advantages, they still lack the ability to detect organisms in ‘real time’ due to the requirement of enrichment (Velusamy, 2010). Immunological detection is also limited by low sensitivity of the assays, low affinity of the antibody to the pathogen, and potential interference from contaminants (Meng, 2002). Additionally, they provide limited information regarding the biophysical properties of target analytes (Pashadazeh, 2017).

Nucleic acid-based techniques, such as polymerase chain reaction (PCR) or DNA probing, significantly reduce assay times while maintaining a high level of sensitivity and specificity (Pashazadeh, 2017; Nugen, 2008). These methods can distinguish between closely related species; and in the case of multiplex PCR – screen for multiple pathogens at once (Nugen, 2008; Lopez-Campos, 2012). However, assays based on nucleic acids alone cannot distinguish between live and dead cells and are therefore used as a screening technique prior to traditional culturing methods (Mahmoud, 2012; Nugen, 2008). Additionally, the sample matrix (ex. Gelatin, high fat content) may inhibit the PCR reaction, and issues such as incorrect PCR ratios and/or poor DNA polymerase activity
may interfere with the results (Dwivedi, 2011). Other methods of nucleic-acid based detection, including nucleic acid-based sequence amplification (NASBA) and loop-mediated isothermal amplification (LAMP), have also been used (Law, 2015).

Culture, immunological, and nucleic-acid based methods are all limited by the requirement of enrichment, which may vary from 6-48 hours depending on the pathogen of interest (Lopez-Campos, 2012). Microbiological analysis of food remains a challenging task for virtually all assays due to complexity of food matrices, the heterogenous distribution of low levels of pathogens, stress suffered by microorganisms during food processing, and the presence of bacteria from normal microbiota (Lopez-Campos, 2012).

The goal of the work in this thesis is to overcome the limitations of traditional food pathogen detection via development of a novel biosensor based on conjugative plasmid transfer. Initial candidates for development of this proposed microbial biosensor assay are *Salmonella enterica* serovar typhimurium and *Campylobacter jejuni* – two significant foodborne pathogens. *Salmonella* and *Campylobacter* are the most frequently isolated foodborne pathogens, predominantly found in poultry, eggs, and dairy products (Silva, 2011). Unfortunately, this assay design proved impractical for *C. jejuni* (see Supplementary). Therefore, this thesis centers on developing a conjugative biosensor for *S. typhimurium*.

### 1.3 *Salmonella* spp.

#### 1.3.1 Background

*Salmonellae* are gram-negative, flagellated, facultatively anaerobic bacteria (Gianella, 1996). These bacteria are non-spore forming, rod-shaped, and belong to the Enterobacteriaceae family (Fabrega & Villa). The genus *Salmonella* is made up of two species - *Salmonella enterica* and *Salmonella bongori*. One species, *S. enterica*, is responsible for 99% of *Salmonella* infections in humans and other mammals (Eng, 2015). *S. enterica* is an extremely diverse species that is made up of six subspecies: *S. enterica* subsp. enterica, *S. enterica* subsp. salamae, *S. enterica* subsp. arizonae, *S. enterica* subsp. diarizonae, *S. enterica* subsp. houtenae and *S. enterica* subsp. Indica. There are over
2,600 serological variants (serovars) which differ in their antigenic presentation, and their host range. These serovars can be broadly classified into either typhoidal or non-typhoidal *Salmonella* (NTS). Typhoidal serovars, including Typhi, Sendai, and Paratyphi exclusively infect humans and are responsible for causing enteric (AKA typhoid) fever (Gal-Mor, 2014). NTS, including serovars Typhimurium and Enteriditis, can infect both humans and animals, and are a common cause of gastroenteritis (Gal-Mor, 2014). *S. typhimurium* strain LT2 was first isolated in the 1940s and remains the principal strain for cellular and molecular biology in *Salmonella*, and thus is the strain of interest for the present study (Neidhardt, 1987).

### 1.3.2 Clinical presentation

The infectious dose for *Salmonella* gastroenteritis is 15-20 colony forming units (CFU), which typically results in self-limiting acute illness (Velusamy, 2010; Eng, 2015). Salmonellosis has an incubation period of approximately 12-72 hours, with symptoms generally resolving within 4-7 days (CDC, 2013). As part of its pathogenesis, *Salmonella* invades the epithelial cells lining the intestinal wall utilizing type III secretion systems (Eng, 2015). The most common symptom of NTS is watery diarrhea, but patients may also experience nausea, vomiting, abdominal pain, and fever (Gal-mor, 2014; McGovern, 1979). Many factors can increase susceptibility to Salmonellosis, such as high gastric pH, gastric/gastrointestinal surgery, antibiotic administration, hemoglobin abnormalities, cancers, diabetes, and compromised immunity (CDC, 2013). Most infections are uncomplicated and rarely require antimicrobial treatment. Research by Aserkoff and colleagues even indicates that antibiotic therapy could be harmful - resulting in prolonged fecal excretion and emergence of resistant strains in treated patients (Aserskoff, 1969). In well-resourced, developed nations mortality due to NTS infection is rare (<2%), whereas in resource-constrained nations mortality can reach 18-24% (Chimalizeni, 2010).

In up to 5% of cases, NTS infection can become invasive and result in extra-intestinal disease (Gal-Mor, 2014). Invasive NTS can have systemic impacts such as bacteremia and focal systemic infections and has been linked to long-term morbidities such as inflammatory bowel disease (IBD) (Gal-Mor, 2014; Gradel, 2009). Though *S. typhimurium* has relatively low virulence compared to other serovars, it is one of the
most common with extraintestinal disease (Wilkins, 1988). The risk factors for invasive NTS include HIV infection for adults, and malaria, HIV, and malnutrition in children (Feasey, 2012). As exploring the microbiome has become of increasing interest in the science community, it has been hypothesized that gut microbiota could also represent a risk factor for invasive NTS (Ferreira, 2011). A 2019 study found that the inner mucus layer and a component of the microbiota, *Mucispirillum schaedleri*, may confer resistance to invasive NTS (Herp, 2019). Treatment for invasive NTS depends on the focus of infection, the age of persons, severity of illness, presence of underlying immunosuppression, and local pattern of antimicrobial resistance (Chimalizeni, 2010).

**1.3.3 Epidemiology**

The WHO identified *Salmonella* as one of the four major global causes of diarrheal diseases and have identified emergence of antimicrobial-resistant serotypes (WHO, 2018). After *Campylobacter*, *Salmonella* is the most identified bacterial pathogen when lab diagnosis of diarrhea is sought (Acheson, 2001). It is difficult to estimate the burden of *Salmonella* infections, as surveillance programs are used as the main evidence guiding public health decisions (Majowicz, 2010; Kuchenmuller, 2009). Capturing an accurate number from these systems is challenging however because the affected person needs to seek medical care, provide a specimen, obtain a laboratory test, and these test results must be reported to relevant health authorities (Majowicz, 2010; Kuchenmuller, 2009). This ultimately underestimates the true burden of disease. Global incidence estimates by Majowicz et al. estimated 93.8 million cases of NTS gastroenteritis every year, with 85.6% being foodborne in origin, resulting in 155,000 deaths. The Public Health Agency of Canada estimates NTS gastroenteritis affects 87,500 Canadians annually, resulting in roughly 17 deaths (PHAC, 2015). The relative risk of NTS varies geographically - even regionally. A study by Varga and colleagues looked at spatial and space-time clustering of NTS serovars in Toronto, Canada to compare seasonal and demographic factors influencing prevalence. They found that the relative risk for S. Typhimurium clustered in the south-central region of Toronto, with significantly higher risk ratios during the summer (Varga, 2020).
Salmonellosis is a nationally notifiable disease in all provinces and territories, and cases are reported to provincial or territorial departments of health, and the federal government (Canada, P. H. A. of, 2016). These cases are monitored via three different surveillance systems – the National Enteric Surveillance Program, FoodNet Canada, and Canadian Notifiable Disease Surveillance System (Canada, P. H. A. of, 2016). Since 2014, there have been 17 reported Salmonella outbreaks reported by the Public Health Agency in Canada. A summary of all reported outbreaks, including the outbreak sources and number of affected individuals, are summarized in Table 1 (Canada, P.H.A. of, 2021; Canada, P.H.A. of, 2020, Canada, P.H.A. of, 2021; Canada, P.H.A. of, 2019; Canada, P.H.A. of, 2018; Canada, P.H.A. of, 2017; Canada, P.H.A. of, 2015; Canada, P.H.A. of, 2014). Four of these outbreaks were identified as S. Typhimurium, and were related to pet snakes and rodents, hedgehogs, and pig ear dog treats (Canada, P. H. A. of, 2014; Canada, P. H. A. of, 2020; Canada, P. H. A. of, 2021; Canada, P. H. A. of, 2020).

**Table 1: Public Health Agency of Canada-Reported Salmonella Outbreaks in Canada from 2014-2022.** Outbreak data was collected from Public Health Agency of Canada’s public health notices on Salmonella outbreaks and summarized below.

<table>
<thead>
<tr>
<th>Food Products</th>
<th>Outbreak Dates</th>
<th>Salmonella strain</th>
<th>Cases</th>
<th>Hospitalizations</th>
<th>Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frozen, whole-kennel corn</td>
<td>2021-2022</td>
<td>Enteritidis</td>
<td>118</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Eggs</td>
<td>2020-2021</td>
<td>Enteritidis</td>
<td>80</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>Peaches</td>
<td>2020</td>
<td>Enteritidis</td>
<td>57</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Red Onion</td>
<td>2020</td>
<td>Newport</td>
<td>515</td>
<td>79</td>
<td>0</td>
</tr>
<tr>
<td>Raw poultry</td>
<td>2017-2020</td>
<td>Reading</td>
<td>130</td>
<td>39</td>
<td>1</td>
</tr>
<tr>
<td>Dry-cured sausage products</td>
<td>2019</td>
<td>Litchfield</td>
<td>13</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Profiteroles, mini chocolate</td>
<td>2018-2019</td>
<td>Enteritidis</td>
<td>85</td>
<td>22</td>
<td>3*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.3.4 Transmission and Reservoirs

*Salmonella* spreads via fecal-oral route, and can be transmitted to humans by food, water, contact with animals, and from person-to-person in rare cases (CDC, 2013). The main mode of transmission for most NTS infections is ingestion of contaminated food, as *Salmonellae* can survive in products that are not thoroughly cooked (Gianella, 1996).

Animals are the main reservoir for NTS, especially poultry and livestock, however other domestic and wild animals can also harbor *Salmonella* (Gianella, 1996). While typhoidal strains are host-specific to humans, generalist strains such as S. Typhimurium along with other NTS can cause disease in a broad range of hosts (Hoelzer, 2011). Food-producing animals can act as reservoirs for transmission to humans. FoodNet surveillance of a sentinel site in Waterloo, Canada found that at the farm level, *Salmonella* was found on 93% of broiler (poultry production), 81% of swine, 32% of beef, and 30% of dairy farms.
While strains of importance to public health were found at all sites, they were more common in broilers, and bovine and swine production were more likely to be contaminated if poultry production was present (Flockhart, 2016). Additionally, insects including flies, mealworms, cockroaches, and beetles have been identified as reservoirs for *Salmonella* (Olsen, 2000; Crippen, 2018; Devi, 1991; Skov, 2004).

The wildlife-human interface represents another potential source for human infection. Hunting of, and contact with, wild game may represent a direct source. The reported prevalence of *Salmonella* in fecal samples from wild game varies from 0-18.7%, with reports indicating a prevalence of <1% on meat cuts (Paulsen, 2012). Factors such as location of the shot wound, time between killing and evisceration, hygiene during evisceration, and onset of refrigeration all impact potential for bacterial contamination (Paulsen, 2012). Direct contact with other wildlife species has been indicated as well. Reptiles represent a significant reservoir - a U.S. study found that up to 6% of NTS infections can be attributed to contact with reptiles or amphibians (Mermin, 2004).

Wildlife may indirectly transmit *Salmonella* to humans through food-producing animals. Organic and free-range farming of livestock requires animals to have access to grazing fields and access to outdoor spaces ‘whenever weather conditions permit’ (Organic Federation of Canada, 2020). This may allow for interaction between food-producing animals and wildlife, along with potential bacterial transmission. However, the link between organic farming of livestock and *Salmonella* contamination remains controversial. While some studies have suggested higher *Salmonella* contamination in organic farming compared to conventional farming (Hoogenboom, 2006; Lee, 2013), other studies have found no significant difference between agricultural practices (Fissler, 2004; Zheng, 2007; Siemon, 2007).

Contamination in animal feed and treats can also represent a reservoir for domestically acquired infection *Salmonella* (Hoelzer, 2011; Ellis, 1969). In fact, S. typhimurium found in dried pig ear dog treats resulted in an outbreak in humans across three provinces in 2020 (Public Health Agency of Canada, 2020). Furthermore, a Canadian study sampling commercial ‘raw food’ animal diets found that 21% of products *Salmonella*-positive, in which 67% of these had chicken as an ingredient (Finley, 2008). A systematic review of
Salmonella prevalence in livestock feed from 1955-2020 found that Salmonella detection was highest in raw feed components compared to finished feed and milling equipment (Parker, 2022). However, the prevalence of Salmonella in raw feed has been decreasing over time, potentially due to factors such as enhanced safety accreditation programs, greater participation in accreditation programs (Parker, 2022).

Salmonella is ubiquitous and persists in dry environments as well as water for days to months (Jajare, 2019). A prospective case-control study of Salmonella infections from 2010-2012 from Norway found that eating snow, dirt, sand, or playing in a sandbox was associated with salmonellosis (MacDonald, 2018). Contaminated water sources may be an important source of contamination for fruits and vegetables. In Virginia, two separate outbreaks across two years were associated with eating tomatoes has been linked to S. Newport isolated from pond water being used for irrigation (Greene, 2007). An Australian outbreak of S. Litchfield following papaya consumption found that at one of the farms, the source of the outbreak was untreated river water used for washing papaya (Gibbs, 2009). An important carrier of S. Typhimurium is agricultural runoff from poultry waste, bovine manure, and feces of asymptomatic animals (Jyoti, 2011). Jyoti et al found high levels of S. Typhimurium contamination in green algae, aquatic macrophytes, and river bed sediments in the Ganges River in India – which provides water for drinking and domestic use to nearby populations (Jyoti, 2011).

An often-overlooked source of Salmonella is persistent Salmonellosis in humans. Although long-term carriage is well documented for typhoidal Salmonella infections, persistent NTS infection is not well characterized. A meta-analysis of 48345 culture confirmed NTS infections found that 2.2% of all cases were persistent, ranging from 30 days to 8.3 years (Marzel, 2016). Of these, 65% were symptomatic with relapsing diarrhea (Marzel, 2016). Long term carriage may be an issue particularly in children under 5 (CU5), with 18% of CU5 remaining culture positive after 6 months, in contrast to older children where this number drops to 0.3% (Mermin, 2004).
1.4 Biosensors as an alternative to traditional food pathogen detection

Biosensors have emerged as alternative diagnostic techniques for food, clinical, and environmental monitoring due to their rapidity, specificity, ease and cost of mass production, and field applicability (Thakur, 2013). The definition of a biosensor is almost as broad as its applicability. Biosensors are protein, DNA, or RNA molecules that can sense various metabolites or environmental changes and generate measurable or actionable responses (Teng, 2022). Biological recognition and physical transducer elements are the major components of biosensors that play a vital role in biosensor application (Saravanan, 2021). Commonly used biorecognition elements include nucleic acids, enzymes, and antibodies (Saravanan, 2021). The key component of the biosensor makes use of a physical change accompanying the reaction (Thakur, 2013). These changes may include calorimetric, electrochemical, amperometric, piezo-electric, or optical changes (Thakur, 2013). Two intriguing characteristics of biosensors for the application of food pathogen testing include a naturally involved selectivity to biological or biologically active analytes, and the capacity to respond to analytes in a physiologically relevant manner (Meng, 2002).

Bacterial-based biosensors are of relevance for the work in this thesis. Bacterial-based biosensors have biologically integrated transducers that generate a measurable signal that includes the analyte concentration – allowing for rapid, accurate, and sensitive detection of a target (Lim, 2015; Thakur, 2013). Like other biosensors, sensing and recognition mechanisms of microbial biosensors include various optical, electrochemical, and sensory-regulated devices (Lim, 2015). Bacterial biosensors have been based on bacterial immobilization in a membrane or gel matrix linked to a sensing device, and more recently, combining reporter genes with a contaminant-sensing component to conduct quicker, more sensitive analysis (Ma, 2022). Some commonly used reporter genes include lacZ, lux, luc, and gfp (Ma, 2022).

