THE GENOMIC AND PHENOTYPIC CHARACTERIZATION OF PHAGE-LIKE PLASMIDS AND THEIR ABILITY TO HORIZONTALLY TRANSFER ANTIBIOTIC AND HEAVY METAL RESISTANCE GENES TO BACTERIA OF FOODBORNE IMPORTANCE

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Dedicated to all women in Science

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ABBREVIATIONS

AAC	Acetyltransferases
ABC	Adenosine triphosphate (ATP)-binding cassette
ACSSuT	Ampicillin, Chloramphenicol, Streptomycin, Sulfomides, and Tetracycline
ANT	Nucleotidyltransferase
APH	Phosphotransferases
AR	Antibiotic Resistance
ARGs	Antibiotic Resistance Genes
attB	Bacterial Attachment Site
attP	Phage Attachment Site
BRIG	BLAST Ring Image Generator
CDF	Cation Diffusion Facilitators
CDS	Coding Sequence
CFR	Chloramphenicol-florfenicol resistance
CIPARS	Canadian Integrated Program for Antimicrobial Resistance Surveillance
copA	Copper-Exporting P-type ATPase
CTns	Conjugative Transposons
DprA	DNA Processing Protein A
dsDNA	Double-Stranded DNA
EPS	Extracellular Polymeric Substance
Erm	Erythromycin Ribosome Methylase
ESBL	Extended-Spectrum β-lactamases
HGT	Horizontal Gene Transfer
HME	Heavy Metal Efflux
HMR	Heavy Metal Resistance
HsdM	Host Specificity for DNA, Modification or Methylation
HsdR	Host Specificity for DNA, Restriction
HsdS	Host Specificity for DNA, Specificity
HUS	Hemolytic Uremic Syndrome
ICE	Integrative Conjugative Elements
ICTV	International Committee on Taxonomy of Viruses
IMP	Imipenemase
InC	Incompatibility
int1	Class 1 Integrons
IR	Inverted Repeat
IS	Insertion Sequences
iTOL	Interactive Tool of Life
KPC	*Klebsiella pneumonaie* carbapenemase
LBA	Luria-Bertani agar
LBB	Luria-Bertani broth
LPS	Lipopolysaccharide
MATE	Multidrug and Toxic Compound Extrusion

MDR	Multidrug Resistance
MFS	Major Facilitator Superfamily
MGE	Mobile Genetic Elements
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-resistant *S. aureus*
NARMS	National Antimicrobial Resistance Monitoring System
NCBI	National Center of Biotechnology Information
NTS	Non-typhoidal *Salmonella*
ORF	Open-Reading Frames
oriT	Origin of Transfer
OS	Oligosaccharide
OXA	Oxacillinase
PBP	Penicillin Binding Protein
PLP	Phage-like plasmid
PRP	Pentapeptide Repeat Proteins
QAC	Quaternary Ammonium Compounds
QRDR	Quinolone Resistance Determining Region
R-M	Restriction Modification System
RND	Resistance-Nodulation-Division
ROS	Reactive Oxygen Species
SalFos	*Salmonella* Foodborne Syst-OMICS Database
SCCmec	Staphylococcal Cassette Chromosome mec
SMR	Small Multidrug Resistance
SOC	Super Optimal broth with Catabolite repression medium
ssDNA	Single-Stranded DNA
STEC	Shiga toxin-producing *Escherichia coli*
Stx	Shiga Toxin
T4SS	Type IV Secretion System
TMP-SMZ	Trimethoprim-Sulfamethoxazole
Tn	Transposon
TolC	Outer Membrane Protein Channel
traG	Conjugal Transfer Protein G
VIM	Verona integron encoded metallo β-lactamase
WGS	Whole Genome Sequence
WHO	World Health Organization

ABSTRACT

Antibiotic resistance (AR) is emerging as a major public health problem with global implications. Each year, approximately 700,000 deaths worldwide are attributed to AR, and this number is predicted to rise to 10 million by 2050. The misuse of antibiotics in human and animal medicine, as well as their use as growth promoters in livestock and aquaculture farming are major contributors of AR. These practices impact the environment by disseminating antibiotic resistance genes (ARGs) and antibiotic resistant bacteria in bodies of water, in agriculture soil and crops, and in wildlife, which act as carriers and further contribute to the dissemination of ARGs throughout the environment. The selective pressure exerted by antibiotics facilitates the emergence and horizontal gene transfer (HGT) of ARGs to different bacteria including foodborne bacteria. Antibiotic resistance genes can be found on mobile genetic elements (MGEs), including bacteriophages (phages), plasmids, integrative and conjugative elements (ICE), and transposons, and, as such, can be horizontally transferred to foodborne bacteria present in the environment, soil, and gastrointestinal tracts of mammals including livestock and humans.

Phage-like plasmids (PLPs) are a novel MGE that are part phage and part plasmid, and exist extrachromosomally within bacterial cells. Phage-like Plasmids are emerging as an important contributor to the spread of antibiotic resistance, because they harbour ARGs and/or heavy metal resistance (HMR) genes, and may have the potential to perform HGT by phage-mediated mechanisms, such as transduction, or plasmid-mediated mechanisms, such as transformation and conjugation. The objective of this study was to develop a better understanding of PLP genomic structure and biology, by focusing on two main objectives including genomic characterization of PLPs to determine their taxonomic structure and the types of ARGs and HMR genes that they carry, and phenotypic analysis of the potential of PLPs to horizontally transfer ARGs and HMR genes by transduction, transformation, and conjugation in order to determine whether they can confer resistance to antibiotics and heavy metals.

A total of 57 PLPs (18 reported in the scientific literature, 31 PLP genome sequences identified within Genbank, and 8 PLPs that were isolated from bacterial strains of bovine and food origin) were characterized in this study. Genomic analysis revealed that 29% of the PLPs carried ARGs that are known to confer AR resistance to β-lactams, carbapenems, colistin, and

aminoglycosides, and that 10% of the PLPs carried genes that have been associated with resistance to either mercury or tellurite. The replicon type was identified for 75% of PLPs, with the major groups being IncFIB (33%) and IncY (30%). Additionally, the results from this genomic analysis suggested that 54 of 57 PLPs (93%) had nucleotide sequence identity to the Siphophage SSU5 or the prototypical PLP P1, and, therefore, could be classified into two distinct lineages, the SSU5-like and P1-like lineages. The SSU5-like PLP AnCo1 (encoding CTX-M-15), P1-like PLP SJ1 (encoding a mercury resistance operon), and P1-like PLP MA725 (encoding *terB*) were successfully transduced to 3 different *E. coli* strains with lipopolysaccharide (LPS) glycosyltransferase enzyme deletions including PLP AnCo1 to strain CWG310 which has a *waaW* deletion, PLP SJ1 to strain CWG297 which has a *waaQ* deletion, and PLP MA725 to CWG309 which has a *waaT* deletion. This is the first reported observance of PLP-mediated transduction. These results suggested that the PLPs AnCo1, SJ1 and MA725 use carbohydrate residues including D-galactose I (AnCo1), L-glycero-D-manno-heptose II (SJ1) and D-glucose II (MA725) within the core lipopolysaccharide, respectively, as receptors. Furthermore, PLP SJ1 was capable of infecting 18 different strains, including 2 commensal (lab) *E. coli* strains, 4 *E. coli* O157 strains, and 12 *Salmonella* strains, suggesting that this PLP may have a broad host range. Phage-like-Plasmids were also found to be capable of HGT through plasmid-mediated mechanisms, as demonstrated by the fact that the PLPs AnCo1, SJ1, and MA725 were successfully transformed to *E. coli* DH10B. Furthermore, while the PLPs lack genes necessary for conjugation, AnCo1 and SJ1 were successfully conjugated to *E. coli* J53 in the presence of the helper plasmid pRK2013. Phenotypic characterization of the ability of the PLPs to confer antibiotic and heavy metal resistance was conducted through minimum inhibitory concentration (MIC) experiments that demonstrated that, regardless of the HGT mechanism, individual PLPs conferred the same levels of resistance to their bacterial hosts. The PLPs AnCo1 and SJ1 conferred 3 mg/ml of resistance to cefotaxime and 50 μg/ml to mercury chloride, respectively. Of note, it was demonstrated for the first time that *terB* harboured by PLP MA725 conferred 40 μg/ml of resistance to potassium tellurite and conferred cross-resistance (10 μg/ml) to colistin sulphate, which is of clinical significance, as colistin is considered to be an antibiotic of last resort.

Collectively, the genomic and phenotypic characterization of the PLPs in this study demonstrated that PLPs are disseminated globally from farm-to-fork, can be classified into two

lineages (SSU5-like and P1-like), can horizontally transfer ARGs and HMR genes to *E. coli and Salmonella enterica* and confer the same level of resistance to antibiotics and heavy metals regardless of the HGT mechanism. Phage-like plasmids represent a versatile MGE, as they can mobilize ARGs and HMR genes via all 3 mechanisms of HGT. Taken together, the results of this study highlight the contribution of PLPs to the dissemination of AR within the agricultural and clinical environments.

RÉSUMÉ

La résistance aux antibiotiques (AR) est reconnue comme un problème majeur de santé publique. Chaque année, environ 700 000 décès dans le monde sont attribués à l'AR, et ce nombre devrait atteindre 10 millions d'ici 2050. L'utilisation abusive d'antibiotiques en médecine humaine et animale et comme facteurs de croissance dans l'élevage et l'aquaculture contribuent à ce phénomène. Ces pratiques ont un impact sur l'environnement car elles permettent de disséminer des gènes de résistance aux antibiotiques (ARG) ainsi que des bactéries résistantes aux antibiotiques dans l'eau, les sols, les produits agricoles et la faune sauvage. Ces dernières agissent comme vecteurs et contribuent aussi à la dissémination de ces ARGs dans l'environnement. La pression sélective exercée par les antibiotiques facilite l'émergence des ARGs et le transfert horizontal de gène (HGT) vers d'autres bactéries, y compris des bactéries d'origines alimentaires. Des gènes de résistance aux antibiotiques peuvent être trouvés sur des éléments génétiques mobiles (MGE) comme des bactériophages (phages), des plasmides, des éléments intégratifs et conjugatifs (ICE) ou des transposons; ils peuvent donc être transférés horizontalement à des bactéries d'origines alimentaires présentes dans l'environnement, le sol et le tractus gastro-intestinal des mammifères, y compris du bétail et des humains.

Les plasmides de type phage (PLP) sont un nouveau MGE composé à la fois de phage et de plasmide et existent de manière extrachromosomique au sein des cellules bactériennes. Les plasmides de type phage sont en train de devenir un contributeur important dans la propagation de la résistance aux antibiotiques, car ils contiennent des ARG et/ou des gènes de résistance aux métaux lourds (HMRs), et peuvent potentiellement utiliser la HGT grâce aux mécanismes propres au phage, tels que la transduction ou des mécanismes propres aux plasmides, tels que la transformation et la conjugaison. L'objectif de cette étude était d'acquérir une meilleure compréhension de la structure génomique et de la biologie des PLPs en se concentrant sur deux objectifs principaux : (1) la caractérisation génomique des PLPs pour déterminer leur structure taxonomique et les types de ARGs et gènes HMRs qu'ils portent et (2) l'analyse phénotypique de ces structures incluant le potentiel des PLPs à transférer horizontalement les ARGs et les gènes HMRs par transduction, transformation et conjugaison et ainsi déterminer si les PLPs peuvent ou non conférer une résistance aux antibiotiques et aux métaux lourds .