Sensory regulation mechanisms are essential to survival and have evolved in all biological systems to regulate the optimization of cell growth, behavior, and transduction of bio chemicals (Lim, 2015). Quorum sensing (QS) represents an example of a microbial
sensory mechanism in which bacteria release and subsequently respond to signal molecules as a mechanism for sensing population density (Fuqua, 1994). Bacterial populations can synchronously control gene expression in response to changes in cell density and species complexity at a community level (Ng, 2009). The outcome of synchronous gene expression modifies functions critical to survival such as biofilm formation, virulence factor secretion, bioluminescence, antibiotic production, sporulation, and competence for DNA uptake (Ng, 2009).

In the traditional signaling pathway, low molecular weight molecules are synthesized intracellularly and secreted by bacterial cells at low concentrations (Ng, 2009). The extracellular concentration of these molecules increases with population size until they accumulate above a minimal threshold (Ng, 2009). At threshold, cognate receptors bind the autoinducer and trigger signal transduction cascades resulting in changes to gene expression throughout the population.

The most common bacterial QS system in gram-negative bacteria is the luxI/luxR system, which utilize n-acyl homoserine lactones (AHLs) as the autoinducer (Patel, 2013). The luxI/luxR operon was first characterized in Vibrio fischeri - a marine, gram-negative, facultative anaerobic bacteria which naturally emits a blue-green light under favorable environmental conditions (Teng, 2022; Erzinger, 2018). V. fischeri forms a symbiosis with the Hawaiian Bobtail Squid, which provides nutrients and a high oxygen concentration that allows V. fischeri to grow to a high cell density, resulting in light that the squid can utilize as an anti-predation strategy (Ruby, 1996).

In the canonical luxI/luxR system, AHLs are recognized by members of the luxR family of transcription factors, which are often encoded adjacent to luxI AHL synthases (Fuqua, 2006). LuxI directs synthesis of AHLs which can bind luxR to form a transcriptional activator of the pLux promoter (Eberhard, 1981; Teng, 2022). LuxR has two functional domains - a cytoplasmic autoinducer receptor and a DNA-binding transcriptional activator (Engebrecht, 1983). At a critical concentration of AHLs, the luxR-AHL transcriptional activator drives activation of the pLux promoter and downstream induction of luciferase, resulting in observable bioluminescence by V. fischeri (Eberhard,
Bioluminescence intensity has been correlated with variations in auto-inducer production (Nealson, 1977).

The *luxI/luxR* system has been widely applied for induction of gene expression in metabolic engineering (Teng, 2022). The work in this thesis aims to utilize the *luxI/luxR* QS pathway in a microbial biosensor for rapid detection of foodborne pathogens. The proposed mechanism uses the *luxI/R* quorum sensing pathway from *V. fischeri* for identification of pathogens and subsequent fluorescent readout. In this model, *luxI* and *luxR* are separated amongst two plasmids which are hosted in non-pathogenic *E. coli* Epi300 which acts as a microbial biosensor (Figure 2). The initial candidate for fluorescent readout is thermal green protein (TGP), which is an extremely stable, non-aggregating form of green fluorescent protein (GFP) (Close, 2015). GFP-based microbial biosensors have previously been useful in assessing heterogeneity of iron bioavailability in plants, as well as assessing several compounds such as arsenite, galactosides, toluene, etc. (Thakur, 2013).

### 1.4.1 Applying AHL-induced quorum sensing to food pathogen detection

The assay design explored in this thesis relies on the division of the *luxI/luxR* operon between two plasmids, which have been assigned the monikers ‘pDETECT’ and ‘pREADOUT’. pDETECT would contain the *luxI* gene under control of a pathogen-specific promoter, which would become active upon entry to the pathogen of interest. In initial experiments, *luxI* is expressed under a constitutive promoter from the Anderson promoter collection (BBa_J23106).

pREADOUT contains the *luxR* gene regulated by a pBAD promoter. pBAD is constitutively active in various concentrations of arabinose but repressed in glucose, which allows us to easily control *luxR* expression. pREADOUT also contains a gene for thermal green protein (TGP) under the pLux promoter.

Ideally, pDETECT and pREADOUT would be both be hosted in *E. coli* Epi300 (Figure X). Contaminated food samples would be co-cultured with the *E. coli* host, which would pass pDETECT to the pathogen of interest via conjugative plasmid transfer.
Reconstitution of pDETECT in the pathogen of interest and subsequent activation of a pathogen-specific promoter would drive luxI expression and downstream expression of AHL autoinducers. AHL would diffuse across the cell membranes back to the E.coli host and form a transcriptional activator with luxR. This ultimately would allow for transcriptional activity of pLux and TGP expression which may be quantified.

**Figure 2. Overview of proposed fluorescent biosensor assay design.** a) Non-pathogenic E.coli Epi300 harboring pREADOUT and pDETECT is cocultured with a pathogen of interest b) pDETECT is passed to the pathogen of interest via conjugative plasmids transfer c) pDETECT reconstitutes in pathogen of interest d) a pathogen specific promoter activates in pDETECT which drives transcription of luxI e) luxI production leads to downstream production of n-acyl homoserine lactones (AHLs) f) arabinose binds araC in pREADOUT which in turn activates the pBAD promoter g)
pBAD activation drives transcription of \textit{lux}R h) AHLs diffuses through to pREADOUT and binds \textit{lux}R i) \textit{lux}R-AHL complex activates pLux j) pLux drives transcription of thermal green protein (TGP) which can be measured via detection of fluorescence.
Chapter 2

2 Rationale, Hypothesis, and Objectives

2.1 Rationale

Food safety remains a significant challenge complicated by an increasingly complex chain of food production, climate change altering ambient temperatures, and emerging antimicrobial resistance. The identification of pathogens in food production represents a modifiable risk factor we can leverage to reduce the burden of foodborne illness. The conventional culturing methods can take up to a week for confirmation of bacterial contamination in food samples. Rapid methods, including immunological and nucleic-acid based detection, have the advantage of high specificity and screening for multiple pathogens at once, but are limited by the requirement for expensive supplies out of reach for smaller producers, as well as cultural enrichment. Biosensors provide a potentially useful alternative, based on their rapidity, specificity, cost effectiveness, and field applicability. Using a microbial-based biosensor based on conjugative plasmid transfer also gives us the power to detect live, metabolically active cells. In this thesis, I sought to characterize and optimize a microbial biosensor based on conjugation for the detection of pathogens in food samples. This work focuses on the potential application for use in detecting Salmonella, a significant foodborne pathogen with major implications for global health.

2.2 Hypothesis

An amplifiable biosensor based on conjugative plasmid transfer can be designed for the rapid detection of S. typhimurium.

2.3 Objectives

The work described in this thesis targets three main objectives:

1. Generate and optimize a fluorescent readout signal by E.coli + pREADOUT in response to pDETECT in S. typhimurium
2. Optimize conjugation of pDETECT from a non-pathogenic *E.coli* donor to *S. typhimurium*

3. Assess potential utility of the assay in detecting *S. typhimurium* isolated from artificially spiked food samples
Chapter 3

3 Materials and Methods

3.1 Bacterial strains and growth conditions

All strains were grown at 37°C, with 225rpm shaking for liquid cultures unless otherwise indicated. *E.coli* Epi 300 was grown in Lennox broth (LB) or on 1.5% LB agar supplemented with antibiotics as indicated by Table 2. *Salmonella enterica* sub. species typhimurium LT2 (ΔhilA::KanR) was grown in low-salt Lennox broth (LSLB) or on 1.5% LSLB agar supplemented with appropriate antibiotics. Antibiotics used for selection include kanamycin (Km), chloramphenicol (Cm), and gentamycin (Gm) – whose working concentrations are summarized in Table 2.

Table 2. Summary of bacterial strains, characteristics, and antibiotic selection.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Features</th>
<th>Selection</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella enterica</em> sub.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species typhimurium LT2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(ΔhilA::KanR)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DE2426</td>
<td></td>
<td>Kanamycin resistance, base strain</td>
<td>Km (50ug/mL)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>used for all experiments</td>
<td></td>
</tr>
<tr>
<td>DE4601</td>
<td></td>
<td>Plasmid of interest: pDETECT(Anderson)</td>
<td>Km (50ug/mL)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cm (25ug/mL)</td>
</tr>
<tr>
<td>DE4602</td>
<td></td>
<td>Plasmid of interest: pNULL, negative</td>
<td>Km (50ug/mL)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>control in fluorescent assay</td>
<td>Cm (25ug/mL)</td>
</tr>
<tr>
<td>DE4723</td>
<td></td>
<td>Plasmid of interest: pDETECT(rpsM)</td>
<td>Km (50ug/mL)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cm (25ug/mL)</td>
</tr>
</tbody>
</table>
### E.coli Epi 300

<table>
<thead>
<tr>
<th></th>
<th>DE4333</th>
<th>Plasmid of interest: pDETECT(1)</th>
<th>Km (50ug/mL)</th>
<th>Cm (25ug/mL)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>DE4264</td>
<td>Contains pDETECT(Anderson)</td>
<td>Km (50ug/mL)</td>
<td>Cm (25ug/mL)</td>
</tr>
<tr>
<td></td>
<td>DE4870</td>
<td>Has dapA gene deletion, requires addition of diaminopimelic acid (DAP) to media (60ug/mL)</td>
<td>Cm (25ug/mL)</td>
<td>Gm (40ug/mL)</td>
</tr>
</tbody>
</table>

|    |          | Plasmid of interest: pDETECT(rpsM) and pTA-mob | Cm (25ug/mL) | Gm (40ug/mL) |

### 3.2 Plasmids

#### 3.2.1 Transformation of plasmid DNA into E.coli and Salmonella

Plasmid DNA was transformed into E.coli or Salmonella hosts via electrotransformation. Plasmid DNA was isolated from overnights using the Monarch® Plasmid Miniprep Kit, (New England Biolabs) according to manufacturer’s instructions. Miniprep DNA was quantified using the Qubit broad-range dsDNA assay with a Qubit 2.0 fluorometer prior to further use (ThermoFisher Scientific).

Briefly, 40uL of electrocompetent cells were added to a 0.2 cm elect rocuvette along with 1-2 uL of miniprepped plasmid DNA. Samples were electroporated at 2.5 kV, 25 uF, and 200 ohm, followed by recovery for 90 minutes in super optimal broth with catabolite repression (SOC) media at 37°C with 225 rpm shaking. 100uL of recovered samples were plated on selective media to screen for transformants, and incubated overnight at 37°C. Bacterial cells treated identically, without the addition of plasmid DNA, served as a
negative control for transformations. Strains were stored for long-term storage in glycerol at -80°C.

3.2.2 Plasmids of interest

Plasmids were designed and constructed by Thomas Hamilton (PhD Candidate) of Dr. David Edgell’s lab, Department of Biochemistry, Western University. Brief descriptions of the plasmids used in this thesis are summarized below. Plasmids have been identified with monikers (e.g. pREADOUT) for discussion in this thesis, but their corresponding lab identification code is listed in brackets (e.g. pCBS5).

3.2.2.1 pREADOUT

pREADOUT (pCBS5) is a 5313bp vector derived from plasmid pBR322-Δrom (Figure 3a). pREADOUT contains luxR regulated under a pBAD promoter. Genes for araC are constitutively expressed, which results in downstream activation of the pBAD promoter when arabinose is supplied in the media. A gene for thermal green protein (TGP) is regulated by a pLuxR promoter. pREADOUT has Amp resistance conferred by the AmpR promoter.

3.2.2.2 pDETECT

pDETECT (pCBS4) is a 3693bp vector derived from plasmid pNuc-trans (Hamilton, 2019) which contains a luxI gene expressed under a J23106 (Anderson) promoter (Figure 3b). It has Cm resistance conferred by chloramphenicol-acetyltransferase. pDETECT(1) (pCBS9), optimized for C. jejuni, is 7866 bp and integrated with the backbone of shuttle vector pGU0202 (Alfredson, 2003). pDETECT(1) harbors an additional Campylobacter-derived Km resistance gene (aph(3’)-III) (Figure 3c).

3.2.2.3 pNULL

pNULL (pACYC) is the 4008 bp vector known as pACYC Duet-1 (Figure 3d). pACYC Duet-1 contains the p15a replicon, lacI gene, and a chloramphenicol resistance gene (Addgene, #71147). pNULL was used primarily as a negative control as it does not have any genes expected to induce fluorescence by pREADOUT.
Figure 3. Plasmid maps. The plasmid size and genes of interest are summarized for a) pREADOUT b) pDETECT c) pDETECT(1) and d) pNULL. Plasmid maps were created in BioRender.
3.3 Fluorescent biosensor assay

The fluorescent biosensor assay was initially designed by Thomas Hamilton, PhD candidate in Dr. David Edgell’s lab, Department of Biochemistry, University of Western Ontario. The goal of the experiments described in this thesis was to optimize this assay for fluorescent readout in Salmonella. Briefly, overnights of E.coli + pREADOUT and Salmonella + pDETECT (or pDETECT variations) were diluted 1:50 and 1:30, respectively, in antibiotic-free LSLB and grown to a mid-log growth phase (A600 = 0.5). 5uL of each culture were subsequently co-cultured in 240uL LSLB supplemented with 0.2% arabinose in a 96-well plate (n=3). Co-cultures were incubated in a Synergy H1 plate reader (O’Donoghue Lab, Department of Biochemistry, University of Western Ontario; BioCore Facilities, Department of Biochemistry, University of Western Ontario) at 37°C, and fluorescence (480/520nm) and absorbance (600nm) were read every 5 minutes as a measure of TGP fluorescent output and culture density, respectively. Plate reader measurements were done using Biotek Gen5 software (Version 3.12).
Figure 4. Overview of fluorescent biosensor assay for detection of *Salmonella*. Overnights of *Salmonella* + pDETECT and *E.coli* + pREADOUT were diluted and grown to mid-log growth phase. 5uL of each culture were added to 240uL LSLB supplemented with 0.2% L-arabinose, along with a 0.2% glucose control. Fluorescence (480/520nm) and absorbance (600nm) were recorded every 5 minutes in Biotek Gen 5 from a Synergy H1 plate reader.

Controls for this experiment were *E.coli* + pREADOUT co-cultured with *Salmonella* + pNULL, as pNULL has no genes expected to induce transcription of TGP by pLux.
Additionally, blank wells without the addition of either bacterial species were used to determine the base fluorescence and absorbance of the co-culture media. The mean fluorescence of blank wells was calculated in Excel (Version 2210) and subtracted from each replicate at each timepoint to calculate the relative fluorescence (RFU) \[ \text{RFU} = \text{fluorescence}_{\text{replicate}} - \text{mean fluorescence}_{\text{blank}} \]. The same was done for absorbance values to calculate the relative absorbance (RA) \[ \text{RA} = \text{absorbance}_{\text{well}} - \text{mean absorbance}_{\text{blank}} \].

Fluorescent readout induced by pNULL was used to calculate the time to detection of the assay. The limit of detection was set as the meanRFU plus 3x the standard deviation for each timepoint for pNULL (LOD=mean_{control}+3(SD)). Fluorescent readout was considered ‘detectable’ for a sample once the mean RFU crossed, and consistently remained above, the LOD for subsequent timepoints.

To assess the impact of arabinose concentration in the media on fluorescent output by pREADOUT, the fluorescent output assay was performed as described above in LSLB with varied arabinose concentrations – 0.2%, 0.02%, 0.0002%, or 0.00002%. Coculturing were also added to LSLB + 0.2% glucose as a negative control. For comparison of media conditions, the RFUs were normalized for each arabinose media concentration by dividing the RFU for each timepoint by the glucose control. Conditions were then compared using a one-way ANOVA with Tukey’s HSD test for multiple comparisons. A p<0.05 was considered significant.

3.3.1 Optimization of pDETECT for *Salmonella*

pDETECT has an Anderson(J23106) promoter, a constitutive promoter from the Anderson series of promoters. The 5’ region of the rpsM promoter (PrpsM) is a constitutive promoter commonly used in *Salmonella* which encodes for the ribosomal protein S13 (Cooper, 2017; Valdivia, 1996). Although rpsM does not have *Salmonella*-specific expression, it has relatively high (>1,000 transcripts per million) absolute expression under a wide range of conditions (Kroger, 2013; SalCom v.10). We replaced the Anderson promoter in pDETECT with rpsM as described below.
Overnight culture of DE4264 (*E. coli* + pDETECT) was grown at 37°C and pDETECT backbone DNA was isolated via Monarch® Plasmid Miniprep Kit, (New England Biolabs) as described above. The rpsM promoter insert was amplified directly from pFPV25.1 (Plasmid #20668, Addgene; pub: [https://www.addgene.org/browse/article/3945/](https://www.addgene.org/browse/article/3945/)). Primers used for amplification of pDETECT backbone and rpsM insert are listed below in Table 3.

**Table 3. Primer sequences for Gibson Assembly of the rpsM promoter into pDETECT backbone.**

<table>
<thead>
<tr>
<th>Primer target</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDETECT – forward</td>
<td>5’-cttgaagacgaaggATCTTTTCTAGAAGATCTCCTACAATATTCC-3’</td>
</tr>
<tr>
<td>pDETECT – reverse</td>
<td>5’-gaaggagatatacatATGACGATTATGATCAAAAAATCGG-3’</td>
</tr>
<tr>
<td>rpsM – forward</td>
<td>5’-TTTGATCATAATCGTATatgtatatctttcttttaaatcttagg-3’</td>
</tr>
<tr>
<td>rpsM – reverse</td>
<td>5’-ATCTTTCTAGAAGATcctttgctttcaagaatcgc-3’</td>
</tr>
</tbody>
</table>

Amplification products were digested with DPNI (New England Biolabs) according to manufacturer’s protocol to eliminate non-PCR gene products. DNA was purified using Monarch® PCR & DNA Cleanup Kit (New England Biolabs) according to manufacturer’s protocol, and re-quantified using the Quibit fluorometer. Amplified gene products were combined using the NEBuilder® HiFi DNA Assembly Cloning Kit (New England Biolabs) according to manufacturers protocol and incubated at 50°C for 30 minutes. Immediately following assembly, pDETECT(rpsM) clones were transformed into *E.coli* Epi300 via electroporation as previously described. Confirmation of cloning was obtained by whole-plasmid sequencing by Flow Genomics (London Regional Genomics Center).
Successful pDETECT(rpsM) transformants were stored in 40% glycerol at -80°C for further use (Strain ID: DE4723). A fluorescent biosensor assay was performed as described above to compare the performance of the rpsM promoter to the original Anderson promoter in pDETECT.

3.4 Optimizing conjugation of pDETECT from E. coli to Salmonella

An auxotrophic strain of E. coli Epi300 with a dapA gene deletion was selected as a donor for conjugation of pDETECT to Salmonella. The dapA gene is required for production of diaminopimelic acid (DAP) (Gerdes, 2003). Thus, DAP must be supplemented in the growth media, providing an antibiotic-free method for counter selection of E. coli following conjugation with Salmonella (Brumwell, 2018).

3.4.1 Conjugation time

Overnights of DE4870 (E. coli (dapA-) + pDETECT(rpsM)) and DE2426 (Salmonella recipient) were diluted 1:25 in LSLB, with Dap (60μg/mL) supplementation for DE4870, and grown to an OD600 of 0.5. 100μL of each culture were co-cultured in the well of a 96-well plate and incubated at 37°C for conjugation. To assess for optimal conjugation time, co-cultures were allowed to conjugate for 0.5, 1, 2, or 4 hours (n=4). At each timepoint, replicates were serially diluted tenfold, and 10μL of each dilution were spotted on selective plates. LSLB + Km + Cm agar was used for selection of Salmonella-pDETECT(rpsM) transformants; LSLB + Km was used to screen for Salmonella recipients; LSLB + Dap + Gm + Cm was used for isolation of E. coli donors. Plates were incubated overnight at 37°C, and CFU were counted for each plate the next day.

Conjugation frequencies (CF) were calculated by dividing the CFU present on LSLB + Km + Cm agar by the CFU on the LSLB + Km agar (CF = CFU transformants / CFU recipients only).

3.4.2 Recipient:donor ratio

The next step was determining the optimal recipient:donor (R:D) ratio for efficient delivery of pDETECT(rpsM) to Salmonella. Overnights of DE4870 (E. coli (dapA-) +
pDETECT(rpsM)) and DE2426 (*Salmonella* recipient) were diluted 1:25 in LSLB and grown to an A600 = 0.5. The *E.coli* donor was grown with DAP (60μg/mL) supplementation. Prior to conjugation, donors and recipients were diluted tenfold and 10μL were spotted on selective agar to determine the actual R:D ratios (n=4). Donors and recipients were co-cultured to a final volume of 200μL with the following estimated R:D ratios: 1, 0.5, 0.2, 0.1, 0.01, or 0.001 (n=4). Cocultures were incubated for 30 minutes at 37°C to allow for conjugation, and 10μL were spotted on selective agar as described above. Plates were incubated overnight and the CFU and CF were determined.

### 3.4.3 Optimizing removal of conjugation donor

Overnights of DE4870 (*E.coli* (dapA-) + pDETECT(rpsM)) and DE2426 (*Salmonella* recipient) were diluted 1:25 in LSLB and grown to an A600 = 0.5. Donors and recipients were co-cultured at a R:D ratio of either 0.01 or 0.001 to a final volume of 1mL, and incubated at 37°C for 30 min for conjugation. Following conjugation, cultures were washed via centrifugation at 8000 rpm for 2 minutes to remove excess DAP from the media. The supernatant was replaced with equal volume of fresh LSLB and re-spun – this process was repeated twice to a final resuspension volume of 100μL. For the outgrowth, 100μL of washed co-culture was grown in either liquid LSLB or on LSLB agar (1.5%). Outgrowth time was set at either 30 min, 1 hour, 2 hours, 4 hours, or 6 hours to determine minimum incubation time for elimination of donor *E.coli*. Following outgrowth, samples were serially diluted tenfold and spotted on selective agar as described above. Plates were incubated overnight and the CFU and CF were determined.

### 3.4.4 Measuring fluorescent output following conjugation of pDETECT to *Salmonella* and outgrowth of *E.coli* donor

An overview of the assay workflow is outlined in Figure 5. Briefly, co-cultures of *E.coli* (dapA-) + pDETECT(rpsM) and *Salmonella* recipient were conjugated and washed as described above, and 100μL were added to LSLB agar plates (1.5%) for 6 hours to eliminate donor *E.coli* (n=4). Meanwhile, *Salmonella*-pNULL and *E.coli*-pREADOUT were prepared for a fluorescent assay as described in Section 3.3. Following the outgrowth step, cultures were re-suspended in 1mL LSLB, serially diluted (tenfold), and
10μL was plated on selective agar for cell count (n=4). Control for conjugation was *Salmonella* treated identically without the addition of *E.coli* donors. 5μL *E.coli*-pREADOUT was co-cultured with 5μL *Salmonella* transconjugants, *Salmonella* recipient-only control, or *Salmonella*-pNULL (n=4). Co-cultures were added to LSLB containing 0.2% arabinose or 0.2% glucose, and the fluorescent assay was performed as described above.
Figure 5. Overview of conjugation, outgrowth, and fluorescent biosensor assay for detection of *Salmonella*. *E.coli* (dapA-) + pDETECT(rpsM) was co-cultured with *Salmonella* and allowed to conjugate for 30 min at 37°C (a). Following conjugation, samples were washed via centrifugation, removal of supernatant, and resuspension in fresh LSLB for sufficient removal of DAP from the media(b). This was repeated twice to a final resuspension volume of 100uL, and this was spotted on DAP-free LSLB and
incubated at 37°C for 6 hours (c). Following outgrowth and sufficient removal of E.coli (dapA-) donors, samples were resuspended in 1mL LSLB, and 5uL was co-cultured with E.coli + pREADOUT for the fluorescent biosensor assay. Samples were also diluted tenfold, and 10uL of each dilution was spot plated on LSLB + Km, LSLB + KmCm, and LSLB + DapGmCm for selection of Salmonella recipients, Salmonella transformants, and E.coli donors, respectively.

3.5 Fluorescent detection of Salmonella in artificially spiked leafy greens

To demonstrate proof-of-principle for potential utility of the assay as a test for Salmonella in food samples, leafy greens were artificially spiked with Salmonella to assess detection following conjugation with E.coli (dapA-) + pDETECT(rpsM), outgrowth, and co-culture with E.coli + pREADOUT. Methods for the food spike protocols were loosely adapted from “Procedures for the Development and Management of Food Microbiological Methods” (Committee of the Microbiological Methods, Health Canada). One food was selected from the ‘high-risk’ categories for Salmonella contamination – salad mix (Committee of the Microbiological Methods, Health Canada). All assays used PC Organic Spring Mix (Loblaws, London, Canada), specifically, as the testing medium.

Food samples were aliquoted from the sampling unit into 25g samples per replicate under sterile conditions and added to a Whirl-Pak ® filter bag (Nasco Sampling). One sampling unit is defined as one container of spring mix for experimental purposes. In the case where multiple sampling units had to be used, the two units were mixed prior to dividing into 25g aliquots to control for variation between packages. 100mL of 0.1% peptone water was added to each sample. Samples were spiked with varying concentrations of Salmonella and homogenized in a Bag Mixer ® (INTERSCIENCE) for 1 minute. Following homogenization, samples were incubated at 37°C for 2 hours. Meanwhile, E.coli (dap-) + pDETECT(rpsM) overnights were diluted and grown to A600 = 0.5 in preparation for conjugation. Following initial incubation, spiked food samples were pelleted via centrifugation at 8000rpm for 2 minutes and the supernatant was replaced with fresh LSLB for removal of antibiotic from the media. Samples were then co-cultured
with *E.coli* (dap-) +pDETECT(rpsM) for conjugation, outgrowth, and ultimately fluorescent readout as described above. Samples were diluted ten-fold, and 10uL of each dilution were plated on LSLB + DapGmCm, Km, or KmCm for donors, recipients, and transconjugants respectively. Selective spot plating was done with initial *Salmonella* and *E.coli* cultures, as well as pre- and post- conjugation outgrowth to establish CFU counts.

### 3.5.1 Evaluating autofluorescence by spring mix samples

To determine if spring mix samples were producing auto fluorescent signals, independent of *E.coli* + pREADOUT, 25g lettuce samples were prepared, digested, and incubated for 2 hours as described above (n=4). Following incubation, 5uL of samples were added to a 96 well plate containing LSLB + 0.2% L-arabinose either with or without the addition of *E.coli* + pREADOUT (n=4). *E.coli* + pREADOUT was also co-cultured with *Salmonella* + pNULL as a control (n=4).

### 3.6 Data analysis

All data analysis was completed in Excel (Version 2210) and/or GraphPad Prism 9. Figures were made in GraphPad Prism 9, BioRender.com, and Canva Pro.
Chapter 4

4 Results

4.1 Characterizing TGP fluorescent output by \textit{E.coli} + pREADOUT in response to \textit{Salmonella} + pDETECT

Fluorescent readout by \textit{E.coli} + pREADOUT when co-cultured with \textit{E.coli} + pDETECT(1) in 0.2% arabinose had previously been established by a colleague, so we aimed to adapt this system for \textit{Salmonella}. pDETECT(1) was initially designed for detection of \textit{C. jejuni}, but as plasmid transfer could not be established by any of the transformation or conjugation methods attempted (Appendix A), we moved forward with testing this plasmid in \textit{Salmonella}. \textit{E.coli} + pDETECT(1) was used as a positive control for TGP fluorescence. TGP fluorescence (480/520) and co-culture absorbance (600nm) were recorded for \textit{E.coli} + pREADOUT co-cultured with either \textit{Salmonella} + pDETECT(1), \textit{E.coli} + pDETECT(1) (positive control), or \textit{Salmonella} + pNULL (negative control) over a 10 hour period. All three strains produced fluorescent readout, reaching maximum RFUs of 9909.97 +/- 100.23, 2801.67 +/- 99.28, and 659.00 +/- 46.52 for \textit{E.coli} + pDETECT(1), \textit{Salmonella} + pDETECT(1), and \textit{Salmonella} + pNULL, respectively (Figure 6).
Figure 6. Comparison of maximum fluorescent output by pREADOUT co-cultured with pDETECT in 0.2% L-arabinose and 0.2% glucose. *E.coli* + pREADOUT was cocultured with *Salmonella* + pDETECT(1), *E.coli* + pDETECT(1) (positive control), or *Salmonella* + pNULL (negative control) in 0.2% arabinose or a 0.2% glucose control. Fluorescence (480/520) was recorded every 5 minutes over a 10 hour window in a Synergy H1 plate reader. Relative fluorescence (RFU) was calculated for each replicate by subtracting the mean of blank wells without the addition of bacteria. The maximum relative fluorescence (RFUmax) was calculated for each replicate (n=3), and plotted in GraphPad Prism 9.

In 0.2% arabinose, *E.coli* + pDETECT(1) produced the strongest fluorescent output and had the fastest time to detection at 30 minutes (34.33 RFU +/- 28.68) (Figure 7a). *Salmonella* + pDETECT(1) took 4x longer to become detectable from the pNULL control at 2hr 10 min (40.67 RFU +/- 18.82). Neither strain produced a detectable signal in the 0.2% glucose control (Figure 7b). The absorbance data indicates that all strains had similar growth patterns, reaching mean A600s of 0.52, 0.53, and 0.52 in arabinose (Figure 7c) and 0.62, 0.63, and 0.63 in glucose (Figure 7d) for *Salmonella* + pDETECT(1), *Salmonella* + pNULL, and *E.coli* + pDETECT(1) respectively.
Figure 7. Relative fluorescence and absorbance trends for \textit{E.coli} + pREADOUT co-cultured with \textit{E.coli} + pDETECT(1) or \textit{Salmonella} + pDETECT(1). \textit{E.coli} + pREADOUT was cocultured with \textit{Salmonella} + pDETECT(1), \textit{E.coli} + pDETECT(1) (positive control), or \textit{Salmonella} + pNULL (negative control) in 0.2\% arabinose (a, c) or a 0.2\% glucose control (b,d). Fluorescence (480/520) (a,b) and absorbance (c,d) was recorded every 5 minutes over a 10 hour window in a Synergy H1 plate reader. Relative fluorescence (RFU) and relative absorbance (RA600) was calculated for each replicate by subtracting the mean of blank wells without the addition of bacteria, and plotted for each replicate in GraphPad Prism 9.
4.1.1 Evaluating media conditions for optimal fluorescent output by 
*E.coli* + pREADOUT when co-cultured with *Salmonella* + 
pDETECT

With successful fluorescent readout of pREADOUT by *Salmonella* + pDETECT(1), we 
moved on toward optimizing the conditions of fluorescent readout. *E.coli* + pREADOUT 
was co-cultured with *Salmonella* + pDETECT(1) in 0.2%, 0.02%, 0.002%, 0.0002%, or 
0.00002% arabinose, along with 0.2% glucose control, to determine which condition was 
optimal for fluorescent output. In all conditions, except the glucose control, *Salmonella* + 
pDETECT produced fluorescent output distinguishable from the control (Figure 8a-f). The time to detection appeared to scale inversely with the arabinose concentration, with 
times of 2 hours, 2 hours 35 minutes, 2 hours 40 minutes, 3 hours 15 minutes, and 3 
hours 50 minutes for 0.2%, 0.02%, 0.002%, 0.0002%, and 0.00002% arabinose, 
respectively (Figure 8a, b, c, d, e).
Figure 8. Evaluating various L-arabinose concentrations for optimal fluorescent output by E.coli + pREADOUT when co-cultured with Salmonella + pDETECT. To investigate the optimal media conditions for the fluorescent assay, E.coli-pREADOUT was cocultured at equal ratios with Salmonella-pDETECT(1) or Salmonella-pNULL (negative control) in a) 0.2% arabinose b) 0.02% arabinose c) 0.002% arabinose d) 0.0002% e) 0.00002% or f) 0.2% glucose as a control (n=3). Cocultures were incubated at 37°C, and fluorescence (520/480) were recorded every 5 minutes. Maximum RFU
reached over the 10 hour window was compared to determine which media condition produced the strongest fluorescent readout (g).

The RFUs for all media conditions were normalized to the 0.2% glucose control, and a one-way ANOVA revealed a statistically significant difference between at least two groups for pDETECT(1) (F(5, 720)=21.82, p = <0.0001). Tukey’s HSD test for multiple comparisons found that readout was highest in 0.02% and 0.002% arabinose compared to 0.2% arabinose, and these results were significant (p=<0.001, 95% CI = [56.40, 170.2]; p=<0.001, 95% CI = [69.35, 183.2]). The 0.0002% arabinose condition was produced a higher signal than 0.2% and 0.00002% arabinose, and these results were significant (p=0.0234, 95% CI = 5.128, 119.0); p=0.0211, 95% CI = [-119. -5.80] ). The 0.0002% arabinose condition produced a lower signal than either 0.02 or 0.002%, and this result was significant for the 0.002% condition (p=0.0166, 96% CI = [-121.1, -7.308]). The 0.0002% condition was not significantly different than the 0.02% arabinose condition (p=0.1050). The two highest readout conditions, 0.02% and 0.002% arabinose, were not found to be significantly different than each other (p=0.9870). The lowest readout groups, 0.2% and 0.00002% arabinose, were not found to be significantly different from each other (p=<0.9999).