Au total, 57 PLPs (18 publiés dans la littérature scientifique, 31 séquences de génome de PLP identifiées au sein de Genbank et 8 PLP isolés de souches bactériennes d'origine bovine et alimentaire) ont été caractérisés dans cette étude. L'analyse génomique a révélé que 29% des PLPs portaient des ARGs connus capable de conférer la résistance aux antibiotiques β-lactames, aux carbapénèmes, à la colistine et aux aminoglycosides. De plus, 10% des PLPs portaient des gènes HMRs connus pour conférer la résistance au mercure ou à la tellurite. Soixante-quinze pourcents des PLPs ont pu être classifiés par groupe de plasmide ; les principaux groupes étant IncFIB (33%) et IncY (30%). En outre, les résultats de cette analyse génomique suggèrent que 54 PLP sur 57 (93%) avaient une identité de séquence nucléotidique avec le Siphophage SSU5 ou le PLP prototype P1 et pouvaient donc être classés en deux lignées distinctes, le type SSU5 et le type P1. Le PLP AnCo1 de type SSU5 (codant pour CTX-M-15), le PLP SJ1 de type P1 (codant pour un opéron de résistance au mercure) et le PLP MA725 de type P1 (codant pour *terB*) ont été transférés par transduction à 3 souches différentes d'*E. coli* avec des délétions de l'enzyme glycosyltransférase dans le lipopolysaccharide (LPS). PLP AnCo1 a été transduit à la souche CWG310 qui présente une délétion *waaW*, PLP SJ1 à la souche CWG297 qui possède une délétion *waaQ*, et enfin PLP MA725 à la souche CWG309 qui possède une délétion *waaT*. Ceci est la première observation de la transduction médiée par les PLPs. Ces résultats suggèrent que les PLPs AnCo1, SJ1 et MA725 utilisent les résidus glucidiques D-galactose I pour AnCo1, le L-glycéro-D-manno-heptose II pour SJ1 et le D-glucose II pour MA725 dans le lipopolysaccharide central comme des récepteurs. De plus, PLP SJ1 était capable d'infecter 18 souches différentes, dont 2 souches de *E. coli* commensales (de laboratoire), 4 souches de *E. coli* O157 et 12 souches de *Salmonella* , ce qui suggère que ce PLP pourrait avoir une large gamme d'hôtes. Les plasmides de type phage se sont également avérés capables de transféré horizontalement les gènes par les mécanismes propres par les plasmides, comme le démontre le fait que les PLPs AnCo1, SJ1 et MA725 ont été transformées avec succès en *E. coli* DH10B. De plus, alors que les PLPs ne possèdent pas les gènes nécessaires à la conjugaison, AnCo1 et SJ1 ont été conjugués avec succès à *E. coli* J53 en présence du plasmide auxiliaire pRK2013. La caractérisation phénotypique de la capacité des PLPs à conférer une résistance aux antibiotiques et aux métaux lourds a été réalisée par des d'expériences de concentration minimale inhibitrice (CMI) qui ont montré que, indépendamment du mécanisme de HGT, les PLPs individuels conféraient les mêmes niveaux de résistance à leurs hôtes bactériens. Les PLPs AnCo1 et SJ1 ont

conféré 3 mg/ml de résistance au céfotaxime et 50 µg/ml au chlorure de mercure, respectivement. De même, il a été démontré pour la première fois que le gène *terB* porté par le PLP MA725 conférait 40 µg/ml de résistance au tellurite de potassium et une résistance croisée (10 µg/ml) au sulphate de colistine, ce qui est cliniquement significatif, car la colistine est considérée comme un antibiotique de dernier recours.

Collectivement, la caractérisation génomique et phénotypique des PLPs dans cette étude a démontré que les PLPs sont disséminés dans le monde entier, de la ferme à la fourchette, qu'ils peuvent être classés en deux lignées (de type SSU5 et de type P1), qu'ils peuvent transférer horizontalement les ARGs et les gènes HMRs à *E. coli* et *Salmonella enterica* et qu'ils confèrent le même niveau de résistance aux antibiotiques et aux métaux lourds, quel que soit le mécanisme HGT. Les plasmides de type phage représentent une MGE polyvalente, car ils peuvent mobiliser les ARGs et les gènes HMRs via les 3 mécanismes de la HGT. Pris ensemble, les résultats de cette étude mettent en évidence la contribution des PLPs à la dissémination de la résistance aux antibiotiques dans les environnements agricoles et cliniques.

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STATEMENT FROM THE THESIS OFFICE

This thesis is submitted in the format of papers suitable for journal publication. This thesis format has been approved by the Faculty of Graduate and Postdoctoral Studies, McGill University, and follows the conditions outlined in the Guidelines: Concerning Thesis Preparation, which are as follows:

“As an alternative to the traditional thesis format, the dissertation can consist of a collection of papers of which the student is an author or co-author. These papers must have a cohesive, unitary character making them a report of a single program of research. The structure for the manuscript-based thesis must conform to the following:

1. Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis).

2. The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.

3. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts.

The thesis must include the following

(a) A table of contents;
(b) An abstract in English and French;

(c) An introduction which clearly states the rational and objectives of the research;
(d) A comprehensive review of the literature (in addition to that covered in the introduction to each paper);
(e) A final conclusion and summary;

4. As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.

5. In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers".

CONTRIBUTION OF AUTHORS

The following are the manuscripts prepared for publication:

1. **Colavecchio, A**., Joseph, S., Mui M., Goodridge L., (2018) Factors, Mechanisms and Mobile Genetic Elements that Contribute to the Dissemination of Antibiotic Resistance Worldwide – A Review. **(Draft prepared)**

2. **Colavecchio, A.,** Joseph, S., Amitrano M., Nguyen S., Hurley D., Beech R., Jeukens J., Freschi L., Hamel J., Emond-Rheault J-G., Kukavica-Ibrulj I., Mui M., Fanning S., Levesque R.C., Goodridge L., (2018) Genomic Characterization of 57 Phage-Like Plasmids and Their Potential Role in Antibiotic Resistance. **(Draft prepared)**

3. **Colavecchio, A.,** Joseph, S., Amitrano M., Nguyen S., Hurley D., Fanning S., Goodridge L., (2018) PLPs, the ultimate mobile genetic element, can horizontally transfer antibiotic and heavy metal resitance genes by transformation, conjugation and transduction to *E. coli* and *Salmonella enterica*. **(Draft prepared)**

The work reported in all chapters of this study was performed by Anna Colavecchio, PhD candidate, who planned and conducted all the experiments, in consultation with her supervisor, gathered and analyzed the results, and drafted the thesis and the manuscripts for scientific presentations and in all published, submitted and drafted papers. Dr. Lawrence Goodridge is the thesis supervisor, under whose guidance the research was carried out, and who guided and supervised the candidate in planning and conducting the research, as well as in correcting, reviewing and editing of the thesis and the manuscript drafts for publication. The work reported in this study under the supervision of Dr. Goodridge, was performed in the Diagnostic, Enrichment, Testing and Characterization (D.E.Te.CT) laboratory in the Department of Food Science and Agriculture Chemistry at Macdonald Campus of McGill University, Ste-Anne-de-Bellevue, Québec, Canada.

The minimum inhibitory concentration (MICs) experiments involving the phage-like plasmids (PLPs) MA725, JEC725, SN747 and DH728 and the whole genome sequencing (WGS) and assembly of their hosts was performed at the University College Dublin (UCD) Centre for Food Safety in Dublin, Ireland. Dr. Séamus Fanning was my supervisor under whose guidance this specific component of the research was carried out in the Food Safety Laboratory at UCD, Dublin, Ireland.

The remaining whole genome sequencing (WGS) and assembly of the isolates in this study, with the exception of PLPs MA725, JEC725, SN747 and DH728, was performed under the supervision of Dr. Roger C. Levesque at the EcoGenomics Analysis Platform at the Institute de Biologie Intergrative et des System (IBIS), Université Laval, Québec City, Québec, Canada.

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PUBLICATIONS

Colavecchio, A., Jeukens, J., Freschi, L., Rheault, J. G. E., Kukavica-Ibrulj, I., Levesque, R. C., ... & Goodridge, L. (2017). Complete Genome Sequences of Two Phage-Like Plasmids Carrying the CTX-M-15 Extended-Spectrum β-Lactamase Gene. *Genome Announcements*, *5*(19), e00102-17.

Colavecchio, A., Jeukens, J., Freschi, L., Rheault, J. G. E., Kukavica-Ibrulj, I., Levesque, R., & Goodridge, L. (2017). AnCo3, a New Member of the Emerging Family of Phage-Like Plasmids. *Genome Announcements*, *5*(19), e00110-17.

Colavecchio A., Cadieux B., Lo A., Goodridge L., (2017) Bacteriophages Contribute to the Spread of Antibiotic Resistance Genes among Foodborne Pathogens of the *Enterobacteriaceae* Family– A Review. *Frontiers in Microbiology*

Colavecchio, A., D'Souza, Y., Tompkins, E., Jeukens, J., Freschi, L., Emond-Rheault, J. G., ... & Levesque, R. C. (2017). Prophage integrase typing is a useful indicator of genomic diversity in *Salmonella enterica*. *Frontiers in Microbiology*, *8*

CONFERENCE PRESENTATIONS

Colavecchio, A., Amitrano M., Goodridge L., (2018) Phage-like plasmids are a novel class of temperate bacteriophages that encode antibiotic resistance genes of clinical importance. International Association for Food Protection (IAFP), Salt Lake City, Utah, USA

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Colavecchio, A., Gordon M., Jeukens J., Freschi L., Emond-Rheault J-G., Kukavica-Ibrulj I., Levesque R.C., Goodridge L., (2017) *"Escherichia coli* and *Salmonella* Derby Carry a Novel Family of Temperate Bacteriophages that Encode Extended-Spectrum Beta-Lactamase Genes". International Association for Food Protection (IAFP), Tampa Bay, Florida USA

Colavecchio, A., Tompkins E., Beech R., Jeukens J., Freschi L., Emond-Rheault J-G., Kukavica-Ibrulj I., Levesque R.C., Goodridge L., (2017) "Six Novel Phage-like plasmids detected within *Salmonella* of foodborne origin**".** Genome Canada conference, Québec, Québec, Canada

CHAPTER I

GENERAL INTRODUCTION

The World Health Organization (WHO) estimates that foodborne illnesses affects 600 million people and causes 420,000 deaths globally (World Health Organization, 2015b). In North America (USA and Canada) foodborne outbreaks cause 52 million illnesses, an estimated 139,000 hospitalizations and 3,200 deaths (CDC, 2011; Thomas et al., 2015). The economic impact of foodborne illness in the USA is estimated at approximately $10-83 billion (USD) annually (McLinden et al., 2014). A major contributor to the costs associated with foodborne disease is healthcare costs. The continued emergence of antibiotic resistance (AR), and especially multidrug resistance (MDR), among foodborne pathogens may contribute to unsuccessful treatment outcomes, thereby increasing costs associated with foodborne disease. For example, in the United States (no data in Canada) it is estimated that the additional cost of treating a patient's AR bacterial infection is $1,383. With an estimated 430,000 AR foodborne infections annually in the USA, that's an additional cost of $5.94 million annually on the healthcare system (Centers for Disease Control and Prevention, 2014; Thorpe et al., 2018). Furthermore, it is estimated that by 2050 there will be 10 million deaths associated with AR infections, which exceeds deaths caused by cancer and would cost the global economy a cumulative $100 trillion USD (O'Neill, 2014; Public Health Agency of Canada, 2017). In Canada, the latest report by the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) has highlighted pathogenic bacteria including *Campylobacter*, non-typhoidal *Salmonella* (NTS) and *Escherichia coli* as AR threats (CIPARS, 2012). Many factors contribute to the spread of AR such as the misuse and overuse of antibiotics. Surveys conducted globally suggest that patients believe antibiotics can treat viral infections (World Health Organization, 2015a). Antibiotics are easily accessible through over-the-counter and Internet sales. They are also widely used in animal husbandry, aquaculture and seafood farming as growth promoters, which maintains a selective pressure within the gastrointestinal tract of food-producing animals sustaining the presence of AR bacteria. Humans can be exposed to AR bacteria through improperly cooked meat, crops contaminated by manure and farmyard run-offs

that contaminate groundwater (World Health Organization, 2015a). The contamination of groundwater is harmful to humans because it is used in manufacturing, irrigation, animal farming and washing and drinking water (Government of Canada, 2013). Moreover, groundwater eventually flows into streams and seas contaminating more bodies of water. In Canada and the United States, 30.3% and 39% of the population, respectively, rely on groundwater for domestic and municipal use (Government of Canada, 2013; Dieter et al., 2018).

Proposals to combat AR include reducing the use of antibiotic as agents in human and animal health. However, even their reduction does not necessarily prevent the spread and maintenance of AR bacteria. Despite the ban of growth promoters in animal production in the European Union in 1999, several studies demonstrate the persistence of AR genes in different environments and even in remote communities without selective pressure (Sundqvist et al., 2010; Tamminen et al., 2010; Pallecchi et al., 2012).

Moreover, in Europe copper and zinc are used in animal farming and aquaculture as growth promoters and copper is used in agriculture as a pesticide, which promotes the spread of AR via co-selection mechanisms (Monteiro et al., 2010; Seiler and Berendonk, 2012). In Canada and the United Sates, copper is used in aquaculture as an anti-fouling agent and in agriculture as a pesticide while zinc is used as a growth promoter (US Environmental Protectio Agency (US EPA), 2008; Government of Canada, 2016). Therefore, there is a growing need to better understand the mechanisms, frequency, reservoirs, and vectors governing the transfer of AR determinants in major foodborne pathogens in order to control the dissemination of antibiotic resistant microorganisms.