It was a surprising result that in 0.02% and 0.002% arabinose conditions, Salmonella+pDETECT produced higher RFUmax than the highest concentration (0.2%). This may be explained by differential cell growth in different concentrations of arabinose, as indicated by the relative A600 of the different cultures (Figure 9). In 0.2% arabinose, Salmonella + pDETECT had the lowest final A600, reaching a plateau at the 4-hour mark of 0.491 +/- 0.032 (n=3) (Figure 9a,g). The other media conditions had a final A600 ranging between 0.623 (Figure 9f) to 0.836 (Figure 9c), and followed a linear growth pattern without plateau for the entire 10 hour readout.
Figure 9. Absorbance patterns as an indicator of growth by co-cultures of *E. coli* + pREADOUT and *Salmonella* + pDETECT in various concentrations of L-arabinose.

*E. coli*-pREADOUT was cocultured at equal ratios with *Salmonella*-pDETECT(1) or *Salmonella*-pNULL (negative control) in a) 0.2% arabinose b) 0.02% arabinose c) 0.002% arabinose d) 0.0002% e) 0.00002% or f) 0.2% glucose as a control (n=3). Cocultures were incubated overnight (~10 hours) at 37°C, and absorbance (600nm) was
recorded every 5 minutes. Maximum RA reached over the 10 hour window was compared to determine maximum growth achieved by cultures (g).

4.1.2 Estimating the minimum *Salmonella* + pDETECT concentration necessary to induce fluorescent readout by *E.coli* + pREADOUT

Although 0.2% arabinose produced the quickest time to detection, 0.02% arabinose resulted in over a 100-fold higher RFU with only 35 minutes difference in detection time. 0.02% arabinose was chosen as the concentration for determining the minimum CFU/mL of *Salmonella* + pDETECT(1) necessary to produce readout in the biosensor assay. *Salmonella* overnights were diluted, grown to $A_{600} = 0.5$, and diluted 10-fold from $10^1 - 10^6$ prior to co-culture with *E.coli* + pREADOUT. The initial *Salmonella* + pDETECT(1) and *Salmonella* + pNULL cultures prior to co-culture were roughly equal – with average concentrations in the $10^8$CFU/mL range (n=3).

Sample concentrations of $10^8$, $10^7$, and $10^6$ CFU/mL produced a fluorescent signal distinct from the pNULL control at 25 minutes, 2 hours and 20 minutes, and 3 hours, respectively (Figure 10 a, b, c). Initial concentrations of $10^5$-$10^2$ CFU/mL did not differ from the control (Figure 10 d, e, f, g).
Figure 10. Assessing minimum initial concentration of *Salmonella* + pDETECT to produce measurable TGP fluorescence by *E.coli* + pREADOUT in 0.02% arabinose. Equal initial concentrations of *Salmonella* + pDETECT and *Salmonella* + pNULL were
compared at $10^8$, $10^7$, $10^6$, $10^5$, $10^4$, $10^3$, and $10^2$ initial CFU/mL when co-cultured with *E.coli* + pREADOUT in 0.02% arabinose. Fluorescence (480/520) was recorded every 5 minutes for 10 hours, and the mean relative fluorescence for each timepoint was plotted (+/- SD) (n=3).

### 4.1.3 Evaluating two promoters for optimal induction of fluorescence by *Salmonella* + pDETECT

To further optimize the plasmid readout system for *Salmonella*, we cloned the rpsM promoter in place of the original Anderson promoter. The cloning was deemed successful by whole-plasmid sequencing (Flow Genomics), and we repeated the minimum detection assay in both 0.2% and 0.02% arabinose to determine if pDETECT(rpsM) performed differently than the original promoter.

The limit of detection of pDETECT(rpsM) and pDETECT(Anderson) was $10^4$ CFU/mL for both media conditions (Figure 11, 12). In 0.2% arabinose, the times to detection for the lowest concentration were roughly equal, with times to detection of 4 hours 40 minutes and 4 hours 45 minutes for pDETECT(Anderson) and pDETECT(rpsM), respectively (Figure 11e). At the highest concentration $10^8$ CFU/mL, the time to detection for pDETECT(Anderson) was 25 minutes while pDETECT(rpsM) was 30 minutes in 0.2% arabinose (Figure 11a).
Figure 11. Comparison of fluorescent readout of *Salmonella* + pDETECT(Anderson) and *Salmonella* + pDETECT(rpsM) co-cultured with E.coli + pREADOUT 0.2% arabinose. *Salmonella* + pDETECT(rpsM) or + pDETECT(Anderson) were compared to *Salmonella* + pNULL at concentrations of a) $10^8$ b) $10^7$ c) $10^6$ d) $10^5$ e) $10^4$ or f) $10^3$ CFU/mL when co-cultured with E.coli + pREADOUT in LSLB + 0.2% arabinose. Co-cultures were incubated at 37°C and fluorescence (480/520) was measured every 5 minutes over a 15 hour window in a Synergy H1 plate reader. Relative fluorescence was plotted as mean +/- SD for each plasmid in GraphPad Prism 9.

In the 0.02% arabinose condition, the time to detection for the lowest detectable concentration ($10^4$ CFU/mL) was also roughly equal at 6.5 hours for both plasmids (n=3) (Figure 12e). At the highest concentration tested ($10^8$ CFU/mL), the times to detection were 2 hours 5 minutes for pDETECT(Anderson) and 1 hour 55 minutes for pDETECT(rpsM) (Figure 12a). As the time for detection was faster in 0.2% arabinose in all conditions for pDETECT(rpsM), except for the $10^4$ CFU/mL concentration, the 0.2%
0.02% arabinose condition was adopted for future use in conjugation assays. Additionally, as the two plasmids did not differ significantly in their time to detection, the pDTECT(rpsM) plasmid was used in further assays.

Figure 12. Comparison of fluorescent readout of *Salmonella* + pDTECT(Anderson) and *Salmonella* + pDTECT(rpsM) co-cultured with *E.coli* + pREADOUT 0.02% arabinose. *Salmonella* + pDTECT(rpsM) or + pDTECT(Anderson) were compared to *Salmonella* + pNULL at concentrations of a) $10^8$ b) $10^7$ c) $10^6$ d) $10^5$ e) $10^4$ or f) $10^3$ CFU/mL when co-cultured with *E.coli* + pREADOUT in LSLB + 0.02% arabinose. Co-cultures were incubated at 37°C and fluorescence (480/520) was measured every 5 minutes over a 15 hour window in a Synergy H1 plate reader. Relative fluorescence was plotted as mean +/- SD for each plasmid in GraphPad Prism 9.
4.2 Optimization of conjugation of pDETECT from an
_E.coli_ (dapA-) donor to _Salmonella_

4.2.1 Conjugation time

After demonstrating that fluorescence could be consistently produced by _Salmonella_ + pDETECT(Anderson), and when modified to pDETECT(rpsM), the optimal conditions for conjugation of pDETECT(rpsM) from an _E.coli_ donor to _Salmonella_ were investigated. pDETECT(rpsM) was transformed into an auxotrophic strain of _E.coli_ Epi300 (dapA-) to act as the initial conjugative donor of pDETECT(rpsM) to _Salmonella_.

To investigate optimal time for conjugation, _E.coli_ (dapA-) + pDETECT(rpsM) was co-cultured at equal ratios with _Salmonella_ and conjugated at 37°C for 0.5, 1, 2, 3, or 4 hours (n=4). Of the four conjugation times tested, the 0.5 hour conjugation produced the highest conjugation frequency (0.04%, n=4) whereas the 4 hour conjugation produced the lowest (0.0006%, n=4) (Figure 13). A one-way ANOVA of the mean conjugation frequencies revealed a statistically significant difference in mean CFs in at least two of the conjugation times tested (F(3,12) = [5.58], p=0.0124). Tukey’s HSD Test for multiple comparisons revealed a significant difference between conjugation times of 0.5 hours and 2 hours (p=[0.0302], 95% CI = [2.9510 x 10^{-5}, 0.0006]) or 4 hours (p=[0.0125], 95% CI = [8.061 x 10^{-5}, 0.0007]). Based on the results, a conjugation time of 0.5 hours was used in subsequent experiments.
Figure 13. Evaluating different conjugation times for optimal delivery of pDETECT(rpsM) from *E.coli* (dapA-) donor to *Salmonella*. *E.coli* (dapA-) + pDETECT (rpsM) and *Salmonella* were grown to an A600=0.5, and co-cultured at an equal ratio. Cultures were incubated at 37°C without shaking for 0.5, 1, 2, or 4 hours to allow for conjugation (n=4). CFU counts were established via selective plating, and conjugation frequencies (CF) were calculated for each conjugation time (CF = (CFU\_transconjugants/CFU\_recipients) x 100%). Mean CFs were analyzed via a one-way ANOVA with Tukey’s HSD Test for multiple comparisons, a significant result (p<0.05) is indicated by *.

4.2.2 Recipient:donor ratios

To determine the optimal ratio of recipients to donors (R:D) for efficient conjugation of pDETECT(rpsM) to *Salmonella*, the conjugations were repeated at estimated R:D ratios
of 1.0, 0.5, 0.2, 0.1, 0.01, and 0.001 with a conjugation time of 30 minutes. Initial culture
densities of *Salmonella* recipients and *E.coli* donors were estimated by spot plating to be
1.78 x 10^8 CFU/mL and 1.53 x 10^8 CFU/mL – therefore the actual R:D ratios tested were
1.16, 0.56, 0.23, 0.12, 0.01, and 0.001. The conjugation frequencies were calculated for
each replicate, and their means were compared by a one-way ANOVA with Tukey’s
HSD test for multiple comparisons (Figure 14).

The lowest CF was produced by a R:D ratio of 1 (0.02%), whereas the highest CF was
produced with a R:D of 0.001 (2.10%) (Figure 14). Results of a one-way ANOVA
revealed that there was a statistically significant difference in mean CFs between at least
two of the R:D ratios tested (F(5, 18) = [24.85], p<0.0001). Tukey’s HSD Test for
multiple comparisons found that the mean CF was significantly different between R:D of
0.01 and 1.16 (p=[0.0021], 95% CI = [-0.0234, -0.0045]), 0.56 (p=[0.0032], 95% CI = [-
0.0228, -0.0039]), 0.23 (p=[0.0026], 95% CI = [-0.0231, -0.0042]), and 0.12 (p=[0.0167],
95% CI = [-0.0205, -0.0016]. Tukey’s HSD test also revealed the mean CF was
significantly different between the highest R:D ratio of 0.001 and 1.16 (p=[<0.0001],
95% CI = [-0.0351, -0.0162]), 0.56 (p=[<0.0001], 95% CI = [-0.0345, -0.0157]), 0.23
(p=[<0.0001], 95% CI = [-0.0348, -0.0159]), 0.12 (p=[<0.0001], 95% CI = [-0.0322, -
0.0133]), and 0.01 (p=[0.0104], 95% CI = [-0.0211, -0.0023]). The highest R:D ratios of
0.001 and 0.01 produced conjugation frequencies of 2.10% and 1.42%, respectively,
reflecting a difference of 0.68%.
4.2.3 Outgrowth for sufficient removal of donor *E. coli* (dapA-)

To determine outgrowth time necessary for sufficient removal of the initial *E. coli* (dapA-) + pDETECT(rpsM) donors, donors and *Salmonella* recipients were co-cultured at a R:D ratio of 0.01, conjugated for 0.5 hours at 37°C. Co-cultures were then washed for removal of remaining DAP in the media, and outgrown in either LSLB liquid or LSLB agar.
without antibiotics for 0.5, 1, 2, 4, or 6 hours (n=3). The concentration of *E.coli* (dapA-)
following the initial conjugation was $10^7$ CFU/mL. The average conjugation frequency
for the co-cultures was 1.37% (n=3). The final *E.coli* donor concentrations were divided
by the pre-outgrowth concentration to determine the percentage (%) of remaining *E.coli*
(dapA-) donors following outgrowth in liquid (Figure 15a) or on plates (Figure 15b).

The liquid outgrowth did not fully remove *E.coli* donors within the time periods tested,
with $10^8$ CFU/mL still present at the 6 hour timepoint (Figure 15a). In contrast, the plate
outgrowth saw full removal of the *E.coli* donors following a 6 hour outgrowth (Figure
15b). The plate outgrowth saw a steady decrease of *E.coli* donors, with final
concentrations of $10^7$, $10^6$, $10^3$, $10^2$, and 0 CFU/mL for 0.5, 1, 2, 4, and 6 hour
outgrowths, respectively.

The ratio of *Salmonella* + pDETECT(rpsM) transconjugants to un-conjugated *Salmonella*
recipients steadily decreased as the outgrowth time increased in liquid LSLB outgrowth,
decreasing from 1.36% with a 0.5 hour outgrowth and decreasing to 0.13% following the
6 hour outgrowth (Figure 15c). In the plate outgrowth the ratio of transconjugants to un-
conjugated recipients was more variable, without a distinct trend (Figure 15d). The
highest ratio was 14.76% for the 1 hour plate outgrowth, and the lowest was 1.79% for
the 6 hour outgrowth.

As only the plate outgrowth produced 100% removal of the initial *E.coli* (dapA-) donor,
outgrowth on LSLB agar for 6 hours was set as the standard outgrowth time for
subsequent assays.
Figure 15. Evaluating outgrowth conditions for sufficient removal of *E.coli* (dapA-) + pDETECT(rpsM) following conjugation with *Salmonella*. *E.coli* (dapA-) + pDETECT(rpsM) and *Salmonella* were grown to 108CFU/mL and co-cultured at a
recipient:donor ratio of 0.01. Following a 30 minute conjugation, co-cultures were washed via centrifugation for removal of excess DAP from the media. Co-cultures were then incubated in liquid LSLB (a, c) or on LSLB agar (b, d) for 0.5, 1, 2, 4, or 6 hours. CFU counts were established via selective plating, and the ratio of *E. coli* (dapA-) donors pre- and post- outgrowth (a, b) was calculated. The ratio of *Salmonella* transconjugants and un-conjugated recipients (c, d) was also calculated and compared for the two outgrowth conditions.

### 4.2.4 pDETECT produces measurable fluorescent readout when conjugated to *Salmonella* from a donor *E. coli* (dapA-)

Fluorescent readout of pDETECT(rpsM) in *Salmonella* was previously established, along with the optimal time and R:D ratio for conjugation, and the conditions necessary for elimination of the initial *E. coli* (dapA-) pDETECT donor. To determine if *Salmonella* + pDETECT(rpsM) transconjugants could produce fluorescence in the biosensor assay, *Salmonella* was co-cultured with *E. coli* (dapA-) + pDETECT(rpsM) at a 0.01 R:D ratio for 0.5 hours, and outgrown for 6 hours on antibiotic-free LSLB agar. Control for this experiment was *Salmonella* recipients without the addition of *E. coli* (dapA-) + pDETECT(rpsM) donors. Following the outgrowth, samples were diluted tenfold, selectively plated and co-cultured with *E. coli* + pREADOUT.

Following conjugation and outgrowth, 0 CFU/mL of the initial *E. coli* donors remained. For the experimental group, the ratio of transconjugants to un-conjugated *Salmonella* recipients was 1.89% - 0% for the *Salmonella*-recipient only control (n=4). The resuspended outgrowths had a transconjugant concentration of $10^7$ CFU/mL, so the estimated concentrations of the dilutions tested was $10^7$, $10^6$, and $10^5$ CFU/mL transconjugants. Both the $10^7$ and $10^6$ CFU/mL transconjugant ranges produced measurable fluorescence above the *Salmonella* + pNULL control, while the $10^5$ CFU/mL condition did not (Figure 16a). The time to detection for the $10^7$ CFU/mL condition was 2 hours and 15 minutes, and reached a max RFU of 2584 +/- 468 RFU after 15 hours (n=4) (Figure 16a, b). The $10^6$ CFU/mL condition had a time to detection of 4 hours and 25 minutes, reaching a max RFU of 1359 +/- 436 RFU (n=4) (Figure 16a, b).
Figure 16. Relative fluorescence and absorbance of \textit{Salmonella} + pDETECT(rpsM) transconjugants co-cultured with \textit{E.coli} + pREADOUT following conjugation and outgrowth of a donor \textit{E.coli} (dapA<sup>-</sup>). \textit{E.coli} (dapA<sup>-</sup>) + pDETECT (rpsM) and
Salmonella were grown to an A600=0.5, and were co-cultured at a recipient:donor ratio of 0.01. Following a 30 minute conjugation, co-cultures were incubated at 37°C on LSLB agar for 6 hours for removal of the E.coli donor. Following outgrowth and re-suspension in LSLB, the transconjugant concentrations were $10^7$, $10^6$, and $10^5$ CFU/mL prior to co-culture with E.coli + pREADOUT in LSLB + 0.2% arabinose. A) Fluorescence (480/520) and b) absorbance (A600) were measured every 5 minutes over 15 hours in a Synergy H1 plate reader. Relative fluorescence (RFU) and absorbance (RA) were calculated by subtracting the mean of bacteria-free wells and the mean RFU (a) and RA (c) were plotted (+/- SD). The maxRFU (b) and maxRA (d) were determined for each replicate. Graphs were made in GraphPad Prism 9.