Bacteriophages (phages), which are viruses that infect bacteria, are one mechanism by which antibiotic resistance genes (ARGs) can be transferred to foodborne pathogens. Phages are the most abundant organism in the biosphere, and are found in diverse environments including oceans, lakes, soil, urban sewage, potable and well water and plant microbial communities (Clokie et al., 2011) Most phages are either virulent or temperate and follow two distinct lifecycles, lytic and lysogenic, respectively. Virulent phages do not integrate their DNA into the host chromosome but induce immediate formation of phage particles and lysis of the host cell. Temperate phages integrate their DNA into the host chromosome and the prophage may remain dormant in the host until some stress will lead to induction and excision of the phage from the chromosome leading to subsequent formation of phage particles and lysis of the host cell (Feiner

et al., 2015). Temperate phages have increasingly been demonstrated to contribute to the spread of AR via horizontal gene transfer (HGT) mechanisms (Muniesa et al., 2013). Conjugation, transformation, and transduction are the primary HGT mechanisms by which dissemination of ARGs occur (von Wintersdorff et al., 2016). The idea that phages are a major driver of HGT of ARGs between foodborne pathogens, as well as from the environment to animals and humans, is increasingly becoming recognized (Muniesa et al., 2014). Antibiotic resistance genes are often found on various mobile genetic elements (MGEs), such as plasmids, genomic islands and transposons, and, as such, can be horizontally transferred by phage-mediated mechanisms. Recently, phage-like plasmids (PLPs), has been shown to carry ARGs and heavy metal resistance (HMR) genes. Due to their phage/plasmid hybrid nature, PLPs can potentially transfer ARGs and HMR genes via conjugation, transduction and transformation (Octavia et al., 2015b). Thus, in this study, emphasis was placed on detecting and characterizing PLPs carrying ARGs and HMR genes and their ability to horizontally transfer these resistance determinants to foodborne pathogens.

1.1 General Hypothesis

Phage-like plasmids are reservoirs of antibiotic and heavy metal resistance genes, which may be transferred to and contribute to the emergence of antibiotic and heavy metal resistant bacteria of foodborne importance.

1.2 Objectives

1.2.1 Main Objective

The identification and phenotypic and genomic characterization of phage-like plasmids within bacteria of foodborne importance and the determination of their potential to horizontally transfer antibiotic and heavy metal resistance genes to other foodborne pathogens via transduction, transformation and conjugation.

1.2.2 Specific Objectives:

1. The identification and genomic characterization of phage-like plasmids carrying antibiotic and heavy metal resistance determinants.

2. The phenotypic characterization, such as minimum inhibitory concentrations (MICs) and host range, of phage-like plasmids carrying antibiotic and heavy metal resistance determinants.

3. The determination of the potential for PLP-mediated horizontal gene transfer via transformation, conjugation and transduction of antibiotic and heavy metal resistance genes to different bacteria of foodborne importance.

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Chapter II

Factors, Mechanisms and Mobile Genetic Elements that Contribute to the Dissemination of Antibiotic Resistance Worldwide – A Review

2.1 The global emergence of antibiotic resistance (AR)

The over prescription of antibiotics globally contributes to AR, increases the severity and length of diseases, increases the risk of complications, healthcare costs and mortality rates (Llor and Bjerrum, 2014). In Europe, it is estimated that 80–90% of general practitioners prescribe antibiotics, with one study finding that on average, 30.1% of patients were prescribed at least one antibiotic per year (Shallcross et al., 2017). Moreover, the availability of over-the-counter antibiotics promotes self-medication, incomplete courses or regimens, and storage of leftover antibiotics that further contribute to the problem of AR (Morgan et al., 2011). This practice is common in Asia, Africa, South and Central America and in Southern European countries such as Italy, Greece, Malta and Spain (Llor and Bjerrum, 2014). These Southern European countries as well as China have the highest consumption rates of antibiotics in the world due to over-the-counter practices (Llor and Bjerrum, 2014), and it has been demonstrated that countries with higher consumption rates also have higher AR rates (Morgan et al., 2011).

In addition to the use of antibiotics in human medicine, they are also used in agriculture, aquaculture, and animal farming as growth promoters (Figure 2.1). This in turn creates a selective pressure and maintains the presence of AR-bacteria within the microflora of the animals. Although Canadian data is unavailable, an estimated 80% of antibiotics in the USA and Canada are used in animal farming and aquaculture, of which 70% are from classes of antibiotics that are medically important to human medicine (Martin et al., 2015). Thus, the development of bacteria resistant to antibiotics used in animal husbandry can lead to infections that are resistant to the same antibiotics clinically used in humans.

The widespread use of antibiotics in community settings, hospitals, animal farming, and aquaculture all contribute to the cycle of AR, where the spread of resistant bacteria can occur in many different ways (Figure 2.2). More specifically, foodborne pathogens carrying ARGs can be

transferred from livestock to humans through improperly cooked meat, cross-contamination, and occupational contact of farmers and slaughterhouse workers (Centers for Disease Control Prevention, 2002; Schneider et al., 2011)(Klous et al., 2016). Additionally, irrigation water and manure application can contaminate crops, leading to AR foodborne illnesses. Finally, pathogens carrying ARGs can return to the environment through urban sewage and wastewater effluents and the cycle begins again. The emergence and persistence of antibiotic-resistant bacteria impacts the environment through wastewater treatment plants, farm and slaughterhouse runoffs, hospital effluents, manure applications, and aquaculture; consequently perpetuating the cycle of AR, which has the potential to greatly compromise the effectiveness of antibiotic therapeutics.

2.1.1 Antibiotic resistance in foodborne pathogens *Salmonella enterica* and Shiga toxin-producing *Escherichia coli* (STEC)

Nontyphoidal *Salmonella* (NTS) is the most prevalent bacterial foodborne pathogen resulting in hospitalization in the western world and the most multi-drug resistant (MDR) pathogen associated with outbreaks of foodborne illness (Doyle, 2015; Thomas et al., 2015).

Although most NTS infections do not require antibiotic treatment, in 5% of gastroenteritis cases caused by NTS, patients develop bacteremia or other extraintestinal complications, such as urinary tract infections, pneumonia, endocarditis, meningitis and cellulitis (Eng et al., 2015). In the 1980s, NTS was treated with ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole (TMP-SMZ); but by the 1990s, widespread resistance emerged (Acheson and Hohmann, 2001). Currently, fluoroquinolones, such as ciprofloxacin, and third-generation cephalosporins, such as ceftriaxone, are mainly administered for NTS treatment. However, resistance to these newer antibiotics is also emerging (Eng et al., 2015). From 2004–2012, *Salmonella* isolates resistant to ceftriaxone increased from 9% to 22%. It is estimated that 6,200 culture-confirmed *Salmonella* isolates responsible for infections are resistant to ampicillin, ceftriaxone, and ciprofloxacin annually in the US (Medalla et al., 2017). Canadian data for the prevalence of third-generation cephalosporin resistance in Canada is unavailable. However, in 2013 and 2014, a study identified a strong correlation between third-generation cephalosporin resistant *Salmonella* Heidelberg from clinical infections and retail chicken meat (Public Health Agency of Canada, 2016). Concern grew when multidrug resistant strains of

Salmonella Typhimurium phage type DT104 emerged, as this strain has been associated with poultry, cattle and swine, and notably, is defined by penta-resistance to ampicillin, chloramphenicol, streptomycin, sulfomides, and tetracycline (ACSSuT) (Threlfall, 2000). Data from the National Antimicrobial Resistance Monitoring System (NARMS) indicated that 8.5% of NTS isolated from humans between 1999 and 2004, and 3.8% of retail meat isolates from 2002 to 2004, possessed the ACSSuT phenotype, where *Salmonella* Typhimurium and *Salmonella* Newport were the most common serovars with the penta-resistance pattern (Whichard et al., 2010). In fact, *Salmonella* was the most prevalent foodborne pathogen involved in antibiotic-resistant outbreaks between 1973 and 2011, with *S.* Typhimurium being the most frequent serovar (DeWaal and Grooters, 2013).

After NTS, Shiga toxin-producing *Escherichia coli* (STEC) accounts for the largest number of MDR isolates from poultry and retail meat in the US (Doyle, 2015). Antibiotic treatment for STEC has been controversial, with some studies demonstrating efficacy with certain classes of antibiotics, while others induce prophages present in STEC to increase the production of Shiga toxin (Stx), potentially triggering complications such as hemolytic uremic syndrome (HUS) (Kurioka et al., 1999; Bielaszewska et al., 2012). Nevertheless, AR among STEC strains has increased significantly since the first identification of STEC as a foodborne pathogen in 1982 (Kim et al., 1994; Meng et al., 1998; Galland et al., 2001; Schroeder et al., 2002; Vidovic and Korber, 2006), likely due to overuse of antibiotics in food-producing animals such as ruminants, which are the animal reservoir for STEC (Hunt, 2010).

One study in 1988 indicated that 2.9% of *E. coli* O157:H7 isolates (the predominant STEC serotype accounting for the majority of STEC cases in North America) from human cases were resistant to antibiotics (Ratnam et al., 1988). As of 2007, 79.8% of *E. coli* O157:H7 isolates from bovine and human feces, bovine milk products, ground beef, and cider carried one or more ARGs (Srinivasan et al., 2007). In 2015, 83.3% of *E. coli* O157 isolates from healthy sheep were resistant to at least one antibiotic (Ferreira et al., 2015). In another study, antibiotic profiles of 95 fecal isolates of *E. coli* O157:H7 collected from two commercial dairy farms in South Africa demonstrated that the ARGs bla_{ampC} (90 %), *str*A (80 %), *tet*A (70 %), bla_{CMY} (70 %) and $bla_{\text{CTX-M}}$ (65 %) resistance genes were predominant (Iweriebor et al., 2015). El-Shatoury and colleagues also demonstrated that of 44 *E. coli* O157:H7 isolates, 100% were resistant to amoxicillin and 77% were resistant to clarithromycin (El-Shatoury et al., 2015) .

2.2 Bacterial mechanisms of antibiotic resistance (AR)

Bacterial resistance to antibiotics may result from either intrinsic resistance or acquired resistance. Intrinsic resistance is the ability of bacteria to resist an antibiotic due to inherent functional resistance genes or structural mechanisms, whereas acquired resistance is conferred via mutations in chromosomal genes and horizontal gene transfer (HGT) (Munita and Arias, 2016).

Antibiotic resistance through HGT is acquired by the gain of mobile genetic elements (MGEs) carrying ARGs for different classes of antibiotics. It has also been proposed that phages (viruses that infect and replicate within bacteria) harbouring ARGs might be the primary mechanism by which mobilization and transfer of such resistant genes to humans and animals through pathogens may occur (Muniesa et al., 2013). Present in different environmental niches, these phages may mobilize ARGs to commensal bacteria of animal and human biomes. From commensal bacteria, ARGs can be further transferred to pathogens of the same genetic exchange community. Under conditions of selective pressure exerted by antibiotics, ARGs may be incorporated into MGEs and continue their mobilization to animal and human biomes through HGT—of which there are several mechanisms: conjugation, transformation, and transduction. Additionally, ARGs within MGEs may also develop point mutations under the selective pressure of antibiotics, thus creating more variations of ARGs. Ultimately, the maintenance and persistence of these ARGs within certain bacteria and pathogens can lead to eventual mobilization back to environmental bacteria, as well as animals and humans (Muniesa et al., 2013).

In contrast, resistance acquired by mutations is established when a susceptible population develops mutations in genes that inhibit the activity of the antibiotic, thus allowing the bacteria to survive in this environment. This is followed by selection of a resistant mutant, which eliminates the susceptible population. These mutations can arise through the modification of the antibiotic target, the activation of efflux pumps, and the alteration of metabolic pathways.

Bacteria can resist a single class of antibiotics by multiple mechanisms at any given time due to the existence of multiple mechanisms of AR; thus producing an additive effect and often increasing the levels of resistance. The classification for AR mechanisms are based on 4 biochemical mechanisms: i) prevention of access to antibiotic targets, ii) changes in antibiotic

targets by mutation, iii) modifications and protection of antibiotic targets, iv) direct modification of antibiotics (Munita and Arias, 2016).

2.2.1 Prevention of access to antibiotic targets

Bacterial efflux systems are a major contributor to intrinsic antibiotic resistance, and confer resistance to antibiotics of clinical importance when overexpressed. The presence of antibiotics triggers the overexpression of efflux pumps and their broad substrate specificities underlie their roles in MDR (Sun et al., 2014). There are five major families of efflux pumps: the resistance-nodulation-division (RND) family, the major facilitator superfamily (MFS), the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily, the small multidrug resistance (SMR) family, and the multidrug and toxic compound extrusion (MATE) family. MFS, ABC, SMR and MATE are present in Gram-positive and Gram-negative bacteria, while the RND family is exclusive to Gram-negative bacteria (Blanco et al., 2016).

The RND family includes 7 protein families and is present in all kingdoms of life. The main functions of the RND family are to transport heavy metals, hydrophobic compounds, amphiphiles, and nodulation factors (Tseng et al., 1999; Nies, 2003). They are composed of a transporter in the inner membrane (AcrB), a periplasmic accessory protein (AcrA) and an outer membrane protein channel (TolC). Their tripartite composition allows them to exclude various antibiotics from the cytoplasm. Clinically, the RND family has been extensively associated with AR, such as AcrB in *E. coli* and *Salmonella* , MexB in *Pseudomonas. aeruginosa*, and cmeB in *Campylobacter jejuni*. AcrAB efflux pumps have been associated with resistance to fluoroquinolones, ciprofloxacin, tetracycline, ceftazidime, and ciprofloxacin in clinical *Klebsiella pneumonaie* strains (Pakzad et al., 2013; Sato et al., 2013).

The MFS family represents 25% of all known membrane transport proteins in prokaryotes. NorA and PmrA are major MFS family efflux members in Gram-positive bacteria, and provide clinical antibiotic resistance in *Staphylococcus aureus* and *Streptococcus pneumoniae*, respectively (Piddock, 2006).

The ABC transport family are involved in the uptake or secretion of a wide variety of substrates, as well as different cellular functions such as DNA repair and regulation of gene

expression. Lmr in *Lactococcus lactis* and VcaM from *Vibrio cholerae* are known to pump a variety of antibiotics from different classes (Lubelski et al., 2007).

The SMR family are small integral inner membrane proteins that confer resistance to a variety of antibiotics such as β-Lactams, cephalosporins, and aminoglycosides. Unlike other efflux families, they frequently provide co-resistance to quaternary ammonium compounds (QAC) (Heir et al., 1999; Bay et al., 2008).

Finally, the MATE family has been described in various Gram-negative bacteria such NorM in *Vibrio parahaemolyticus*, VcmA in *V. cholerae*, and PmpM in *P. aeruginosa* as being involved in the extrusion of fluoroquinolones, norfloxacin, and ciprofloxacin (Piddock, 2006).

2.2.2 Changes in antibiotic targets by mutation

Antibiotics bind to their targets with high affinity; thus, bacteria have developed mechanisms to change the targets to prevent antibiotic activity (Blair et al., 2015). One mechanism by which this occurs is the induction of a single point mutation that can be passed to a population of bacteria in order to confer resistance (Blair et al., 2015). For example, *S. pneumoniae* and *S. aureus* have developed resistance against linezolid, a first generation oxazolidinone antibiotic, by a point mutation in the 23S rRNA ribosomal subunit. Consequently, a population selecting for the presence of this point mutation was rapidly produced, and now confers resistance to these antibiotics (Gao et al., 2010). Another well-characterized example is the mutational mechanism of fluoroquinolone resistance. DNA gyrase and topoisomerase IV enzymes are responsible for removing the negative supercoiling ahead of the replication fork and separating the replicated DNA strands, respectively (Drlica et al., 2008). Fluoroquinolone antibiotics inhibit DNA replication by binding to subunit A of the gyrase (*gyrA*) and subunit C of the topoisomerase IV (*parC*) (Drlica et al., 2008). Bacteria have developed chromosomal mutations in regions of *gyrA* and *parC*, termed quinolone resistance determining regions (QRDRs), which decreases the affinity of fluoroquinolone antibiotics on the bacteria (Munita and Arias, 2016).

2.2.3 Modifications and protection of antibiotic targets

Resistance to antibiotics by modification of the target through mechanisms including enzymatic alteration or complete replacement of the target site, results in decreased affinity for clinically important antibiotics. Examples of enzymatic modifications of the target site include the erythromycin ribosome methylase (*erm*) family, which methylate 16S rRNA preventing the binding of macrolides, lincosamines and streptogramins (Weisblum, 1995); as well as chloramphenicol-florfenicol resistance (*cfr*) provides resistance to phenicols, streptogramins, lincosamines, and oxazolidinones by methylating 23 rRNA (Long et al., 2006). Both *erm* and *cfr* are encoded on plasmids promoting their dissemination. Examples of complete replacement of the target site include the quinolone resistant genes, *qnr*. Also located on plasmids, they encode pentapeptide repeat proteins (PRPs), which bind the topoisomerase IV and DNA gyrase and protect them from fluoroquinolone antibiotic action. The PRPs also promote the release of the antibiotics which permits topoisomerase IV and DNA gyrase activity to resume (Drlica et al., 2008). Another example of the acquisition of a gene homologous to the original target is the acquisition of the staphylococcal cassette chromosome mec (SCCmec) element in methicillin-resistant *S. aureus* (MRSA). This particular element contains the *mecA* gene, which encodes PBP2a, a penicillin binding protein (PBP) that resists β-Lactams by protecting the bacterial cell wall structure (Katayama et al., 2000).

2.2.4 Direct modification of antibiotics

In addition to preventing antibiotics from binding to their targets, modifying targets by mutations, and transporting antibiotics out of the cell by efflux, resistance can also be achieved by directly inactivating or modifying the antibiotic itself.

Enzymatic modification of antibiotics is a major mechanism of AR, and was first observed by the emergence of β-lactamase TEM-1 in the early 1960s (Datta and Kontomichalou, 1965). β-Lactamases can inactivate penicillins, cephalosporins, clavams, carbapenems, and monobactams by cleaving their cyclic amide ring. β-Lactamases that cleave third and fourth generation β-Lactam antibiotics are known as extended-spectrum β-lactamases (ESBLs). These enzymes including imipenemase (IMP), Verona integron encoded metallo β-lactamase (VIM), *K.*

pneumoniae carbapenemase (KPC) and oxacillinase (OXA) are resistant to all β-lactam antibiotics, which has severe implications for clinical treatment (Blair et al., 2015). There are also hundreds of variants of β-lactamases with specific activity for cefotaxime called CTX-M that encodes resistance to third-generation cephalosporins. Most notable of the CTX-M variants are CTX-M-14 and CTX-M-15 because they have become the most widely disseminated ESBLs worldwide (Cantón et al., 2012). All of these ESBLs can be easily maintained and readily disseminated among bacteria through plasmids, integrons (further discussed in section 2.4.1), and other MGEs.

Aminoglycosides are also susceptible to modification by three different classes of modifying enzymes: acetyltransferases (AACs), phosphotransferases (APHs), and nucleotidyltransferases (ANTs) (Blair et al., 2015), which catalyze the acetylation of NH_2 groups, the transfer of a phosphate group, and the transfer of an AMP group to the aminoglycoside molecule, respectively (Ramirez and Tolmasky, 2010). These enzymes are also disseminated by MGE such as plasmids, transposons (further discussed in section 2.4.1), genomic islands, and integrons.

2.3 Bacterial resistance to heavy metals

Sub-lethal concentrations of heavy metals are often used in animal farming, aquaculture, and agriculture and studies have demonstrated that they can promote the spread of AR bacteria. Heavy metals ions such as copper (Cu^{2+}) and zinc (Zn^{2+}) are incorporated into animal feed, particularly in pig and poultry production, for growth promotion and therapy of intestinal disease (Wales and Davies, 2015). Other heavy metals ions such as iron (Fe^{2+}), cobalt (Co^{2+}), and manganese (Mn^{2+}) are also added to livestock, as well as aquaculture feed as nutritional additives (Seiler and Berendonk, 2012). Like antibiotics, heavy metals are used at sub-lethal concentrations, which allow the development of resistance because of the constant selective pressure. However, unlike antibiotics, Cu^{2+} and Zn^{2+} are very persistent in the environment and can accumulate in soil, water, and sediments (Wales and Davies, 2015). It has been observed that 90–95% of in-feed Cu^{2+} and Zn^{2+} is shed in the feces of livestock and contaminates the environment (Medardus et al., 2014). Furthermore, in developing countries, due to high nutritional content and low cost of wastewater treatment, wastewater and sewage sludge

containing accumulated heavy metal ions are converted to fish feed. This practice re-introduces heavy metal ions to farmed fish and the aquatic environment.

In agriculture, copper sulfate is applied to crops as an organic pesticide due to its bactericidal and fungicidal properties. In different regions worldwide, it is also common practice to apply fertilizers, sewage sludge, and liquid manure to agricultural soils, which are contaminated with Cu^{2+}, Zn^{2+}, mercury (Hg^{2+}), lead (Pb^{2+}), and cadmium (Cd^{2+}). Nicholson et al. (2003), study observed that the application of livestock manure and sewage sludge was responsible for 37–40% and 8–17% of total Zn^{2+} and Cu^{2+} inputs in the soil, respectively.

In aquaculture, Cu^{2+}-based antifouling paints are applied on fish cages and netting to prevent the growth of marine organisms that decrease the durability and flotation of the netting and oxygen flow (Braithwaite et al., 2007). Consequently, Cu^{2+} is released into the water and accumulates in sediments of the aquaculture cages (Burridge et al., 1999). Elevated concentrations of Cu^{2+} that exceed regulations for sediment quality are commonly found in sediments near aquaculture (Burridge et al., 1999; Burridge and Zitko, 2002; Chou et al., 2002; Brooks and Mahnken, 2003). Additionally, metals such as Zn^{2+} are added to fish feed for nutritional purposes or growth promotion. Like Cu^{2+}, studies conducted in Canada and New Zealand measured elevated Zn^{2+} levels in sediments near aquaculture facilities (Morrisey et al., 2000; Parker and Aube, 2002; Brooks et al., 2003; Dean et al., 2007). This is of concern because depending on geographical location the concentrations of heavy metal ions in sediments do not always reduce to background levels (Smith et al., 2005). These persistently elevated levels of Cu^{2+} and Zn^{2+} are eventually taken up into the environment by currents and become incorporated into all aspects of the ecosystem.

2.3.1 Bacterial co-resistance of heavy metal and antibiotic resistance

Various reports of co-resistance of AR and heavy metal resistance (HMR) phenotypes of environmental isolates from manure, aquaculture, and feed have been demonstrated. A study of the New York Bight involving areas where sewage sludge, dredge soil acid waste, and cellar dirt were discharged demonstrated that *Bacillus* strains resistant to Hg^{2+} and ampicillin were 6 times more frequent than control strains from non-sediment areas. The authors hypothesized that mercury resistance and β-lactamases were simultaneously selected for (Timoney et al., 1978).

Stepanauskas *et al.,* (2006), exposed naïve freshwater bacterioplankton to various concentrations of Cd^{2+}, nickel (Ni^{2+}), ampicillin, and tetracycline to investigate whether co-resistance mechanisms can select for multi-resistant bacterioplankton. The authors observed that 50–100% isolates exposed to Cd^{2+} and Ni^{2+} were resistant to at least one antibiotic, and 50–60% of isolates exposed to tetracycline and ampicillin were resistant to at least one of the two heavy metal ions compared to 20% of control isolates (Stepanauskas et al., 2006). Another study observed that AR levels for β-Lactams and sediment Cu^{2+} levels in the Almendares river in Cuba were 3 times higher downstream of pharmaceutical factories (Graham et al., 2010). Copper-resistant Gram-negative isolates were observed to be 7 times more resistant to more than 4 different antibiotics including ampicillin, chloramphenicol, nalidixic acid, olaquindox, streptomycin, sulphanilamide, and tetracycline than copper-sensitive isolates, suggesting the existence of co-selection or cross-resistance mechanisms (Berg et al., 2005). Another study also observed that the level of copper in soil strongly influenced ARG abundances in the soil such as tetracycline, β-lactamases, ESBLs, and erythromycin (Knapp et al., 2011). Cu^{2+} and Zn^{2+} were often associated with an increase in resistance to ampicillin, amoxicillin+clavulanate, and piperacillin in *E. coli* isolated from pig manure (Hölzel et al., 2012).