4.3 Demonstrating proof-of-principle for the fluorescent biosensor assay in detecting Salmonella in artificially spiked food samples

To evaluate the fluorescent biosensor’s potential utility in detecting Salmonella in contaminated food samples, 25g samples of PC Organic Spring Mix (Figure 17a) were spiked with various concentrations of Salmonella, digested in a lab blender, and incubated for 2 hours at 37°C. Prior to co-culture with E.coli (dapA-) + pDETECT(rpsM), the two spike groups corresponded to $10^6$ and $10^5$ CFU/mL Salmonella (n=3). These samples were co-cultured with $10^6$ CFU/mL E.coli (dapA-) + pDETECT(rpsM) – making the R:D ratios 1 and 0.1 for the high and low spike condition. The conjugation frequencies were 0.93% and 1.16% for the high and low spike, respectively (Figure 17b).

Following conjugation and outgrowth, the ratio of transconjugants/un-conjugated recipients for the high and low spike condition were 0.46% and 0.29%, respectively (Figure 17b) (n=3). The low spike had $10^4$ CFU/mL transconjugants while the high spike had $10^6$ CFU/mL transconjugants prior to the fluorescent biosensor assay (n=3). The unspiked control had a transconjugant/un-conjugated recipient ratio of 0%, and 0 CFU/mL transconjugants produced (n=3).
All three conditions (high spike, low spike, unspiked control) had fluorescent output distinct from the *Salmonella* + pNULL control (Figure 17c). The high spike had a time to detection of 1 hour, while the low spike was 2 hours and 15 minutes (Figure 17c). This was a surprising result, as the time to detection was ~1 hour faster in this assay than in the previous conjugation assay for $10^6$ CFU/mL transconjugants. Another unexpected result was that the highest fluorescent output was seen by the unspiked control condition, with the fastest time to detection of 5 minutes (Figure 17c).
Figure 17. Fluorescent detection of *Salmonella* + pDETECT(rpsM) transconjugants in artificially spiked spring mix samples. 25g samples of Pc Organic Spring Mix (a) were spiked with overnights of *Salmonella*, along with an unspiked control group. and
incubated for 2 hours at 37°C (n=3). Post-incubation, *Salmonella*-spiked spring mix was co-cultured with *E.coli* (dapA-) + pDETECT(rpsM) at recipient:donor ratios of 1 and 0.1, for the high and low spike respectively. Co-cultures were incubated for 30 minutes, followed by a 6 hour outgrowth on antibiotic-free LSLB for removal of the donor *E.coli*. Post-outgrowth, samples were resuspended and co-cultured with *E.coli* + pREADOUT in 0.2% arabinose, and fluorescence was read every 5 minutes for 15 hours (c). Samples were selectively plated pre- and post- outgrowth, and CFU counts of *Salmonella* recipients and *Salmonella* transconjugants were compared (b).

This assay was repeated with a broader range of spike conditions, and with the addition of Km selection to the original spike media (0.1% peptone water) prior to the initial incubation step to reduce any bacteria that may have been present in the spring mix samples. In addition to selective plating for initial CFU counts, the unspiked control was also plated on antibiotic-free LSLB, which revealed a heterogeneous mixture of cells naturally present from the spring mix (Figure 18).

**Figure 18. Unspiked spring mix samples spotted on antibiotic-free LSLB.** 25g samples of PC Organic Spring Mix were blended in 100mL of sterile 0.1% peptone water for 1 minute and incubated for 2 hours at 37°C. Following incubation, samples were diluted tenfold and 10μL of each dilution was plated on antibiotic-free LSLB per replicate (n=3).
Prior to conjugation with *E. coli* (dapA-) + pDETECT(rpsM), the spiked samples had *Salmonella* concentrations of $10^7$, $10^6$, $10^5$, $10^4$, & $10^3$ CFU/mL, which corresponded to recipient:donor ratios of 1, 0.1, 0.01, 0.001, and 0.0001 for the conjugation step. Additionally, the unspiked controls all produced colonies on LSLB + Km, indicating some naturally present bacteria was antibiotic resistant.

The average conjugation frequencies for the spiked samples were 0.32%, 0.13%, 0.17%, 6.24%, and 3.99% from the highest to lowest spike condition (Figure 19a) (n=3). The unspiked control had a CF of 0%. Following outgrowth, the average ratio of *Salmonella* + pDETECT(rpsM) transconjugants to unconjugated recipients were 0.007%, 0.009%, 0.004%, 0.003%, 0.002% from the highest to lowest spike condition, respectively (Figure 19b) (n=3). These conjugation frequencies resulted in $10^4$, $10^4$, $10^3$, $10^3$, and $10^2$ CFU/mL transconjugants from the highest to lowest spike conditions, respectively, and 0 CFU/mL for the unspiked control.

**Figure 19.** Ratios of *Salmonella* transconjugants to un-conjugated *Salmonella* recipients pre- and post-outgrowth for removal of *E. coli* (dapA-) + pDETECT(rpsM). 25g samples of PC Organic Spring Mix were spiked with *Salmonella* and digested in a lab blender for 1 minute with 0.1% peptone water + Km selection prior to incubation for 2 hours at 37°C. The *Salmonella* concentrations of the spiked samples
prior to conjugation were $10^7$, $10^6$, $10^5$, $10^4$ or $10^3$ CFU/mL (n=3). Control for this experiment was an unspiked control group. Post-incubation, spring mix samples were co-cultured with *E. coli* (dapA-) + pDETECT(rpsM) resulting in recipient:donor ratios of 1, 0.1, 0.01, 0.001, or 0.0001 for the highest to lowest spikes, respectively. Co-cultures were incubated for 30 minutes, followed by a 6 hour outgrowth on antibiotic-free LSLB for removal of the donor *E. coli*. Pre- and post- outgrowth, samples were diluted tenfold and selectively plated to determine the a) conjugation frequency of *E. coli* (dapA-) + pDETECT(rpsM) to spiked samples and b) ratio of transconjugants to unconjugated *Salmonella* recipients remaining post outgrowth.

Initial spikes of $10^7$ and $10^6$ CFU/mL did not produce a signal distinct from the control (Figure 20a, b). $10^5$, $10^4$, and $10^3$ CFU/mL spikes produced detectable signals at 9 hours 40 minutes, 10 hours 35 minutes, and 10 hours 40 minutes, respectively (Figure 20c, d, e). The unspiked control again had the quickest time to detection of 1 hour 55 minutes (Figure 20).
Figure 20. Fluorescent biosensor assay for detection of *Salmonella* + pDETECT(rpsM) transconjugants from *Salmonella*-spiked spring mix. 25g samples of PC Organic Spring Mix were spiked with *Salmonella* and digested in a lab blender for 1 minute with 0.1% peptone water + Km selection prior to incubation for 2 hours at 37°C. The *Salmonella* concentrations of the spiked samples prior to conjugation were $10^7$, $10^6$, $10^5$, $10^4$ or $10^3$ CFU/mL (n=3). Control for this experiment was an unspiked control.
group. Post-incubation, spring mix samples were co-cultured with *E. coli* (dapA-) + pDETECT(rpsM) resulting in recipient:donor ratios of 1, 0.1, 0.01, 0.001, or 0.0001 for the highest to lowest spikes, respectively. Co-cultures were incubated for 30 minutes, followed by a 6 hour outgrowth on antibiotic-free LSLB for removal of the donor *E. coli*. Post outgrowth, samples were re-suspended in LSLB and co-cultured with *E. coli* + pREADOUT in 0.2% arabinose, and fluorescence (480/520) was read every 5 minutes for 15 hours in a Synergy H1 plate reader (n=6). *E. coli* + pREADOUT was co-cultured with *Salmonella* + pNULL as a control for fluorescence.

**4.3.1 Optimizing outgrowth conditions for reduction of background fluorescence by unspiked spring mix samples**

We repeated the assay with starting spike concentrations of 10^7 and 10^6 CFU/mL *Salmonella* spikes, with the goal of reducing off-target conjugation to bacteria in the spring mix by adding antibiotic selection to the outgrowth media. We tested the 6 hour outgrowth in LSLB only, LSLB + Km, or LSLB + KmCm (n=3).

Prior to outgrowth, the conjugation frequencies were 0.11% and 0.60% for the 10^7 and 10^6 CFU/mL spikes, respectively. Following outgrowth on LSLB only, the ratio of transconjugants to un-conjugated recipients were 0.01% and 0.11% for the 10^7 and 10^6 CFU/mL spikes, and 0.07% for the unspiked control (n=3) (Figure 21d). This correlated with 10^5 and 10^6 CFU/mL transconjugants for the high and low spike, respectively, and 10^2 CFU/mL transconjugants for the unspiked control. LSLB + Km outgrowth had similar final ratios, with the ratio of transconjugants to un-conjugated recipients were 0.08% and 0.15% for the 10^7 and 10^6 CFU/mL spikes, whereas the unspiked control had a higher ratio at 0.33% (n=3) (Figure 21e). These produced the same concentration of transconjugants as the LSLB only outgrowth condition. Conversely, in the LSLB + KmCm outgrowth the ratio of transconjugants to un-conjugated recipients was much higher - 31.00% and 61.11% - for the 10^7 and 10^6 CFU/mL spikes, while the unspiked control was 0% (Figure 21f). These corresponded to transconjugant concentrations of 10^5 and 10^4 CFU/mL for the high and low spike, respectively, and there were 0 CFU/mL transconjugants for the unspiked control.
The LSLB outgrowth had the highest fluorescence produced by the unspiked control sample, which was in line with previous experiments (Figure 21a). The time to detection for the unspiked sample was 5 minutes, considerably faster than the $10^7$ and $10^6$ CFU spikes which were detectable within 1 hour 30 minutes and 2 hours 10 minutes, respectively (Figure 21a). In the LSLB + Km outgrowth, the $10^7$ and $10^6$ CFU/mL spikes did not produce a detectable signal above the pNULL control (Figure 21b). However, the unspiked control had a time to detection of 3 hours and 20 minutes (Figure 21b). The LSLB + KmCm outgrowth was the only condition where the $10^7$ and $10^6$ CFU/mL spikes produced a faster time to detection, and stronger RFU output than the unspiked control (Figure 21c). The higher spike concentration had the fastest time to detection at 3.5 hours, whereas the $10^6$ CFU/mL concentration had a time to detection of 3 hrs 50 minutes.
Figure 21. Fluorescent biosensor assay for detection of *Salmonella* + pDETECT(rpsM) transconjugants under various outgrowth conditions for removal of donor *E.coli* (dapA-). 25g samples of PC Organic Spring Mix were spiked with
Salmonella, and digested in a lab blender for 1 minute with 0.1% peptone water + Km selection prior to incubation for 2 hours at 37°C – resulting in initial Salmonella concentrations of $10^7$ or $10^6$ CFU/mL (n=3). Control for this experiment was an un-spiked control group. Post-incubation, spring mix samples were co-cultured with E.coli (dapA-) + pDETECT(rpsM) and incubated for 30 minutes at 37°C to allow for conjugation. Post conjugation, samples were outgrown on LSLB (a, d), LSLB + Km (b, e), or LSLB + KmCm (c, f) agar for 6 hours for removal of the E.coli donor (n=3). Following outgrowth, cultures were resuspended and co-cultured with E.coli + pREADOUT, and fluorescence (480/520) was read every 5 minutes for 15 hours in a Synergy H1 plate reader (n=6) (a, b, c). E.coli + pREADOUT was also co-cultured with Salmonella + pNULL as a control for fluorescent readout. Resuspended samples were also diluted tenfold and 10uL were selectively plated to determine the remaining ratio of transconjugants to un-conjugated recipients for each respective outgrowth type (d, e, f) (n=3).

In addition to selective plating on LSLB + Km + Cm for selection of Salmonella transformants, we plated on LSLB + Cm as well to capture all bacteria with pDETECT(rpsM), and compared the two to investigate off-target conjugation to off-target bacteria in the spring mix (Figure 22). The $10^7$ CFU/mL spike had relatively equal CFU for Salmonella transconjugants (KmCm) and all transconjugants (Cm only) in all outgrowth conditions tested (Figure 22a, b). Concentrations of $10^5$ CFU/mL, $10^6$ CFU/mL, and $10^5$ CFU/mL were present from the LSLB, LSLB + Km, and LSLB + KmCm outgrowths in both. The $10^6$ CFU/mL spike had a similar pattern – with $10^6$ CFU/mL for both LSLB (Figure 22a) and LSLB + Kan outgrowths (Figure 22c). However, on LSLB + KmCm the concentrations were $10^4$ and $10^5$ CFU/mL for Salmonella transconjugants and all transconjugants, respectively (n=3). In the control condition, the LSLB outgrowth produced CFU/mL of $10^2$ and $10^5$ for selective plating on KmCm and Cm respectively (Figure 22a). When outgrown on LSLB + Km, this ratio evened out, with $10^2$ CFU/mL on both KmCm and Cm selection (Figure 22d). With double antibiotic selection for the outgrowth (LSLB + Km Cm), there were 0 transformants on either the KmCm selection or Cm only selection.
Figure 22. Measuring pDETECT(rpsM) transconjugants in Salmonella-spiked spring following removal of E.coli (dapA-) under various outgrowth conditions. 25g samples of Pc Organic Spring Mix were spiked with Salmonella, digested in a lab blender with 0.1% peptone water + Km for 1 minute, and incubated for 2 hours resulting in initial Salmonella concentrations of b) $10^7$ or c) $10^6$ CFU/mL (n=3). Control for this experiment was an unspiked control group (n=3) (d). Post-incubation, spring mix samples were co-cultured with E.coli (dapA-) + pDETECT(rpsM) and incubated for 30 minutes to allow for conjugation. Post conjugation, samples were outgrown on LSLB (a), LSLB + Km, or LSLB + KmCm for 6 hours for removal of the E.coli (dapA-) donor. Following outgrowth, samples were resuspended, diluted tenfold, and 10uL was plated on LSLB + KmCm and LSLB + Cm for identification of Salmonella transconjugants and all transconjugants, respectively. Mean concentration (CFU/mL) and standard deviation were plotted in GraphPad Prism 9.
The LSLB + KmCm produced higher final ratios of transconjugants to un-conjugated recipients, and less background fluorescence from the unspiked control group. A potential benefit of outgrowth on double selection was reduction of outgrowth time. To investigate this potential benefit, *E.coli* (dapA-) + pDETECT(rpsM) and *Salmonella* were conjugated at a 0.01 R:D ratio, and outgrowth on LSLB agar or LSLB + KmCm for 0.5, 1, 2, or 4 hours.

The conjugation frequency of pDETECT(rpsM) to *Salmonella* was 0.25% (n=3). Following outgrowth, the ratio of transconjugants to un-conjugated recipients in LSLB was 0.26% after 4 hours- reflective of the initial conjugation frequency (Figure 23c). In LSLB + KmCm, the ratio of transconjugants/recipients after 4 hours was 32.38% (Figure 23d). Although the absolute number of transconjugants was in the 10^5 CFU/mL range for both conditions, the overall ratio of transconjugants to un-conjugated recipients was higher in the LSLB + KmCm outgrowth.

In examining the number of remaining *E.coli* donors, it was found on the LSLB agar that none of the outgrowth times tested sufficiently killed the donors. Even at the longest outgrowth (4 hours) 104 CFU/mL (0.07% of initial *E.coli* donors) remained (Figure 23a). In the LSLB + KmCm outgrowth, 100% of the initial *E.coli* donors were removed by the 4 hour timepoint (Figure 23b).
Figure 23. Comparison of outgrowth for removal of *E.coli* (dapA-) + pDETECT(rpsM) donors in antibiotic free or double antibiotic selection. *E.coli* (dapA-) + pDETECT (rpsM) and *Salmonella* were grown to an $A_{600}=0.5$, and were co-cultured at a recipient:donor ratio of 0.01. Following a 30 minute conjugation, co-cultures were incubated at 37°C on LSLB agar (a, c) or on LSLB + KmCm agar (b, d) for 0.5, 1, 2, or 4 hours (n=3). CFU counts were established via selective plating, and the ratio of final *E.coli* donor to pre-conjugation concentrations (a, b) and ratio of transconjugants to un-conjugated recipients remaining (c, d) were calculated for each conjugation time.
To further investigate the source of off-target fluorescence by unspiked spring mix samples, 25g samples were prepared by digestion and incubation for 2 hours. To assess potential autofluorescence, samples were tested in the fluorescent biosensor assay with and without the addition of *E.coli + pREADOUT*.