2.3.2 Bacterial co-selection and cross-resistance mechanisms

Heavy metals can promote the spread of AR bacteria via co-selection and cross-resistance mechanisms (Figure 2.3). Co-selection occurs when AR and HMR genes expressing a resistant phenotype are located on the same genetic element, such as a transposon, integron, or plasmid (Baker-Austin et al., 2006). Co-selection has been well documented in the literature. For example, a mercury resistance (*mer*) operon, a class 1 integron containing *aadA7*, and another AR cassette containing *floR, tetA, sulII, strA/strB* were all identified on one conjugable plasmid within an isolate of *Aeromonas salmonicida* subsp. *salmonicida* from a farmed Atlantic salmon (McIntosh et al., 2008). Another study also identified a *mer* operon located within a transposon on a conjugable plasmid containing *aadK* for streptomycin resistance (Davis et al., 2005). Another group identified a novel copper resistance gene, *tcrB*, in *Enterococcus faecium* strains isolated from livestock and human isolates (Hasman and Aarestrup, 2002). Copper resistance conferred by *tcrB* in 91% and 31% of the copper-resistant isolates, were resistant to macrolide

and macrolide+glycopeptide, respectively. *TcrB* as well as the ARGs were found to be present on a plasmid; hence, co-resistance was conferred by co-selection mechanisms (Hasman and Aarestrup, 2002).

Cross-resistance is a physical mechanism that is initiated to reduce and expel heavy metal ions and antibiotics (Baker-Austin et al., 2006). There are three general mechanisms by which this occurs. The first is the sequestration of heavy metal ions to reduce their toxicity in the cytoplasm. This is facilitated by cell membranes, cell walls, and extracellular polymeric substance (EPS) of biofilms that act as barriers (Seiler and Berendonk, 2012). Second is detoxification by the reduction of intracellular ions. A well-characterized example of this is the reduction of mercury by the *merA* gene from Hg^{2+} to Hg^{0}, which will diffuse out of the bacteria due to its low evaporation point (Seiler and Berendonk, 2012). The third mechanism is the extrusion of heavy metal ions through efflux systems.

There are three types of efflux systems for heavy metal transport: antiporters, cation diffusion facilitators, and ATPases (Nies, 2003). These three main efflux systems are present in over 64 species of bacteria and foodborne pathogens including *Bacillus subtilis, L. monocytogenes, S. aureus, Clostridium perfringens*, *C. jejuni*, *E. coli,* and *S. enterica*.

The main efflux system for antiporters is the RND protein family, described previously. Of the 7 RND protein families, there are 4 transport proteins named heavy metal efflux (HME) (HME1/HME2, HME3B, HME4, HM5) that exclusively transport heavy metal ions (Nies, 2003). HME1/HME2 were the first known RND family-related heavy metal transporter proteins, and encode CzcA, a protein capable of exlcuding cadium (Cd^{2+}), zinc (Zn^{2+}), nickel (Ni^{2+}), and copper (Cu^{2+}) from the cytoplasm (Nies and Silver, 1989). A strong correlation between AR sul2 and *czcA* has been demonstrated on a class 1 integron from wastewater treatment plant isolates, thus supporting cross-resistance as well as co-selection mechanisms (Di Cesare et al., 2016). Gillings *et al.,* also observed that class 1 integrons are closely located to *czcA*, further indicating co-selection (Gillings et al., 2008). Another study demonstrated that a mutation in *czcS*, a sensor protein part of the *czc* operon, conferred cross-resistance to Zn^{2+} and imipenem in isolates of *P. aerugionasa* (Perron et al., 2004). HME4 encodes the CusCFBA operon on the bacterial chromosome for transport of Cu^{2+} and silver (Ag^{2+}) from the cytoplasm. In addition to the RND family, a study also observed that the protein OprD2, a membrane porin responsible for the

passive uptake of amino acids and resistance to carbapenem in *P. aeruginosa,* was responsible for the increased resistance to this antibiotic in the presence of Zn^{2+} (Conejo et al., 2003).

The cation diffusion facilitators (CDF) family of proteins transport Zn^{2+}, Cu^{2+}, Ni^{2+}, Cd^{2+}, and iron (Fe^{2+}) and is propelled by a concentration gradient. The main operon is *czcD*, which is encoded on the bacterial chromosome. *P. aeruginosa* deletion mutants for *czcD* demonstrated low zinc sensitivity and a 4- to 8-fold increased sensitivity to imipenem and ciprofloxacin, as well as a 2-fold increased sensitivity to chloramphenicol and gentamycin; thus demonstrating the importance of *czcD* in cross-resistance (Salusso and Raimunda, 2017).

The third family of efflux pumps are P-type ATPases, which export heavy metals such as Cu^{2+}, Ag^{2+}, Zn^{2+}, and $Cd^{2+}$from the cytoplasm to the periplasm, and then to the environment via ATP hydrolysis (Nies, 2003). One of the most well-characterized P-type ATPases is a plasmid-encoded copper resistance determinant in *E. coli* called a copper-exporting P-type ATPase (copA), which transports heavy metal ions from the cytoplasm into the periplasm. Cross-resistance was also observed when a P-type ATPase *pbrA* and *L. monocytogenes mdrL* efflux gene were isolated from a *Pseudomonas sp.* and *Vibrio sp.* Strain, and observed to confer resistance to lead (Pb^{2+}) as well as 10 different antibiotics (Naik et al., 2013).

2.4 Horizontal gene transfer (HGT) mechanisms

Horizontal gene transfer (HGT) is an essential force in the evolution of bacteria. Typically, genes are horizontally transferred to a recipient host to provide a selective advantage either to itself or to the recipient host (Soucy et al., 2015). The three most recognized mechanisms of HGT are transformation, conjugation and transduction.

Transformation is the uptake of naked DNA into a bacterial cell. Unlike conjugation and transduction, this process is entirely driven by the recipient cell and requires genes encoded in the core genome. More than 80 bacterial species have been demonstrated to develop competence in order to acquire DNA through natural transformation (de Vries et al., 2001). The induction of competence is a form of quorum sensing in the bacteria that initiates transformation. Competence is induced by environmental stresses that threaten survival of the bacteria and low availability of nutrients in the environment (Johnston et al., 2014). Once bacterial competence is induced, free double-stranded DNA (dsDNA) is transported across the outer membrane or peptidoglycan by the pilus (PilE) via the secretin channel (PilQ). One strand of the dsDNA is degraded while the

other is internalized across the inner membrane through the ComA channel into the cytosol. The resulting single-stranded DNA (ssDNA) is bound by the DNA processing protein A (DprA), which recruits RecA to polymerize the ssDNA and initiate a search for homologous regions in the chromosome in order to integrate the DNA by homologous recombination (Johnston et al., 2014).

Conjugation is the mobilization of independently replicating genetic elements such as plasmids, integrated conjugative elements (ICEs), and conjugative transposons (CTns) through cell-to-cell contact from a donor cell to a recipient cell linked by a pilus (Frost et al., 2005). Conjugation is initiated by the establishment of a stable mating pair by the transfer gene (*tra)* between the donor and recipient bacterial cells (Frost et al., 2005). The type IV secretion system (T4SS) then initiates pilus assembly by linking a transferosome to a relaxosome via a coupling protein. In order to linearize the dsDNA for conjugative transfer, the relaxosome uses its relaxase (*traI)* to nick the dsDNA at a *nic* site located within the origin of transfer (oriT). A signalling event then triggers DNA transport (Dfr) from the donor cell to the recipient (Frost et al., 2005).

In contrast to conjugation and transformation, transduction is uniquely performed by phages. Two types of transduction mechanisms have been described: generalized and specialized. Generalized transduction refers to the mispackaging of bacterial DNA into the phage capsid. As a result, the phage may then infect another susceptible host, thereby transferring genetic material to another bacterial cell where it will be integrated by homologous recombination. Specialized transduction is the improper excision of a prophage from the bacterial chromosome that results in packaging of the bacterial DNA into phages. Specialized transduction occurs at a higher frequency than generalized transduction (Griffiths, 2002).

2.5 Mobile genetic elements

Mobile genetic elements (MGEs) are DNA sequences encoding proteins and enzymes that mediate the transfer of DNA within the genomes of bacteria (intracellular mobility) and between bacterial cells (intercellular mobility) (Partridge, 2011). Intracellular mobility is mediated by transposons and integrons while intercellular mobility is mediated by conjugative plasmids, integrative and conjugative elements (ICEs), and phages.

2.5.1. Intracellular mobility

2.5.1.1. Transposons

Transposons are segments of DNA capable of moving within the genome and can be either simple or composite. Simple transposons are insertion sequences (IS) that do not carry ARG, but instead mobilize and can inactivate genes through the presence of short inverted repeat (IR) sequences that flank the coding region of the IS (Frost et al., 2005). Composite transposons on the other hand can carry ARGs, and are flanked on either side by identical or non-identical IS sequences (Partridge, 2011). On the left of the IS coding region, relative to the direction of transcription, is IR_L and on the right is IR_R. The IR sequences that flank the transposon are recognized by transposases, which mobilize the transposon using a "cut and paste" mechanism.

There are 3 families of transposons commonly associated with ARGs: Tn*3*-like, Tn-*21* like, and Tn*5053*. The first 2 are both bound by 38-bp IR and are mobilized by transposase TnpA, while Tn5053 has three transposase genes*, tniA, tniB* and *tniQ* that provide added mobility. Transposon Tn5053 is important in the dissemination of ARGs as it targets integration sites in the partitioning (*par*) regions of plasmids, mercury resistance operons, and class 1 integrons (Partridge, 2011).

2.5.1.2. Integrons

Like composite transposons, integrons also carry ARG, but their integration into the chromosome is mediated by an integrase rather than a transposase. The *intI* gene encodes the integrase, which integrates the integron by site-specific recombination at chromosomal *attI* recognition sites. Integrons are usually part of larger transposons that are present in plasmids or ICEs (Partridge, 2011). Class 1 integrons are the most extensively studied due to their association with ARGs, and are responsible for the mobilization of the penta-resistant genomic island SGI1 in *S.* Typhimurium DT104, which was a prevalent MDR *Salmonella* strain in Europe and North America between 1970 and 2000 (Boyd et al., 2002).

2.5.2. Intercellular mobility

2.5.2.1. Conjugative plasmids

Plasmids are self-replicating replicons that exist extrachromosomally as covalently closed dsDNA segments that are either circular or linear. Although plasmids can contain genes that encode for non-essential cellular functions (Frost et al., 2005), they can also encode for genes involved in maintenance and stability, as well as genes that provide bacterial fitness, such as virulence, antibiotic resistance (AR), and heavy metal resistance (HMR). Shintani *et al.,* (2015), determined that of 4602 known bacterial plasmids, of which the average size is 80 kb and approximately 14% can be transferred by conjugation. Due to the explosion of next-generations sequencing, these characteristics have certainly changed since there are currently over 8000 known complete sequences of plasmids in the National Center of Biotechnology Information (NCBI) RefSeq database (Roosaare et al., 2018). Conjugable plasmids can be classified into 6 mobilization groups based on their *mob* genes. However, plasmids are primarily characterized based on their replicon gene. Plasmids having the same replicative mechanism cannot exist within the same bacterial cell forms the basis for the classification termed incompatibility (Inc). There are 27 Inc groups within the *Enterobacteriaceae* family, 14 Inc groups in *Pseudomonas*, and 18 Inc groups in *Staphylococcus* (Shintani et al., 2015).

2.5.2.2. Integrative and Conjugative Elements

Integrative and Conjugative Elements (ICEs), ranging in size from 20 kb to 500 kb, are the most abundant conjugative elements in prokaryotes, present in a diverse array of Gram-negative and Gram-positive bacteria (Johnson and Grossman, 2015). In contrast to plasmids, ICEs are chromosomally integrated plasmids that encode a tyrosine integrase enzyme, for integration into the bacterial chromosome, and a type IV secretion system, for conjugation to other hosts (Johnson and Grossman, 2015). ICEs encode genes that are grouped into modules, which can be exchanged among ICEs as well as to other MGEs (Wozniak and Waldor, 2010). In addition to the core genes that mediate regulation and conjugation, ICEs encode various fitness factors such as genomic islands, ARGs, HMR genes, virulence genes, carbon-source utilization genes, restriction-modification systems, bacteriocin synthesis genes, and biofilm formation genes (Wozniak and Waldor, 2010). One of the most extensively studied groups of ICEs is SXT.

Originally identified in *V. cholerae* O139, SXT confers resistance to numerous antibiotics, most notably sulfamethoxazole and trimethoprim, which have been used to treat cholera. Approximately 30 ICEs have been identified within the SXT group amongst different genera (Wozniak et al., 2009).

Under certain conditions, ICEs will excise from the chromosome and transfer to another host via conjugation (Guglielmini et al., 2011). When ICEs are induced, they circularize and their proteins assemble to form a mating pore. Once ICEs-encoded proteins and the recipient host recognize the origin of transfer (oriT), a relaxase covalently binds to ICEs and nicks it, causing linearization (Johnson and Grossman, 2015). As the relaxase remains bound to the linearized DNA, ICEs initiate rolling circle replication (Wozniak and Waldor, 2010). The relaxase then interacts with the conjugal transfer protein G (traG) to initiate conjugal transfer at the mating pore, where the mating machinery pumps the ICE ssDNA into the recipient host (Johnson and Grossman, 2015). ICEs then re-circularize and integrate into the recipient host chromosome via the action of an integrase. Finally, a Holliday junction mediates the recombination of ICEs into the recipient chromosome via the ICEs attachment site (*attP*) and bacterial attachment site (*attB*).

2.5.2.3. Phages

Phages are the most abundant life form on earth, with an estimated 10^{31} phages in the biosphere. Due to their ubiquitous nature, they can be found in soil, sediments, volcanoes, bodies of water, the air, and sewage (Hatfull, 2008). Phage genomes can be composed of ssDNA, dsDNA, ssRNA or dsRNA, and their genome size can vary from 3.5 kb to several 100 kb. The majority (96%) of phages are dsDNA tailed phages belonging to the order Caudoviridae and consist of a head, neck and tail (Casjens, 2008). The head is assembled by the co-polymerization of capsid and scaffolding proteins to form a procapsid. Once the phage nucleic acid is replicated, it is translocated into the procapsid, and a maturation process transforms the procapsid to a capsid. The portal protein, tail tube, and capsid completion proteins then ensure the attachment of the phage tail, which is constructed via polymerization by late phase proteins to form a mature virion (Fokine and Rossmann, 2014).

Phages can adopt three distinct life cycles: lytic, lysogenic, and pseudolysogenic. Virulent phages adopt the lytic lifecycle where upon infection, they replicate their genome and synthesize the capsid and tail proteins using the host machinery to produce dozens to thousands of progeny before lysing their host. In contrast, temperate phages adopt the lysogenic cycle, in which the phage does not induce the lysis of their host nor the production of phage particles. Instead, the phage genome is dormant within the host and replicates with the bacterial host chromosome, thus generating a prophage in each daughter cell upon bacterial division (Suzuki and Griffiths, 2000; Feiner et al., 2015). During this cycle, the phage represses the lytic cycle genes; however, under the action of a stressor, the phage can switch to the lytic cycle by inactivating the repressor (Howard-Varona et al., 2017). Finally, under unfavourable growth conditions, some phages can adopt a pseudolysogenic state, where their genome does not degrade, but instead exists as a plasmid within the cytoplasm that becomes incorporated into only 1 daughter cell during bacterial cell division (Feiner et al., 2015).

Phages are known to carry and disseminate ARG and MGE via transduction. For example, phage-mediated transduction of tetracycline, chloramphenicol, ampicillin, and kanamycin resistance genes has been observed in laboratory strains of *E. coli* (Serra-Moreno et al., 2006; Battaglioli et al., 2011; Shousha et al., 2015). Generalized transduction of genes resistant to third-generation cephalosporins, which are used to treat invasive *Salmonella* infections, has also been observed in different *Salmonella* serovars (Schmieger and Schicklmaier, 1999; Zhang and LeJeune, 2008). Evidence of phage-mediated transduction events has also been investigated in different environmental niches harbouring phages and in different AR bacteria. Wastewater, urban sewage, and river water have been demonstrated to contain high levels of phages, as well as β-lactamases, ESBLs, and fluoroquinolone resistance genes (Colomer-Lluch et al., 2011) (Colomer-Lluch et al., 2014). Marti et al. (2014), demonstrated that phages carrying ARG persist longer in the environment than their bacterial hosts. Ross and Topp (2015), also observed this in agricultural soils after treatment with manure and biosolids. These treatments reduced the bacterial load but phages carrying ARGs continued to persist in the treated soil demonstrating that phages are reservoirs for ARGs.

2.6 Phage-like plasmids (PLPs)

Phage-like plasmids (PLPs) are phage/plasmid hybrids that exist extrachromosomally as low-copy plasmids and have circular dsDNA genomes (Octavia et al., 2015b). A total of 22 PLPs have been reported in the literature and they are of interest because PLPs are reservoirs of antibiotic resistance genes and heavy metal resistance genes (Table 2.1). To date, PLPs have been isolated from 10 different countries worldwide including Canada, the United-States, Germany, Australia, China, France, Vietnam, South Korea, Japan and the Baltics (Alton and Vapnek, 1979; Lindler et al., 1998; Kidgell et al., 2002; Smeesters et al., 2011; Liu et al., 2012; Uchiyama et al., 2013b; Billard-Pomares et al., 2014; Falgenhauer et al., 2014; Hammerl et al., 2014; Huang et al., 2014; Utter et al., 2014; Octavia et al., 2015a; Shin and Ko, 2015; Li et al., 2016; Bai et al., 2017; Colavecchio et al., 2017c; Colavecchio et al., 2017d; Gillis et al., 2017; Yang et al., 2017; Zhang et al., 2017; Liu et al., 2018; Galetti et al., 2019). The majority of the reported PLPs (12 of 22) were isolated from clinical sources while the remaining PLPs were isolated from animal and environmental sources including the sea, wildlife feces, slaughterhouse, pig fecal samples and avian sources. The majority (20 of 22) PLPs have been isolated from foodborne pathogens including *E. coli, Salmonella enterica, Klebsiella pneumonaie, Vibrio vulnificus* and *Bacillus cereus* (Table 2.1).

2.6.1 Genomic characterization

Phage-like plasmid genome size can range from 26–369 kb, but a considerable proportion of PLPs characterized so far (14 of 21) are between 97–120 kb (Table 2.1). The plasmid region of PLPs consist of various bacterial genes and 3 essential plasmid maintenance encoded genes. Plasmid-partitioning genes, termed *parA* and *parB*, ensure the accurate inclusion of the low-copy plasmid prophages into their host daughter cells (Bignell and Thomas, 2001). The *repA* gene is responsible for PLP replication and may also contain the replication initiation site (*ori*). In addition to the plasmid-partitioning genes and *repA*, PLPs also encode toxin/anti-toxin modification systems such as *phd/doc*, or restriction modification systems that inhibit the growth of any bacterial daughter cells that emerge without the PLP (Łobocka et al., 2004).

The phage region consists of genes encoding for the major capsid protein, portal protein, phage tail proteins, sheath protein, tail fibers, the terminase for genome replication, host lysis proteins, such as holins and endolysins, and lysogeny-related proteins, such as a lysogeny repressor and the integrase. In addition to phage and plasmid genes, PLPs also encode ARGs, heavy metal resistance genes, and MGEs (section 2.6.3).

Based on the use of the phage integrase as a signature gene (Colavecchio et al., 2017b), and whole genome alignments of PLPs (Chapter III, Figure 3.3), two PLP lineages have been identified (Chapter III, section 4.1). The PLPs pSTM_Phi, pECHOH89, pHCM2, pMT1, pKHS1, AnCo1, AnCo2, AnCo3, and ABTJ2 all have nucleotide sequence identity to phage SSU5, a virulent phage that infects rough strains of *S.* Typhimurium (Kim et al., 2012a; Octavia et al., 2015b). The PLPs SJ46, RCS47, pKP12226, pVv01, pMCR-1-P3, and pSLK172-1 all have nucleotide sequence identity to phage P1, a temperate extrachromosomal phage and the prototypic phage-like plasmid (Łobocka et al., 2004; Kim et al., 2012b; Billard-Pomares et al., 2014; Hammerl et al., 2014; Shin and Ko, 2015; Bai et al., 2017; Yang et al., 2017; Zhang et al., 2017). The remaining five PLPs (*Bacillus spp,* vB_BceS-IEBH and pBtic235, *Helicobacter pylori* KHP30, *S. aureus* φBU01 and *Pseudomonas aeruginosa* pBH6::Phage BH9) do not have nucleotide sequence similarity to phage SSU5 or phage P1 nor do their integrase genes cluster within the two lineages (Chapter III, Figure 3.2). In addition, two putative PLPs have been identified within *Clostridium botulinum* strain Hazen and strain 202F, which also have no nucleotide sequence identity to SSU5 or P1 (Unpublished Cadieux et al., 2018).

2.6.2. Phenotypic characterization

Since PLPs are phage/plasmid hybrids, they may have the ability to infect different hosts via the phage-mediated mechanism, transduction, and plasmid-mediated mechanisms, transformation and conjugation, due to their phage/plasmid nature. Despite their large genomes, two PLPs, RCS47 and SJ46 (115 Kb and 103.4 Kb), were transformed by electroporation into competent *E. coli* cells (Billard-Pomares et al., 2014; Yang et al., 2017), suggesting that other PLPs also have the potential to be transformed into different hosts. Three PLPs (pBtic235, pKP12226 and pSLK172-1) were observed to be conjugative, however, pBtic235 could only be transferred by conjugation when a pXO16 conjugative plasmid was present within the donor host

cell. When plasmids do not contain their own conjugative system, they can use either co-resident conjugal plasmid machineries or host chromosome machineries (Yin and Stotzky, 1997). Thus, non-conjugative PLPs may have a conjugative ability if mobilization genes are present elsewhere within the donor host. The potential for PLPs to be transferred by transduction has not been previously investigated, however, half (11 of 22) of the reported PLPs in the literature were observed to be inducible, whereas 1 (PLP pABJT2) was not inducible, and the inducible capacity of the remaining 10 was not investigated. Furthermore, all 11 of the inducible PLPs carry ARGs suggesting they may have the ability to be transferred by transduction and contribute to the spread of antibiotic resistance.

2.6.3. Antibiotic resistance and heavy metal resistance in phage-like plasmids

Of 22 reported PLPs, 12 encode for ARGs and 1 for tellurite resistance determinants (Table 2.2). The majority of antibiotic resistant encoding PLPs (8 of 12) carry ESBLs, which confer resistance to third-generation cephalosporins (Chaubey et al.,2017; Leangapichart et al.,2017). Cephalosporins are a class of broad-spectrum β-lactam antibiotics, which are the most prescribed class of antibiotics worldwide and last line of treatment for Gram-negative infections (Bennett et al., 2014; Bozcal and Dagdeviren, 2017). Fourth and fifth generation cephalosporins are effective mainly against *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* (MRSA), respectively (Bennett et al., 2014). Rates of resistance to third-generation cephalosporins in the *Enterobactericeae* family are consistently over 10% in the USA and can be up to 70% in various countries in Europe (Sader et al., 2014; Rosenthal et al., 2016).

One PLP RSC47 encodes SHV-2, while seven other PLPs encode CTX-M variants. SHV-2 is one of 189 SHV allelic variants and confers resistance to third-generation cephalosporins (Tzouvelekis, 1999). Of over 1,000 known β-lactamase enzymes, CTX-M is one of the most globally disseminated and variants CTX-M-14 and CTX-M-15 are the most predominant worldwide (Cantón and Coque, 2006). These two determinants have been detected in pets, farm animals, the environment, sewage, food and clinical samples and have been detected on plasmids, transposons and integrons as well as PLPs (Potron et al., 2012; Amos et al., 2014; Fischer et al., 2014; Upadhyay et al., 2015; Timofte et al., 2016; Irrgang et al., 2017; Pérez-Etayo et al., 2018). Phage-like plasmids AnCo1, AnCo2, pKP12226, and pECOH89

encode CTX-M-15, pKPHS1 and pSLK172-1 encode CTX-M-14, and SJ46 encodes CTX-M-27 (Table 2.2). These PLPs were isolated from China, South Korea, Germany and the USA, which further demonstrates the global dissemination of CTX-M and ESBL resistance genes (Liu et al., 2012; Falgenhauer et al., 2014; Shin and Ko, 2015; Colavecchio et al., 2017d). Interestingly, AnCo1, AnCo2 and pECOH89 all share a CTX-M-15 resistance cassette with 100% nucleotide sequence homology even though pECOH89 was isolated from a patient in Germany and AnCo1 and AnCo2 were isolated from environmental feedlot isolates in the United States, demonstrating the vast dissemination of the CTX-M-15 gene.