Analysis of the fluorescent readout revealed that unspiked samples, when not co-cultured with *E.coli + pREADOUT*, did not produce any detectable fluorescence (Figure 24) (n=4). However, when co-cultured with *E.coli + pREADOUT*, the unspiked samples produced detectable fluorescence within 4 hours and 4 minutes (Figure 24).

**Figure 24. Evaluating unspiked spring mix samples for autofluorescence in the fluorescent biosensor assay.** 25g lettuce samples were suspended in 0.1% peptone water + Km and digested for 1 minute in a lab blender prior to a 2 hour incubation at 37°C (n=4). After incubation, 5uL samples were added to a LSLB + 0.2% arabinose with or without the addition of *E.coli + pREADOUT*. *Salmonella + pNULL* was co-cultured with
*E. coli* + pREADOUT as a negative control. Samples were incubated at 37°C for 15 hours in a Synergy H1 plate reader and fluorescence (480/520) was measured every 5 minutes. The mean relative fluorescence (RFU) and standard deviation was plotted for each timepoint (n=4).

The assay was repeated with initial *Salmonella* spikes of 10⁷, 10⁶, 10⁵, 10⁴, and 10³ CFU/mL, along with an unspiked control (n=3). These corresponded to a recipient donor ratio of 0.1, 0.01, 0.001, 0.0001, and 0.00001 from the highest to lowest spike concentrations, respectively. The conjugation frequencies after the initial 30 minute conjugation with *E. coli* (dapA-) + pDETECT(rpsM) were 0.08%, 0.42%, 1.11%, 2.61%, and 3.01% from the high to low spike conditions, respectively, and 0% for the unspiked control (Figure 25c). Following outgrowth on LSLB + Km + Cm for 4 hours, the spiked samples had transconjugant concentrations of 10⁵, 10³, 10², 10¹, and 0 CFU/mL from the highest to lowest spike condition, and 0 CFU/ml from the unspiked control.

When compared to the control plasmid pNULL, all groups (including the unspiked control) produced measurable fluorescence between 3 hours and 40 minutes and 4 hours and 25 minutes (Figure 25a, b). Additionally, all spiked samples produced had relative fluorescence above the unspiked control, in contrast to previous assays. The outgrowth with LSLB + Km + Cm appeared sufficient for reduction of background signals from the unspiked spring mix samples. However, these results were interesting given that the lowest spike condition (10³ CFU/mL) still produced measurable fluorescence above the control, despite lack of transconjugants produced during selective plating. This could potentially represent error in plating, as the small volumes used in dilution (20uL) and plating (10uL) may be less accurate. Prior to outgrowth, both the 10³ and 10⁴ CFU/mL cultures had 10² CFU/mL transconjugants and 10³ CFU/mL total *Salmonella*. Following outgrowth, however, both conditions had 10² CFU/mL total *Salmonella*, but the 10⁴ CFU/mL spike still had 10² CFU/mL transconjugants while the 10³ CFU/mL spike did not.
Figure 25. **Fluorescent biosensor assay in spring mix spiked with various concentrations of *Salmonella***. 25g lettuce samples were suspended in 0.1% peptone water + Km and digested for 1 minute in a lab blender prior to a 2 hour incubation at 37°C (n=3). Samples were then co-cultured with *E.coli* (dapA-) + pDETECT(rpsM) and incubated for 0.5hrs at 37°C to allow for conjugation to occur (n=3). Selective plating was done following conjugation to determine conjugation frequencies (c). Samples were washed via centrifugation, and cells were plated on LSLB + Km + Cm and incubated for 4 hours at 37°C for outgrowth of the initial *E.coli* donor. After incubation, 5uL samples were added to a LSLB + 0.2% arabinose with *E.coli* + pREADOUT. *Salmonella* + pNULL was co-cultured with *E.coli* + pREADOUT as a negative control. Samples were incubated at 37°C for 15 hours in a Synergy H1 plate reader and fluorescence (480/520) was measured every 5 minutes. The mean relative fluorescence (RFU) was plotted with (a) and without (b) standard deviation for each timepoint (n=6). Selective plating was
also done with resuspended samples to determine the ratio of transconjugants to unconjugated *Salmonella* recipients (d).
Chapter 5

5 Discussion

Development of new, rapid methods of detection of pathogens in food samples is of great concern to food producers and processors. Rapid, sensitive detection of foodborne pathogens in food samples is a feasible approach for prevention of foodborne illness in consumers. I aimed to overcome the limitations of traditional food pathogen detection, namely the speed and sensitivity for live, metabolically active cells, via generation of an amplifiable biosensor based on conjugation to *Salmonella*. By relying on *luxI* production by pDETECT in *Salmonella*, this assay is suited to detection of live, metabolically active cells that can cause disease.

5.1 *Salmonella* + pDETECT induces TGP fluorescence by *E.coli* + pREADOUT

I established that *Salmonella* + pDETECT can successfully produce a fluorescent signal distinct from our pNULL plasmid control. These results were expected, as *E.coli* + pDETECT had previously been shown to produce fluorescence in the biosensor assay. *E.coli* + pDETECT was therefore used as a positive control in this experiment, and we found that it produced ~3.5x higher relative fluorescence than its *Salmonella* counterpart. This could be explained by the promoter used, the Anderson promoter in pDETECT(1) is cited for constitutive expression in *E.coli* and ‘potentially other prokaryotes’, and there is a lack of research on Anderson promoter efficiency in *Salmonella* (Registry of Standard Biological Parts). Nevertheless, *Salmonella* + pDETECT(1) still produced measurable fluorescence within ~2 hours. Fluorescence emitted from the *Salmonella* + pNULL plasmid control was also detected, which likely represents leaky fluorescence by *E.coli* + pREADOUT.

It was previously determined by another lab member that arabinose concentrations exceeding 0.2% did not produce a change in fluorescent readout, so instead I assessed tenfold dilutions of 0.2% arabinose to determine the optimal conditions for fluorescent readout by pREADOUT in the presence of *Salmonella* + pDETECT(1). Despite having equal initial concentrations of *Salmonella* + pDETECT(1), 0.02% and 0.002% arabinose
produced the highest fluorescent readout compared to the other media conditions tested. However, the 0.2% arabinose condition had the fastest time to detection of 2 hours, with the 0.02% and 0.002% groups having 35 and 40 minute longer detection times. These results were surprising, as we expected that higher concentrations of arabinose would lead to more efficient production of TGP by pREADOUT. However, in examining the absorbance data as a measure of culture growth, the 0.2% arabinose condition had a lower final absorbance (A600 = 0.5) than the other conditions, including glucose (0.6-0.8). This may reflect poorer growth of *Salmonella* + pDETECT(1) in the 0.2% arabinose condition. A study by Vasicek et al. (2021) exploring L-arabinose metabolism in wild-type S. Typhimurium mutants found that there was a reduction of pH in the presence of 0.2% and 2.0% arabinose, which was associated with slight reductions in biofilm formation overall (Vasicek, 2021). Acidification of 0.2% arabinose could potentially play a role in the reduced growth we see in this condition, but further testing with comparison of the media pH pre- and post- biosensor assay is needed to assess this possibility.

The sensitivity of the assay for *Salmonella* + pDETECT(1) was assessed using 0.02% arabinose containing media and sample concentrations of $10^8$-$10^3$ CFU/mL. When co-cultured with E.coli + pREADOUT, this correlated to a concentration of $10^6$-$10^3$ CFU/mL *Salmonella*. This assay revealed an initial detection limit of $10^6$ CFU/mL within 3 hours – an encouraging result to see such high sensitivity prior to optimization of the assay. I sought to improve this further by swapping out the Anderson promoter in pDETECT with the rpsM promoter.

The rpsM promoter is reported to have an expression level of >1000 transcripts per million, and is resistant to environmental changes (Kroger, 2013; SalCom v.10). Resistance to environmental conditions is favorable for a promoter in the detection plasmid as food matrices are complex and could potentially alter the transcript readout otherwise. I wanted to compare pDETECT(Anderson) and pDETECT(rpsM) to assess if one produced stronger fluorescent output or a faster time to detection. Ultimately, there was no significant differences between the two plasmids under the standard assay conditions. Both plasmids produced the same level of sensitivity ($10^4$ CFU/mL) in both 0.2% and 0.02% arabinose, and their time to detection at the highest and lowest
detectable concentrations in both media conditions was within 10 minutes of each other. An unexpected result was the large difference (~1.5hrs) in detection time between 0.2% and 0.02% arabinose, when the previous assay indicated a ~0.5hr difference in detection time at the highest concentrations of *Salmonella* + pDETECT(Anderson). As both assays showed reduced time for 0.2% arabinose vs 0.02% arabinose, I returned to using the original 0.2% concentration for the conjugation and food spike assays.

Based on the results of the initial optimization assays, the detection limit ranged from $10^4$-10$^6$ CFU/mL between 3-5 hours. This is a broad estimate as the optimization of the media conditions and determination of the limit of detection of *Salmonella* + pDETECT in the biosensor assay are limited by small sample sizes and high variability between the reported assays. Future work should focus on collecting more data on the assay conditions, as well as looking at factors such as media pH pre- and post-assay, to determine whether 0.2% or 0.02% L-arabinose are optimal for the assay.

### 5.2 Conjugation of pDETECT to *Salmonella* by an *E.coli* (dapA-) donor produces detectable transconjugants in the fluorescent biosensor assay

The ideal detection plasmid for this assay would have selectivity for *Salmonella* such that luxI is only expressed when the plasmid enters *Salmonella*. In this model, both pDETECT(rpsM) and pREADOUT could be harbored in the same *E.coli* Epi300 host, given plasmid compatibility, and pDETECT(rpsM) would selectively drive transcription of luxI following uptake by a *Salmonella* host. However, such a *Salmonella*-specific marker has not yet been identified, so as proof of principle for the assay design I used *E.coli* (dapA-) + pDETECT(rpsM) as the initial donor of pDETECT(rpsM) to *Salmonella*. As this *E.coli* donor strain has a knockout of the dapA gene, and thus cannot produce DAP required for their metabolism, the *E.coli* donor can be easily removed by starving them of DAP without harming the *Salmonella* recipient. To minimize complexity of the overall assay, I opted for liquid conjugations. Further work on this project could potentially integrate techniques such as filter mating or heat shock-enhanced conjugation as they may improve conjugation frequency of pDETECT(rpsM) to *Salmonella*. 
For optimization of conjugation of pDETECT(rpsM) to Salmonella, I was interested in 1) conjugation time, 2) recipient:donor ratio, and 3) the conditions necessary for removal of the E.coli donor. I tested conjugation times of 0.5, 1, 2, or 4 hours to see what duration produced the highest conjugation frequency. I found that as the conjugation time increased, there was a decline in the conjugation frequency as well, ranging from 0.04% for the 30 minute conjugation to 0.0006% for the 4 hour conjugation. The 30 minute conjugation had a significantly higher conjugation frequency than all other groups, with the exception of the 1 hour conjugation time.

Using the 30 minute conjugation time as the standard, I investigated the relationship between E.coli donor and Salmonella recipient concentrations and found that as the concentration of E.coli donors increased with constant Salmonella concentrations, the conjugation frequency increased. Recipient:donor ratios of 0.001 and 0.01 were found to produce significantly higher conjugation frequencies than ratios of 0.1, 0.2, 0.5, or 1. This was expected, as higher concentrations of the donor provides more opportunities for conjugation with the recipient. Future work could investigate if, at very low recipient:donor ratios, this trend still continues to determine optimal culture concentrations for practical use.

Although achieving conjugation of pDETECT(rpsM) to Salmonella was an important first step, I needed to sufficiently remove the donor E.coli for the fluorescent biosensor assay to avoid false signals derived from readout stimulated by leftover E.coli donors harboring the pDETECT(rpsM) plasmid. I evaluated various outgrowth times as well as outgrowing in liquid LSLB or LSLB agar, and found that 100% donor removal was found only after 6 hours on LSLB agar. Interestingly, in the LSLB liquid media, the concentration of pre-outgrowth donors increased 1000-4000% following outgrowth in the liquid media, despite being starved of DAP in both media conditions. Compared to growth on solid agar, liquid media is generally better for growth of organisms as nutrients, oxygen, and waste can move more freely and the temperature is more uniform (Zellner, J. ARL Biopharma). These differential growth conditions could explain why despite having the same cellular concentrations and microbiological growth media (LSLB), the liquid outgrowths were not sufficient for removal of the donor.
With conditions defined for the conjugation and outgrowth, I determined whether I could detect *Salmonella* transconjugants in the fluorescent biosensor assay. *Salmonella* and *E.coli* + pDETECT(rpsM) were co-cultured at a 0.01 R:D ratio of conjugation. The conjugation frequency was ~2%, resulting in a final concentration of $10^7$ transconjugants/mL, which was diluted tenfold twice to get a range of initial input. Fluorescent output was distinguishable from a pNULL plasmid control for $10^7$ and $10^6$ CFU/mL transconjugant concentrations, but not for the $10^5$ CFU/mL and donor-free recipient control. The time to detection was ~2hrs for the $10^7$ CFU/mL condition and ~4.5 for the $10^6$ CFU/mL condition – which was in line with previous data for pDETECT(rpsM) in the 0.2% arabinose condition. These results indicated that pDETECT(rpsM) underwent successful reconstitution and replication in *Salmonella* following conjugation from the original *E.coli* donor.

### 5.3 Proof-of-principle for detection of *Salmonella* in food samples using the fluorescent biosensor assay

With evidence that we could establish successful plasmid readout by pREADOUT following conjugation of pDETECT from an *E.coli* donor to *Salmonella*, I aimed to establish proof of principle for detection of *Salmonella* in food samples. The assay revealed the sample concentration of *Salmonella* transconjugants had to be >$10^4$ CFU/mL to produce measurable fluorescence, which could be detected within 3 hours following co-culture with *E.coli* + pREADOUT. A surprising finding was that the un-spiked control condition produced the highest fluorescence, with the fastest time to detection, compared to *Salmonella*-spiked samples. Plating of the control sample on LSLB agar prior to co-culture with *E.coli* (dapA-) + pDETECT(rpsM) revealed that a heterogeneous mixture of bacteria were naturally present on the spring mix, even when the initial enrichment step was completed in peptone water with Km selection.

I predicted that the off-target fluorescence by unspiked spring mix samples could be due to 1) off-target conjugation of pDETECT(rpsM) 2) autofluorescence or 3) induction of pREADOUT fluorescence in the naturally present bacteria. Initially, it was assumed that this was most likely due to off-target conjugation of pDETECT(rpsM), as although designing pDETECT(rpsM) to be selective for *Salmonella* is the end goal, it is not
possible at this stage of assay development. Thus, it was theoretically possible that other
bacteria present on the spring mix could be competent for conjugation of
pDETECT(rpsM). However, spiked samples were compared on LSLB + Km + Cm
(selective for *Salmonella* transconjugants) versus LSLB + Cm (selective for all bacteria
containing pDETECT(rpsM)), the CFU/mL was found to be nearly equal. To further
investigate the source of fluorescence, I assessed if autofluorescence was occurring by
comparing lettuce samples cultured with and without the addition of *E.coli* +
pREADOUT for the fluorescence biosensor assay. I found that without addition of *E.coli*
+ pREADOUT, the lettuce samples did not produce any measurable fluorescence, and it
is therefore unlikely that the samples are auto fluorescing independently. However, when
co-cultured with *E.coli* + pREADOUT, un-spiked lettuce samples produced measurable
fluorescence above the control. As these samples were not initially conjugated to *E.coli*
(dapA-) + pDETECT, off-target conjugation is also likely not the source of fluorescence
produced by unspiked samples. Off-target fluorescence seen by unspiked lettuce samples
may represent induction of pREADOUT by the native bacteria, as quorum sensing via
AHLs has been established in diverse gram-negative bacteria as a means of responding to
population density (Withers, 2001; Greenberg, 2000; Ling, 2019). AHL-mediated QS has
been demonstrated in gram-negative genera including *Agrobacterium, Aeromonas,
Burkholderia, Chromobacterium, Citrobacter, Enterobacter, Erwinia, Hafnia,
Nitrosomonas, Obesumbacterium, Pantoea, Pseudomonas, Rahnella, Ralstonia,
Rhodobacter, Rhizobium, Serratia* and *Yersinia* (Withers, 2001). For example, the
*Agrobacterium* Lysobacter lives in soil and freshwater – two important reservoirs for
contamination of produce, and have been shown to use AHL quorum-sensing
(Reichenbach, 2006; Ling, 2019). Further work should focus on quantifying AHL
produced by unspiked vs. spiked samples to determine the ‘true’ amount of fluorescent
induction by *Salmonella* + pDETECT(rpsM). AHLs have been quantified from small-
scale bacterial samples using Matrix-Assisted Laser Desorption/Ionization Mass
Spectrometry (MALDI MS) (Leipert, 2017). This technique could potentially be utilized
to gain further clarification.