One PLP, pBH6::Phage BH9, also carries the *Klebsiella pneumonaie* carbapenemase (KPC-2), which confers resistance to carbapenem antibiotics, a member of the β-lactam family of antibiotics (Galetti et al., 2019). Carbapenem antibiotics are unaffected by β-lactamases making them a last resort antibiotic for Gram-negative infections. However, since the first emergence of KPC determinants in 2001 in North Carolina, the treatment options of KPC resistant bacteria are limited (Yigit et al., 2001). In fact, *K. pneumoniae* carbapenemase-resistant *Enterobacteriaceae* have been identified as an urgent threat in Canada and the USA (CIPARS, 2012; NARMS, 2013).

Other PLPs including pHYEC7-*mcr1*, p-MCR-1-P3 and pSLK172-1 encode for *mcr-1*, which confers resistance to colistin, a last resort antibiotic for Gram-negative infections. Colistin (polymyxin E) is an antibiotic first introduced in 1959 to treat Gram-negative multi-drug resistant (MDR) infections. Due to neurotoxicity effects, colistin was suspended from use, but the emergence of third-generation cephalosporin and carbapenem resistance, led to the re-introduction of colistin to treat multi-drug resistant (MDR) infections as an antibiotic of last resort. In 2015, a plasmid-mediated colistin resistance gene (*mcr-1*) emerged in China and as of 2018, *mcr-1* has been reported in over 40 countries (Wang et al., 2017; Yi et al., 2017; Mendes et al., 2018; Principe et al., 2018) .

Some PLPs (pKP12226 and pSLK172-1) contain multiple ARGs encoding resistance to as many as 12 ARGs (Table 2.2) (Shin and Ko, 2015; Bai et al., 2017). The pKP12226 PLP encodes ESBLs (CTX-M-15 and TEM-1), macrolide resistance genes (*mph2, mphR* and *mrx*), a sulphonamide resistance gene (*sul1*), an aminoglycoside resistance gene (*aadA4*) and a trimethoprim resistance gene (*dfrA17*) (Shin and Ko, 2015). The PLP pSLK172-1 encodes a colistin resistance gene (*mcr-1*), an ESBL gene (CTX-M-14), sulphonamide resistance genes

(*sul1, sul2 and sul3*), aminoglycoside resistance genes (*aphA1, aadA1, aadA2,* and *aac(3)-IV*), a trimethoprim resistance gene (*dfrA12*), a chloramphenicol resistance gene (*floR*), and a fosphomycin resistance gene (*fosA3*) (Bai et al., 2017).

The diverse array of ARGs carried by PLPs maybe due to the concurrent carriage of mobile genetic elements (MGEs). Interestingly, PLPs that encode antibiotic resistance also encode at least one mobile genetic element that flanks the ARG (Table 2.2). For instance, each CTX-M variant is flanked by the insertion sequence *ISEcp1*, which not only mobilizes the ARG but also encodes the promoter for CTX-M expression (Ma et al., 2011). In PLP SJ46, not only does *ISEcp1* flank the CTX-M variant (CTX-M-27), but both genes are located on transposon Tn1721 (Table 2.2) (Yang et al., 2017). In PLP RCS47, SHV-2 is flanked by IS5 (Billard-Pomares et al., 2014). In PLP pKP12226, macrolide resistance genes are flanked by the tnpA6100 insertion sequence, sulphonamide and aminoglycoside resistance genes are located on transposon Tn3 and TEM-1 is flanked by the IS5075 insertion sequence (Shin and Ko, 2015). In pSLK172-1, the IS5075 insertion sequence flanks *floR, sul2, aac(3)-IV, fosA3* and CTX-M-14, while the Tn3 transposon flanks *aphA1, aadA1, aadA2, sul1, sul2* and *dfrA12* (Bai et al., 2017). Finally, PLPs that encode *mcr-1* are flanked by the composite transposon *ISAPI1* (Wang et al., 2018). This MGE flanks *mcr-1* on one side in pHYEC7-*mcr1,* and on both sides of *mcr-1* in PLPs pMCR-1-P3 and pSLK172-1 (Li et al., 2016; Snesrud et al., 2016; Bai et al., 2017; Liu et al., 2018). The presence of these IS and transposons highlights how MGEs play a pivotal role in the dissemination of antibiotic resistance via PLPs.

In addition to various antibiotic resistance genes, one PLP, pBtic235, encodes *terC, terD* and *terE* that confer tellurite resistance to its host (Table 2.2). Tellurite is an alloy used in the electronic industry as well as a secondary vulcanizing agent for rubber, and its industrial applications has led to an increase in environmental tellurite contamination and the emergence of tellurite resistant bacteria (Ram and Shanker, 2005; Chien et al., 2011; Grudén, 2013; Akhtar and Rehman, 2017). The tellurite resistance genes *terC, terD and terE* are three genes of a plasmid-encoded seven gene operon consisting of *terZ, terA, terB, terC, terD, terE*, and *terF* (Taylor, 1999; Kormutakova et al., 2000). Anantharaman et al., (2012) have demonstrated that only tellurite resistance genes *terB*, *terC, terD and terE* are essential to provide resistance to their host. Gillis *et al*., 2017 did not investigate the resistance of pBtic235 to potassium tellurite, thus, it is unknown whether these three genes conferred resistance to their host.

2.6.4. Mechanism of cyclization for lysogenization

Although the lysogeny mechanism of PLPs has not been investigated, it is hypothesized that they use the same mechanism as P1, the prototypic PLP (Figure 2.4). Upon infection, circularization occurs by a site-specific recombination system that consists of a 34-bp DNA site, *lox*P, at which recombination occurs by facilitation of the cre recombinase (Hamilton and Abremski, 1984; Sternberg et al., 1986; Łobocka et al., 2004). The *lox*P sites are located in the terminally redundant region of the phage P1 genome and consist of two 13-bp regions of IR, which once recombined together, are separated by an 8-bp region that together forms the 34-bp *lox*P site (Hoess et al., 1982). Once Cre recognizes the *lox*P sites, these termini on the linear genome circularize to form a circular genome (Sternberg et al., 1986). As many as 10 monomers of cre bind the *loxP* sites at either end of the genome in anticipation of their alignment for recombination during cyclization to form one *loxP* site of 34 bp (Hamilton and Abremski, 1984).

The *cre* gene is located downstream of the *cra* gene, whose function is unknown. It is hypothesized that *cra* may contain 2 *cre* promoters, P1*cre* and P2*cre*, or that these promoters may flank *cra* with a third promoter, P3*cre.* There is also a fourth promoter, P4*cre*, which is located 500 bp upstream of *cre* and is believed to be the most important promoter for *cre* regulation (Łobocka et al., 2004). In addition to cyclization, the *cre-lox* system is also important for segregation of low-copy PLPs into daughter cells by resolving dimeric P1 plasmids into two P1 monomers. The dimer is formed by P1 replication and homologous recombination, resulting in a dimer that is physically linked within two daughter molecules. Recombination between the two *lox*P sites restores the P1 dimer into two circular monomers so that they may be partitioned into separate daughter cells (Sternberg et al., 1986). The presence of a cre-lox system has not been investigated within the SSU5-like PLPs.

2.6.5 Origin of PLPs

The phage/plasmid nature of PLPs has led to questions on how the hybrid mobile genetic element was formed. Two ideas have been proposed for the origin of SSU5-like PLPs. First, Kim *et al.,* (2012) proposed that the SSU5-like PLP pHCM2 might have evolved from phage SSU5 because of its high nucleotide sequence identity to the virulent SSU5 phage. On the other hand, Octavia *et al.,* (2015) proposed that SSU5 stems from a primordial PLP form that potentially

arose from gene deletions in the plasmid region as well as the integrase gene. This hypothesis is based on the fact that SSU5-like PLPs contain 22 open-reading frames (ORFs) that are absent in phage SSU5.

There are no theories in the in regard to the origin of P1-like PLPs, however, Coren et al. (1995) reported that phage P1 could package multiple plasmids into its head with sizes ranging from 14 kb to 18 kb. This finding supports the notion that recombination events between P1 and the packaged plasmids could therefore potentially occur and lead to the generation of PLPs.

In contrast, PLPs might have arisen from a recombination event between a plasmid and a prophage. Kreuzer and Kreuzer (1994), demonstrated that plasmid pKK032, a derivative of plasmid pBR322, could integrate by homologous recombination into the genome of phage T4. On the other hand, since phages use bacterial attachment sites (*attB*) for integration into the host chromosome, they could integrate into extrachromosomal plasmids if their *attB* site is present. Pogue-Geile *et al.,* (1980), demonstrated that phage λ could integrate into pKPG10, which contains the phage λ's secondary attachment site, *att*. When the phage λ's main bacterial attachment site, *attλ*, is absent from the host, the phage can use secondary attachments sites that have 15-bp long similarities with the *attP* site on the phage λ genome. Furthermore, the authors demonstrated that more than one *att* site on the plasmid could recombine with the phage λ DNA generating different recombinants (Pogue-Geile et al., 1980). This hypothesis is further supported by Galetti et al., (2019) who reported that bioinformatics analysis strongly suggests that the PLP pBH6::Phage BH9 was formed by the integration of phage BH9 into plasmid pBH6. Phage BH9 is a Mu-like phage that is known to integrate via a transposition mechanism (Cazares et al., 2014). The transposition binding sites of the phage flank the insertion site on the pBH6 backbone, strongly suggesting the insertion of the phage into the plasmid (Galetti et al., 2019).

2.7. Summary:

The overprescription of antibiotics, their subtherapeutic use in agriculture, aquaculture and animal farming as growth promoters in North America and the use of clinically important antibiotics for human medicine in animal farming, is contributing to the emergence of antibiotic resistance and is limiting treatment options. Under the selective pressure caused by the subtherapeutic use of antibiotics, bacteria have developed resistance mechanisms such as point mutations, overexpression of efflux pumps, enzymatic alteration or complete replacement of

antibiotic target sites and the direct enzymatic modification of the antibiotic itself. The use of copper sulphate and zinc as growth promoters in Europe as well as copper sulphate as pesticides and antifouling agents on aquaculture cages in North America also contribute to antibiotic resistance because bacteria resist these metal ions by similar mechanisms that bacteria resist antibiotics including co-selection and cross-resistance. Mobile genetic elements (MGEs) play a pivotal role in AR and heavy metal resistance by co-selecting and mobilizing ARGs and heavy metals resistance genes to bacteria. Phage-like plasmids (PLPs) represent another type of MGE that are of significance because they carry ARGs of clinical importance such as carpabenemases and the colistin resistance determinant *mcr-1*. Due to their phage/plasmid hybrid nature, PLPs may have the potential to spread AR and heavy metal resistance genes to bacteria of foodborne importance by plasmid-mediated mechanisms, including conjugation and transformation, as well as by transduction. Phage-like plasmids may represent the first MGE with the ability to transfer ARGs and heavy metal resistance genes via all three mechanisms of horizontal gene transfer to bacteria of foodborne importance. Thus, chapter III and IV will focus on the genomic and phenotypic characterization of PLPs from bacteria of foodborne importance and determine the methods by which they disseminate AR and heavy metal resistance.