To try to correct for fluorescence produced by unspiked samples, the outgrowth
conditions were altered to include either single (Km) or double (Km + Cm) antibiotic
selection. With single (Km) selection, the unspiked control still produced higher fluorescent output than either the high (10^7 CFU/mL) or low (10^6 CFU/mL) Salmonella—spiked samples. However, outgrowth on KmCm reversed this trend, with the 10^7 and 10^6 CFU/mL Salmonella spike conditions producing higher fluorescence, with a faster time to detection compared to the other outgrowth conditions. This result may be due to increased elimination of native bacteria on spiked samples under double antibiotic selection and/or favored growth of transconjugant samples.

Repeating the food spike assay with the modified outgrowth conditions (LSLB + Km + Cm, 4 hours), the range of spikes (10^3-10^7 CFU/mL) all produced measurable fluorescence above the pNULL control, and higher than the unspiked control sample, which was in line with the previous assay. However, the results from this set of samples were interesting, as the lowest spike (10^3 CFU/mL) produced fluorescent output, despite having no transconjugants produced from selective plating. This may be reflective of dilution/plating error as a result of using microvolumes, but could also be reflective of how the LOD is calculated. Calculating the LOD using the pNULL control gives a limit of detection of 10^3 CFU/mL Salmonella cells following conjugation with E.coli (dapA-) + pDETECT(rpsM) and co-culture with E.coli + pREADOUT, however when using food samples, it may be more accurate to determine the LOD based on background fluorescence from the unspiked samples rather than the pNULL plasmid control. The pNULL plasmid control was an appropriate control when the fluorescent biosensor assay was performed in pure culture, but with a more complex matrix (digested spring mix), it may be prudent to base these calculations on the background fluorescence of unspiked samples. When the LOD is calculated by meanRFU_{unspiked} + 3*SD, rather than from the mean/SD of pNULL, detectable fluorescence was only measured by the 10^7 and 10^5 CFU/mL spike conditions at 4 hours 5 minutes and 13 hours 40 minutes, respectively.

Taken together, the results of the present study give an estimated detection limit range of 10^4-10^5 CFU/mL transconjugants between 2-4 hours in spiked spring mix samples, and 10^4 CFU/mL transconjugants in ~4 hours in pure culture. The total assay time, including preparation of samples, conjugation, outgrowth, and fluorescent detection is an estimated 11-13 hours total. A summary of the assay’s complete design as of present, along with an
Figure 26. Overview of total fluorescent biosensor assay and time investment per step. This figure provides an overview of the steps involved in preparation of the biosensor assay. Time estimates of each step (sample preparation, conjugation &
washing, outgrowth, and fluorescent detection) are indicated, with a estimated total assay time ranging from 11-13 hours.

5.4 Limitations

A review by Shen et al on biosensors for rapid detection of *Salmonella* proposed 5 main challenges in developing biosensors – requirement for sample pretreatment, detection of *Salmonella* at low concentrations, discrimination between live and dead bacterial cells, and in-field applications (Shen 2021). Detection of low concentrations of *Salmonella* remains a barrier at this stage in assay development.

While other bacteria, such as *Clostridium* perfringes, are allowed to be present in small concentrations, food safety standards have a zero-tolerance for *Salmonella* in food samples (Health Canada, 2010). With the assays current sensitivity of $10^4$ CFU/mL in pure culture, or $10^{4.5}$ CFU/mL pDETECT transconjugants in spiked lettuce samples, it is not sufficiently sensitive to ensure detection of low concentrations of bacterial cells that are typically present in food samples. With such high cellular concentrations required for detection, cultural enrichment would still be required to produce high enough for detection. However, the measurement of the assay’s sensitivity is limited by small volumes (10uL) used to determine the CFU count. I suspect this plays a role in explaining unexpected results, such as detection of fluorescent output when no *Salmonella* transconjugants were counted via selective plating. The microvolumes used in dilution and plating may have higher error than if the dilutions were completed at higher volumes. The cell concentrations in this thesis can only be considered estimates of the actual range, as the suitable colony counting range is accepted between 20-250 CFU (US FDA, 2001). Although concentration estimates were suitable for the purpose of initial optimization, characterization of the assay’s actual sensitivity should be achieved with more accurate plating methods and larger sample sizes. Additionally, plating was done exclusively on LSLB agar, which only captures microorganisms suited for growth on this type of media. Other organisms may be present that aren’t being identified via the current plating methods, and other nutrient and/or enrichment agars should be used to see if additional bacterial species can be identified.
Each optimization step described in this thesis could be further expanded upon to improve the overall conjugation efficiency of pDETECT to Salmonella, and plasmid reconstitution and communication across the dual plasmid system. One strategy our research team has proposed is duplicating the luxI gene on pDETECT, in theory which would double the transcription of luxI and exponentially increase the downstream signaling by AHLs. In theory, this could produce a stronger, faster response by E.coli + pREADOUT due to higher signal frequencies. It may also improve the sensitivity of the assay, as less Salmonella cells would be necessary to produce the same amount of luxI and AHL expression.

The most challenging limitation of the assay development is optimizing pDETECT to be specific for Salmonella, such that transcription of luxI is only activated when the plasmid is reconstituted in Salmonella. Using AHL signaling as the primary inducer of the pREADOUT promoter is also limited in that it is a signaling mechanism utilized by many gram negative bacteria. As AHL molecules are directly inducing fluorescence via activation of the pLux promoter, driving production of TGP. Therefore, off-target induction of the pLux promoter could be achieved by any bacteria capable of AHL quorum sensing if they were present in the food matrix. Future work could focus on Salmonella-specific proteins or metabolites that could be integrated as inducers of the rpsM promoter for production of luxI. Ultimately, as this assay is an indirect measurement of Salmonella contamination via metabolites, this introduces opportunities for off-target measurements.

Another limitation of the assay design is that it relies on a dynamic process – conjugative plasmid transfer – as the basis for fluorescent output. Conjugative plasmid transfer can be influenced by abiotic factors such as carbon and metal concentrations, temperature, and pH – which were not optimized in these experiments (Alderliesten, 2020). Our assays used consistent growth temperature (37°C) and media (LSLB), but other conditions should be assessed for potential to increase conjugation efficiency. Carbon and metal concentrations, as well as pH, were not directly measured for the experiments in this thesis. More precise control of abiotic factors in future work may potentially help reduce variability between assays. Additionally, the control used in the biosensor assay relies on
consistent growth of *Salmonella* + pNULL, but there can be variation in culture growth between experiments. In future studies, a large sample of fluorescent curves for *Salmonella* + pNULL co-cultured with *E.coli* + pREADOUT could potentially be used to create a set standard so that the control line remains constant for each independent experiment.

With the current design, in-field applicability is also a barrier to use. Currently, the assay requires 11-13 hours for setup, conjugation, and measurement of fluorescent readout – which requires expensive, specialized equipment to measure TGP fluorescence as a measure of *Salmonella* contamination. Future research could include visual methods of evaluation that may reduce this burden. For example, eukaryotic chromoproteins (CPs) are able to absorb visible light, and emit strong colors in ambient light – allowing for instrument-free detection (Liljeruhm, 2018). CPs are homologs of green fluorescent protein (GFP), and could potentially be integrated into pREADOUT as an alternative to TGP in the current system (Liljeruhm, 2018). Another potential area for future investigation could focus on minimizing the preparation time required for the assay to be performed. Currently, *E.coli* cultures used are frozen, which requires ~12-18 hours to grow overnights, followed by ~2.5 hours following dilution and regrowth to a mid-log phase. Dehydrating the *E.coli* donor/sensor could minimize the prep time necessary, which could be explored in future research. *E.coli* can survive dehydration, by freeze- or air-drying, which can be rehydrated for use in a short window of time (Louis, 1994). Future research should focus on identifying ways to reduce total assay time without compromising sensitivity.
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https://doi.org/10.1016/j.chemosphere.2022.135515


https://doi.org/10.1007/0-387-30746-X_37


Appendices

Appendix A: Summary of initial experiments with Campylobacter jejuni

Background

The initial candidate for the proposed assay was Campylobacter jejuni, a prominent foodborne pathogen. Campylobacters are gram-negative, spiral/rod shaped bacterium that require microaerophilic (5% O2, 10% CO2, 85% N2) conditions for growth (Altekuse, 1999; Ketley, 1997; Golz, 2021). Campylobacter jejuni is one of 3 thermophilic Campylobacter species, with optimal growth between 37-42°C (Golz, 2021; Wilson, 2009). This feature may be reflective of adaptation to temperatures within the intestinal tract of warm blooded animals (Ketley, 1997). This is especially true for poultry, as C. jejuni is considered commensal with chickens and other avian species as part of their intestinal microbiota (Young, 2007; Sheppard, 2009).

When consumed, C. jejuni adheres to intestinal epithelium resulting in 1) inhibition of fluid resorption from the intestine via secretion of toxins and 2) inflammation and diarrhea due to bacterial invasion (Snelling, 2005; Johnson, 2017). Like Salmonella, typical C. jejuni infection results in self-limiting gastroenteritis, only requiring antibiotic treatment in severe cases. (Snelling, 2005; Johnson, 2017). In rare cases, C. jejuni infection can be associated with post-infectious complications such as reactive arthritis, Reiter syndrome, and Guillain-Barre syndrome (Snelling, 2005).

Unfortunately, we were unable to achieve successful plasmid reconstitution and replication in C. jejuni via transformation or conjugation methods. Three conjugation methods were attempted to achieve plasmid transfer from E.coli Epi300 to C. jejuni – basic conjugation, heat-shock enhanced conjugation, and filter-mating conjugation. Additionally, two transformation methods were tried to achieve plasmid uptake by C. jejuni – natural transformation and electrotransformation.

Methods
Bacterial strains and growth conditions. A summary of *Campylobacter* strains, and their sources, is summarized in Table A1. All *Campylobacter* strains were grown on Mueller-Hinton (BD™ Difco™) or Trypsin-Soy (BD™ Difco™) in liquid or on 1.5% agar, unless otherwise indicated. Strains were grown in an airtight container with microaerophilic conditions maintained by a GasPak (BD BBL™) at 37°C unless otherwise indicated.

Table A1. *Campylobacter* strains used for conjugation and transformation experiments.

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Strain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. jejuni</em></td>
<td>RM1164</td>
<td>Dr. Michael Rieder, Western University</td>
</tr>
<tr>
<td></td>
<td>NCTC11168</td>
<td>Dr. Carole Creuzenet, Western University</td>
</tr>
<tr>
<td></td>
<td>Human clinical isolates (HC1, HC2, HC3)</td>
<td>London Health Sciences Centre (LHSC)</td>
</tr>
<tr>
<td><em>C. fetus</em></td>
<td>RM4087</td>
<td>Dr. Michael Rieder, Western University</td>
</tr>
<tr>
<td><em>C. upsaliensis</em></td>
<td>RM 3940</td>
<td>Dr. Michael Rieder, Western University</td>
</tr>
<tr>
<td><em>C. coli</em></td>
<td>RM1169</td>
<td>Dr. Michael Rieder, Western University</td>
</tr>
<tr>
<td>C. conscious</td>
<td>RM 5460</td>
<td>Dr. Michael Rieder, Western University</td>
</tr>
</tbody>
</table>
Conjugation experiments. Protocol for initial conjugation experiments was adapted from a paper by Davis (2008). *C. jejuni* RM1164 and *E.coli* Epi300 + pTa-mob+pAC4C-RFP were grown overnight from frozen stocks, on TSA supplemented with Tm or TSB supplemented with Cm and Gm, respectively. *E.coli* were diluted, and incubated at 37°C until cultures reached an A600=0.5. *C. jejuni* was resuspended from agar plates to an OD of 1.0. 0.5mL of donor *E.coli* were washed by centrifugation at 5000rpm for 5 minutes, and the supernatant was removed and replaced with fresh media. Donors were re-pelleted, and resuspended with 1mL of the *C. jejuni* recipient culture. The co-cultures were then spun for 2 minutes at 5000rpm, and the supernatant was removed and replaced with 100uL media. Samples were spotted onto an antibiotic-free agar, and incubated at 37°C under microaerophilic conditions for 5 hours to allow for conjugation. Bacteria were then resuspended, and pelleted. The pellet was resuspended, 100uL was plated on selective agar, and the plates were incubated overnight under microaerophilic conditions to screen for transformants. As a negative control for conjugation, *E.coli* Epi300+pAC4C-RFP was used as it lacks pTa-mob – thus the plasmid is immobilizable for conjugation. Conjugation was attempted with donor: recipient ratios of 0.02, 2.5, 5, 10, 75, and 200 (n=3).

Basic conjugation. An adapted protocol was provided by Nancy Friederich, Dr. Michael Rieder’s lab, Department of Physiology and Pharmacology, University of Western Ontario. 5mL overnight cultures of *E.coli* donors and *C. jejuni* recipients were grown in TSB with antibiotic selection overnight. Overnights were diluted 1:10 into 1-2mL of antibiotic free media, and incubated for 1 hour at 37°C. 500uL aliquots of *C. jejuni* recipients and *E.coli* donors were co-cultured and pelleted at 5000rpm for 2 minutes. Supernatant was removed, with 100uL being reserved to resuspend the pellet. Suspension was spotted on TSB agar for recovery at 37°C for 3.5 hours under microaerophilic conditions. The co-culture was subsequently harvested in 1mL TSB and 100uL was plated on selective agar to screen for transformants. Unless otherwise indicated, negative control for conjugation was *C. jejuni* recipients treated identically without the addition of *E.coli* donors, and positive control was conjugation to an *E.coli* DE3136 tetR test recipient which was known to conjugate with all *E.coli* strains used.
This protocol was first attempted using *C. jejuni* RM1164 and *E.coli* Epi300 + pTa-mob+pAC4C-RFP (n=3). As a negative control for conjugation, *E.coli* Epi300 + +pAC4C-RFP was conjugated to both the *C. jejuni* recipient and the *E.coli* test recipient (n=3).

As human isolates of *C. jejuni* (RM1164) were previously used, I wanted to obtain a better characterized strain for lab use. *C. jejuni* NCTC 11168 is a well-characterized reference strain of *C. jejuni* – which we obtained from Dr. Carole Creuzenet’s laboratory, Department of Microbiology and Immunology, University of Western Ontario (Gundogdu, 2007; Pascoe, 2019). This protocol was repeated using *E.coli* Epi300 + pTa-mob+pAC4C-RFP as the donor and *C. jejuni* NCTC 11168 as the recipient for conjugation (n=6). *E.coli* DE3136tetR was used as a positive control for conjugation (n=6). This was repeated once more, with the addition of the *E.coli* Epi300 + pAC4C-RFP control for conjugation to ensure false positives were not being produced by the *E.coli* DE3136tetR recipient (n=3).

The initial growth conditions were altered for *C. jejuni* – initial overnights were grown on TBS agar instead of in liquid media to see if this could improve conjugation. As in the initial conjugation experiments, cells were harvested from agar plates to an A600=0.5 prior to co-culture with *E.coli* + pTA-mob + pNuc-trans-saCas9-RFP (n=3). *E.coli* DE3136tetR was used as a positive control for conjugation (n=3).

The donor:recipient ratio was previously equal, so recipient:donor ratios of 0.5, 0.04, 0.02, and 0.001 were evaluated using *C. jejuni* RM1164 and *E.coli* + pTA-mob + pNuc-trans-saCas9-RFP (n=3). Control in this experiment was *C. jejuni* RM1164 without the addition of *E.coli* donor.

A new donor strain was obtained from Dr. David Edgell’s lab, Department of Biochemistry, University of Western Ontario. *E.coli* Epi300 + pTA-mob + pGU0202 pGU0202 serves as the backbone for pDETECT(1) – a 6.1kb shuttle vector featuring a *C. jejuni* kanamycin resistance gene [aph(3′)-III], mobilization (mob), replication initiation (repA and repB), and origin of replication (ori) from pCJ419 (Alfredson, 2003). Plasmid pGU0202 was previously shown by Alfredson et al. to transform *C. jejuni*
This donor strain was used in the basic conjugation protocol to conjugate to either *C. jejuni* NCTC11168 or the *E.coli* DE3136tetR test recipient (n=4). This was repeated, with altered conjugation times of either 1, 2, 3, 4, 5, 6, or 7 hours prior to resuspension and selective plating (n=1).

**Heat shock-enhanced conjugation.** There is evidence that applying heat shock to *C. jejuni* samples can enhance plasmid uptake during conjugation (Zeng, 2015). Protocol for heat-shock enhanced conjugation was adapted from Zeng et al (2015). Briefly, overnight cultures of *E.coli* donors and *C. jejuni* recipients were established on plates and in liquid culture (respectively). Following overnight incubation, *C. jejuni* were harvested with TSB to a final A600=1.0–1.2. 500uL aliquots of *C. jejuni* recipients were incubated in a water bath at 50°C for 30 minutes (heat shock), followed by a cooling step in a room-temperature water bath for 2 minutes. 500uL of the donor *E.coli* was washed by pelleting at 5000rpm, supernatant was replaced with fresh media, and the wash was repeated to a final resuspension volume of 500uL. 500uL each of donor and recipients were then co-cultured, pelleted at 5000rpm, and resuspended in 100uL TSB. The co-culture was spotted on a nonselective TSB agar and allowed to conjugate at 37°C under microaerophilic conditions. Cells were harvested with 700uL TSB, serially diluted, and 100uL of each dilution was plated on selective agar to screen for transformants.

This protocol was attempted with donor Epi300+pTA-mob+pGU0202 and recipients *C. jejuni* NCTC 11168, *C. fetus* 4087, *C. lari* 1887, and *C. coli* 1169 (n=3). Recipients were grown overnight on TSB agar supplemented with 5% defibrinated horse blood prior to resuspension and co-culture with donor *E.coli*. The conjugation time for this experiment was assessed at 3.5 or 7 hours.

This was repeated using *C. jejuni* NCTC 11168 and *E.coli* DE3136tetR as recipients for conjugation. Additionally, a non-heat shock condition was tested for both *C. jejuni* NCTC11168 and the *E.coli* test recipient. Conjugation time was set at 3.5 hours for this experiment (n=3).

**Filter-mating conjugation.** Overnights of *E. coli* Epi300+pTA-mob-CBS4-pGU0202 and *C. jejuni* NCTC11168 were grown overnight in selective media, and after ~12hrs
were diluted 1:10 and grown to an A600 = 0.5. 1mL of each culture was then pelleted at 5000rpm and resuspended in 0.5mL TSB media. A 0.2uM polycarbonate filter was placed on an antibiotic-free TSB agar plate and allowed to rest for 10 minutes. The donor and recipient cultures were then co-cultured at recipient:donor ratios of 0.5, 0.1, 0.02, and 0.001 to a final volume of 0.2mL and added to the polycarbonate filter (n=3). After a 5-minute drying period, the samples were incubated at 37°C for 3 or 5 hours under microaerophilic conditions. Following conjugation, the filter was removed with sterile tweezers and placed into a 50mL Falcon tube containing 25mL sterile phosphate buffered saline (PBS). Samples were vortexed gently for 1 minute to separate bacteria from the polycarbonate filter, and the filter was then removed. Samples were diluted tenfold and plated on selective media agar to screen for transconjugants.

This protocol was repeated using 0.1 or 0.02 R:D ratios with conjugation times of either 1, 2, 3, 4, 5, 6, or 7 hours under the conditions described above. Plasmid DNA was isolated using the Monarch® Plasmid Miniprep Kit, (New England Biolabs Inc.) according to manufacturer's instructions. Miniprep DNA was quantified using the Qubit broad range dsDNA assay with the Qubit fluorometer.

**Preparation of DNA for transformation of C. jejuni.** When indicated, plasmid DNA was methylated using Eco RI methyltransferase (New England Biolabs) which modifies the internal adenine residue (N6) of the sequence 5’-GAATTC-3’. Plasmid DNA was mixed with Cori methyltransferase,10x rCutSmart buffer, and S-adenosylmethionine according to manufacturer's instructions (New England Biolabs) and incubated in a thermal cycler at 37°C for 1 hour followed by 65°C for 20 minutes. Methylated DNA was purified using Monarch® PCR & DNA Cleanup Kit (New England Biolabs) according to manufacturer’s protocol, and re-quantified using a Qubit fluorometer.

**Natural transformation.** Protocol for natural transformation of C. jejuni was adapted from a paper by Alfredson (2003). Overnights of C. jejuni were grown on non-selective agar an resuspended in media to A600=0.5. 0.5mL aliquots were added to microcentrifuge tubes containing 2% BHI agar and incubated for 3 hours under microaerophilic conditions. Negative controls were treated identically without the
addition of plasmid DNA. Plasmid DNA was then added to microcentrifuge tubes, and cultures were re-incubated for 4-5 hours at 37°C under microaerophilic conditions. Following incubation, suspensions were diluted tenfold and 100uL was plated on selective agar to screen for transformants.

This protocol was followed using the plasmid-strain pairing outlined in Table A2.

**Table A2. Plasmid-Campylobacter strain pairings attempted for natural transformation.**

<table>
<thead>
<tr>
<th>Plasmid (ug/replicate)</th>
<th>Plasmid details</th>
<th>Campylobacter strain</th>
<th>Replicates (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGU0202 (0.05)</td>
<td>Km/Cm resistance Transforming plasmid used by Alfredson, (2003)</td>
<td>C. jejuni NCTC 11168</td>
<td>4</td>
</tr>
</tbody>
</table>
| pGU0202-met (0.05)     | pGU0202 methylated by Eco RI methyltransferase | C. jejuni NCTC 11168  
C. fetus RM 4087  
C. upsaliensis RM 3940  
C. curvus RM 3269 | 4              |
| pGU0202-met (0.2) or pDETECT(1)-met (0.1) | pGU0202 and pDETECT(1) methylated by Eco RI methyltransferase | C. jejuni NCTC 11168  
C. jejuni RM1164 | 3              |
Modified natural transformation of *C. jejuni* clinical isolates. Four clinical isolates were obtained from London Health Sciences Centre to expand the *C. jejuni* strains used. Of the four obtained, three were successfully re-cultured and used in subsequent experiments. Overnights of *C. jejuni* clinical isolates, along with *C. jejuni* NCTC 11168, were grown on MH agar and resuspended to an A600=0.5. 5ug of pGU0202-met DNA was added to 0.5mL *C. jejuni* suspensions, and immediately spotted on a MH + Km (25ug/mL – half dose) for recovery overnight at 37°C in microaerophilic conditions (n=3). Control was *C. jejuni* suspensions without the addition of plasmid DNA. Following recovery, *C. jejuni* suspensions were re-suspended in 1mL media, and 100uL was spread on MH + Km (50ug/mL) for selection of transformants.

Electrotransformation. Protocols for electro transformation of *C. jejuni* were adapted from Hansen et al (2007) and Miller et al (1988). Overnights of *C. jejuni* were grown from frozen stock in selective media. Cells were diluted 1:50 in 5mL media and grown to an OD600 of 0.2, 0.4, or 0.8. 1.5mL of cells were added to a microcentrifuge tube with 1.5mL ice-cold electroporation buffer (EPB; 272 mmol glucose, 15% glycerol). Cells were pelleted (5000rpm), the supernatant was discarded, and the pellet was resuspended in 500uL EPB. Cells were re-pelleted and resuspended in 100uL EPB, and this was repeated to a final volume of 50uL. Electrocompetent *C. jejuni* was kept on ice until electroporation.

0.05ug or 0.5ug of methylated pGU0202 DNA were added to microcentrifuge tubes containing 50uL aliquots of electrocompetent *C. jejuni* cells with a starting OD600 of 0.2, 0.4, or 0.8 (n=3). Cells and DNA were incubated on ice for 10 minutes, then added to 0.2cm electro cuvettes. Samples were electroporated at 2.5kV at either 200, 400, or 600 ohms of resistance. After electroporation, cells were recovered in 100uL pre-warmed TBS media and spotted on non-selective TSB agar. Plates were incubated overnight at 42oC under microaerophilic conditions. The following day, cells were resuspended in 200uL pre-warmed media and plated on TSB + Km plates for selection of transformants. Plates were incubated overnight at 42oC under microaerophilic conditions.
**Optimizing pDETECT(1) for *C. jejuni*.** pDETECT(1) expresses *luxI* under a constitutive Anderson promoter (J23106). Refer to Section for full plasmid details. The Anderson promoter was successfully swapped with 2 promoters (2A12, 1B7) identified with strong promoters in *C. jejuni* via Gibson assembly (Wosten, 1998). The promoters are identified in Table. The pDETECT(1) backbone was amplified by this method, and double-stranded gBlocks™ Gene Fragments (Integrated DNA Technologies) of the promoter inserts were incubated with the purified pDETECT(1) backbone using the NEBuilder® HiFi DNA Assembly Cloning Kit (New England Biolabs) according to manufacturers instructions.

<table>
<thead>
<tr>
<th>Promoter identifier</th>
<th>Insert length (bp)</th>
<th>Insert sequence (5'→3')</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>2A12</td>
<td>49</td>
<td>aaaaagtaaaaatgaatgtgatacaagattaattagtaaatattatat</td>
<td>GenBank: AJ002421.1</td>
</tr>
<tr>
<td>1B7</td>
<td>48</td>
<td>ttcttttatatatgttatgttatataattgttag</td>
<td>GenBank: AJ002416.1</td>
</tr>
</tbody>
</table>

Immediately following assembly, 40uL of electrocompetent *E.coli* Epi300 was added to a 0.2cm electrocuvette with 1uL of pDETECT(1)-1B7 or pDETECT(1)-2A12 DNA. Positive control was 1uL pCBS9 DNA without the inserted promoter sequences. Negative control was *E.coli* Epi300 treated identically without the addition of plasmid DNA. Samples were electroporated at 2.5kV, 25uF, 200ohm and recovered in 1mL SOC at 37°C and 225rpm for 90 minutes. 100uL of recovered samples were plated on LB + Km for selection of transformants, and incubated overnight at 37°C. Promoter insertion into the pDETECT(1) backbone was further confirmed via whole-plasmid sequencing (London Regional Genomics).

pDETECT(1)-1B7 or pDETECT(1)-2A12 DNA was isolated from *E.coli* and Eco RI methylated as previously described, and natural transformation was attempted using *C. jejuni* NCTC 11168 with 1 or 2 ug of methylated plasmid DNA. This was repeated using
electrotransformation with 2ug methylated plasmid DNA. Control for both conditions was cells treated identically without the addition of DNA.

**Results**

I was unable to achieve successful plasmid reconstitution and replication in *C. jejuni* via any of the transformation and/or conjugation methods attempted. Ultimately, the assay design used in this thesis relies on 1) successful conjugative plasmid transfer to *C. jejuni* and 2) successful reconstitution and plasmid expression by *C. jejuni* – so its potential utility as a screen for *C. jejuni* in food samples could not be achieved.

In the conjugation experiments, conjugation of all plasmids successfully produced transconjugants when tested with the *E.coli* test recipient. This suggests that the conditions used allowed for conjugation from an *E.coli* donor, but not for *C. jejuni* to successfully uptake a plasmid. Three conjugation methods were tested – basic conjugation, heat shock conjugation, and filter-mating conjugation – none of which were successful for conjugation to *C. jejuni* or other *Campylobacter* species used. Three separate plasmids were also used - . Various conjugation times, and donor:recipient ratios were also tested, but ultimately unsuccessful. In all conjugation times, ratios, and strategies used, *Campylobacter* colonies formed on Tm only plates, indicating that they survived the conjugation methods attempted.

The transformation experiments were also unsuccessful at producing transformants for any of the *Campylobacter* strains or plasmids used. A wide range (0.05-5ug) of DNA was used for attempted transformation, along with natural or electro-transformation methods.

**Discussion**

To date, we have yet to have success achieving conjugation and/or transformation with plasmid reconstitution in any of the *Campylobacter* strains tested. Horizontal gene transfer (HGT), a mechanism by which bacteria adapt to their surroundings, can be induced by natural transformation, conjugation, and phage transduction (Gugliemini, 2013; Golz, 2021). For HGT to occur, bacterially must be naturally competent for uptake of macromolecular DNA (Weisner, 2020). *C. jejuni* is naturally competent, meaning it
can take up DNA from the environment via a type II secretion system, and undergo
conjugation via a type IV secretion system which is a driving factor for the vast genetic
diversity seen in *C. jejuni* (Young 2007; Alfredson 2003; Batchelor, 2004). Outside its
species, *C. jejuni* has also been shown to undergo conjugation with other bacteria, such as
*Helicobacter pylori* & *E.coli*, to obtain chromosomally encoded antibiotic resistance
(Oyarzabal, 2020; Zeng, 2015).

I initially aimed to establish conjugation between *E.coli* Epi300 and *C. jejuni* as a starting
point for optimizing the assay design for *C. jejuni*. However, when none of the methods
attempted were able to successfully produce *C. jejuni* transconjugants, I tried to achieve
DNA uptake by transformation of *C. jejuni* using either natural or electro-transformation
methods. If I had been able to transform DNA directly into *C. jejuni*, the fluorescent
biosensor assay could have been performed to see if *C. jejuni* was expressing the plasmid
and successfully inducing fluorescence by pREADOUT. Alfredson et al (2003) were
previously able to express a shuttle vector plasmid – pGU0202 – in *C. jejuni*, conferring
kanamycin resistance to *C. jejuni* transformants. pGU0202 was used for transformation,
as well as the backbone for pDETECT(1), but unfortunately we could not replicate their
results by any of the methods tried.

The barrier of achieving DNA uptake is supported by the literature on *C. jejuni* genetic
modification. Although *C. jejuni* is naturally transformable, and amenable to
electroporation, this only applies to insertion of chromosomal DNA via genetic
recombination (Holt, 2012). Plasmid reconstitution and readout, on the other hand, is
challenging in either method, which conflicts with the assay design (Holt, 2012). *C.
jejuni* limits transformation by selection of DNA from relatives harboring a methylated
RA-m6-ATTY profile, achieved by CtsM methylase in *Campylobacters* (Backert, 2021).
CtsM methylation achieves protection of *C. jejuni* DNA by its own restriction
endonuclease systems (Backert, 2021). Other bacteria are selective with respect to DNA
incorporated during transformation, but this is typically regulated by DNA uptake
sequences (DUS) (Beauchamp, 2017; Goodman, 1988; Mell, 2012). DUS have not been
shown to play a role in *C. jejuni*, as an identical piece of DNA was able to transform *C.
jejuni when isolated from another C. jejuni strain, but not when isolated from E.coli (Weisener, 2006).

I tried to mimic endogenous methylation of plasmid DNA by methylation of plasmids used in transformation experiments with Eco RI methyltransferase. Eco RI MTase recognizes a subset of RAATTY sites, and previous research showed that the methylation of genomic DNA isolated from E.coli by Eco RI MTase transforms C. jejuni as well as gDNA isolated from CtsM MTase + C. jejuni (Beauchamp, 2017). As in-vitro methylation by EcoRI-MTase was shown to be sufficient, I attempted to replicate these results with plasmid DNA for transformation. However, in-vitro methylation of plasmid DNA isolated from E.coli did not successfully produced transformants.

I consulted with Dr. Korolik, a collaborator on Alfredson et al (2003), which showed successful uptake of the pGU0202 shuttle vector by C. jejuni. They recommended that our research team obtain more clinical isolates, as they typically have greater genetic diversity and may be more likely to undergo transformation. I hypothesized that if transformation of C. jejuni with plasmid DNA could be achieved, the DNA could potentially receive CtsM methylation, which could then be re-isolated from C. jejuni to favor transformation and/or conjugation to C. jejuni. However, no transconjugants were produced using hospital isolates either, so this theory could not be evaluated.

Achieving successful uptake of plasmid DNA by C. jejuni remains a significant barrier to adapting the fluorescent biosensor assay for detection of C. jejuni. Future investigations could potentially consider another method of horizontal gene transfer, such as phage transduction, for uptake of plasmid DNA. Additionally, extracellular nucleases have also been shown to inhibit transformation of C. jejuni via hydrolysis of DNA (Gasbeek, 2020). Future work could investigate whether extracellular nucleases play a role in inhibiting gene transfer to the C. jejuni strains used. Ultimately, my results in combination with the literature on plasmid transfer and reconstitution in C. jejuni suggest that future research should consider other alternatives for detection of C. jejuni.

References


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