Table 2.1: Phenotypic characteristics of the phage-like plasmids (PLPs) reported in the literature

Name	Host	Size (kb)	Genome	Inducible	Phage Family	References
pBtic235	*Bacillus thurigiensis*	235	Circular	yes	Possible Myoviridae	(Gillis et al., 2017)
pSTM_Φ	*Salmonella* Typhi	107.7	Circular	unknown	unknown	(Octavia et al., 2015a)
AnCo1	*Escherichia coli* 243	112.2	Circular	yes	unknown	(Colavecchio et al., 2017d)
AnCo2	*Escherichia coli* 244	109	Circular	yes	unknown	(Colavecchio et al., 2017d)
AnCo3	*Salmonella* Derby	106	Circular	yes	unknown	(Colavecchio et al., 2017c)
SJ46	*Salmonella* Derby, *Salmonella* Indiana	103.4	Circular	unknown	Myoviridae	(Yang et al., 2017)
pHYEC7-*mcr1*	*Escherichia coli* HYEC7	97.5	Circular	unknown	unknown	(Li et al., 2016)
p-MCR-1-P3	*Escherichia coli* EMP163	97.3	Circular	unknown	unknown	(Zhang et al., 2017)
RCS47	*Escherichia coli*	115	Circular	yes	Myoviridae	(Billard-Pomares et al., 2014)
pECOH89	*Escherichia coli* H89	111.7	Circular	unknown	unknown	(Falgenhauer et al., 2014)
pABTJ2	*Acinobacter baumanii*	110.9	Circular	no	unknown	(Huang et al., 2014)
vB_BceS-IEBH	*Bacillus cereus* CD555	53.1	Circular	yes	Siphoviridae	(Smeesters et al., 2011)
pHCM2	*Salmonella* Typhi	106.5	Circular	unknown	unknown	(Kidgell et al., 2002)
pSLK172-1	*Escherichia coli*	369.2	circular	unknown	unknown	(Bai et al., 2017)
PVv01 (p48/10)	*Vibrio vulnificus*	79.2	circular	yes	Myoviridae	(Hammerl et al., 2014)

Table 2.1 continued: Phenotypic characteristics of the phage-like plasmids (PLPs) reported in the literature

Name	Host	Size (kb)	Genome	Inducible	Phage Family	References
pKP12226	*Klebsiella pneumonaie*	267.6	circular	unknown	unknown	(Shin and Ko, 2015)
KHP30	*Helicobacter pylori*	26.2	circular	yes	Podoviridae	(Uchiyama et al., 2013b)
PhiBU01	*Staphylococcus aureus*	43.8	circular	yes	Siphoviridae	(Utter et al., 2014)
pKPHS1	*Klebsiella pneumonaie*	122.7	circular	unknown	unknown	(Liu et al., 2012)
pMT1	*Yersinia pestis*	100.9	circular	unknown	unknown	(Lindler et al., 1998)
pMCR_SCKP-LL83	*Klebsiella pneumonaie*	97.4	circular	yes	unknown	(Liu et al., 2018)
pBH6::Phage BH9	*Pseudomonas aeruginosa*	41	circular	unknown	unknown	(Galetti et al., 2019)

Table 2.2: Genomic characteristics of the phage-like plasmids (PLPs) reported in the literature.

Plasmid-like phage	Tyrosine recombinase	tRNA	Mobile elements	Plasmid typing	Resistance genes	References
pBtic235	none	1	none	unknown	Tellurium resistance (*terE*, *terC*, *terD*)	(Gillis et al., 2017)
pSTM_Φ	integrase	2	unspecified	unknown	none	(Octavia et al., 2015a)
AnCo1	integrase	3	*ICEcp1*	unknown	CTX-M-15	(Colavecchio et al., 2017d)
AnCo2	integrase	none	*ICEcp1*	unknown	CTX-M-15	(Colavecchio et al., 2017d)
AnCo3	integrase	1	none	unknown	none	(Colavecchio et al., 2017c)
SJ46	cre	3	Tn1721 containing *ISEcpIB*	untypeable	CTX-M-27	(Yang et al., 2017)
pHYEC7-*mcr1*	cre	none	Tn6330 containing *ISApl1*	IncY	*mcr-1*	(Li et al., 2016)
p-MCR-1-P3	cre	unknown	ISApl1 (2 copies)	IncY	*mcr-1*	(Zhang et al., 2017)
RCS47	cre	none	IS5 (2 copies), IS1	unknown	SHV-2	(Billard-Pomares et al., 2014)
pECOH89	integrase	2	*ICEcp1*	Untypeable	CTX-M-15	(Falgenhauer et al., 2014)
pABTJ2	integrase	1	none	unknown	none	(Huang et al., 2014)
vB_BceS-IEBH	unknown	unkown	Yes, not specified	unknown	none	(Smeesters et al., 2011)

Table 2.2 continued: Genomic characteristics of the phage-like plasmids (PLPs) reported in the literature.

Plasmid-like phage	Tyrosine recombinase	tRNA	Mobile genetic elements	Plasmid typing	Resistance genes	References
pHCM2	integrase	1	none	unknown	none	(Kidgell et al., 2002)
pSLK172-1	cre	none	*ISApl1* (2 copies), Tn3, Tn21	Multi-replicon types HI2, HI2A,Y and N	mcr-1, sul1, aphA1, sul3, aadA1, aadA2, dfrA12, floR, sul2, aac(3)-IV, blaCTX-M-14, fosA3	(Bai et al., 2017)
PVv01 (p48/10)	cre	unknown	unknown	unknown	none	(Hammerl et al., 2014)
pKP12226	cre	unknown	IS26-ISEcp1 tnpA6100 IS5075 Tn3 IS1	unknown	CTX-M-15 *mph*(A), *mrx*, *mph*(R), *chrA, sul1, qacEdelta1, aadA4, dfrA17* TEM-1 *dfrA17*	(Shin and Ko, 2015)
KHP30	integrase	unknown	unknown	unknown	none	(Uchiyama et al., 2013b)
PhiBU01	integrase	none	Yes, not specified	unknown	none	(Utter et al., 2014)

Table 2.2 continued: Genomic characteristics of the phage-like plasmids (PLPs) reported in the literature.

Plasmid-like phage	Tyrosine recombinase	tRNA	Mobile elements	Plasmid typing	Resistance genes	References
pKPHS1	integrase	unknown	yes	unknown	CTX-M-14	(Liu et al., 2012)
pMT1	integrase	none	Yes IS200 IS100	unknown	Murine toxin	(Lindler et al., 1998)
pMCR_SCKP-LL83	integrase	3	*ISApl1*	pO111	*mcr-1*	(Liu et al., 2018)
pBH6::Phage BH9	integrase	none	*Tn3*	unknown	*KPC-2*	(Galetti et al., 2019)

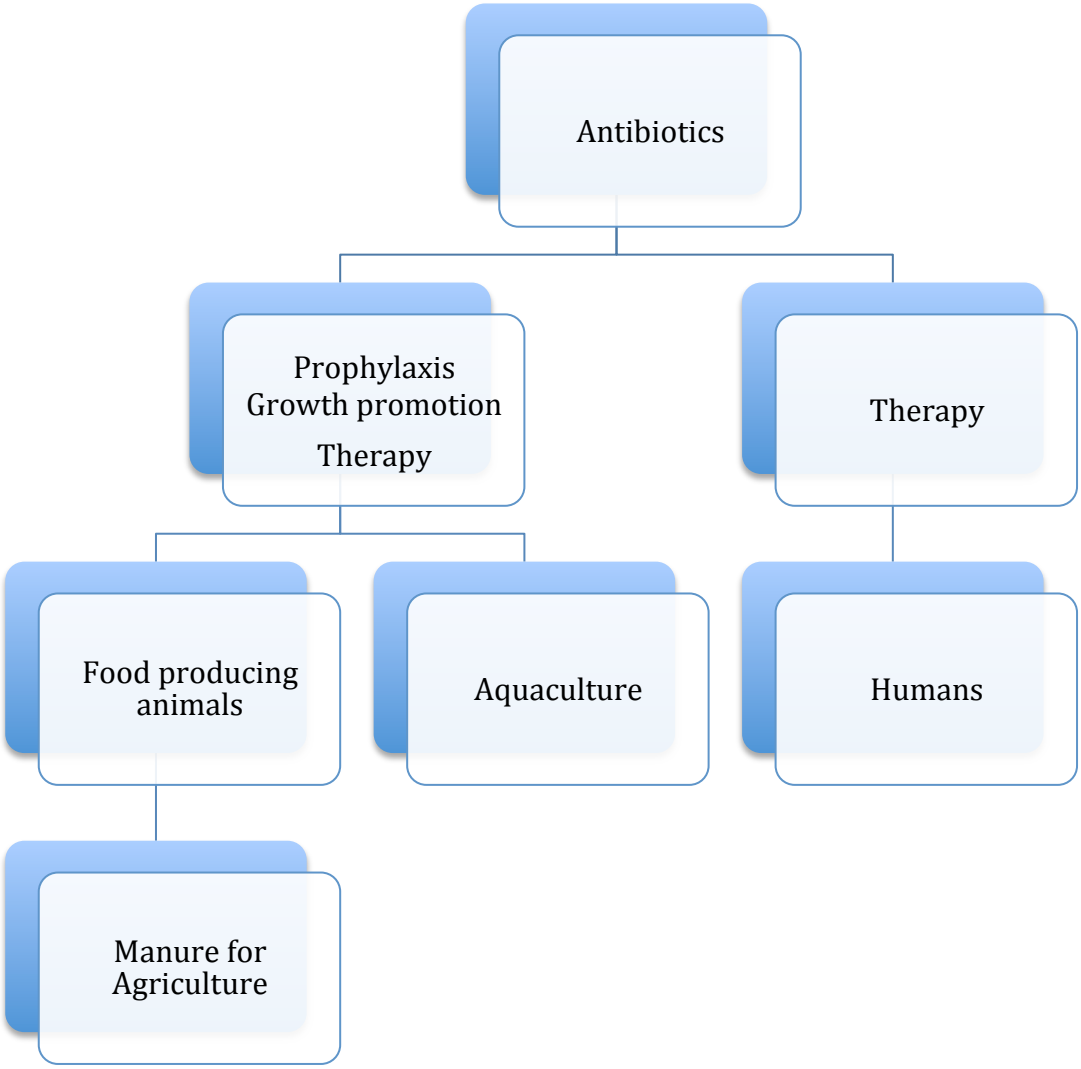

Figure 1.1. Antibiotic consumption in humans and food-producing animals and fish. Antibiotics are used to treat clinical bacterial infections in humans and food-producing animals. In Canada and the USA, antibiotics are also used to promote growth promotion of food-producing animals and aquaculture fish. The feces of food-producing animals is composted and then used as manure to fertilize agricultural soils to grow fresh produce.

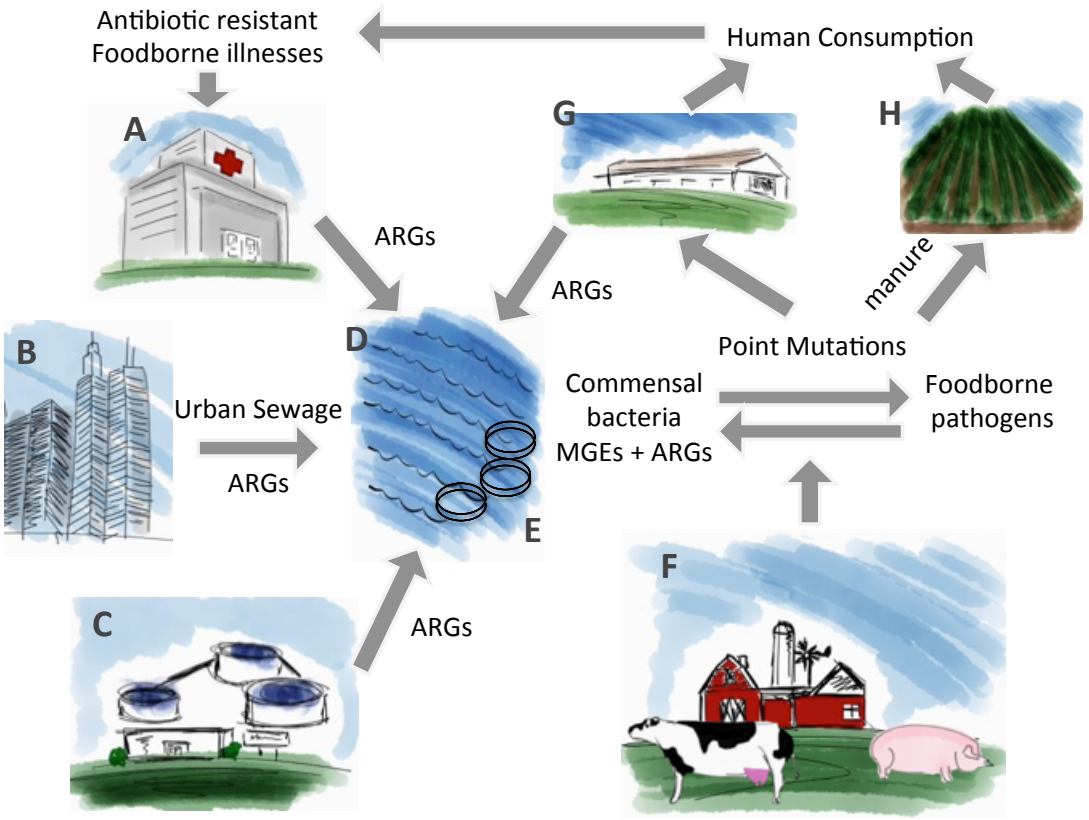

Figure 2.2. Cycle for the dissemination of antibiotic resistance genes (ARGs) from the environment to human consumption. (A) Hospital effluents; (B) Urban sewage; (C) Wastewater treatment plant effluents; (D) Bodies of water such as lakes and oceans; (E) Aquaculture; (F) Food producing animals; (G) Slaughter house; (H) Manure Application. Adapted from (Muniesa et al., 2013).

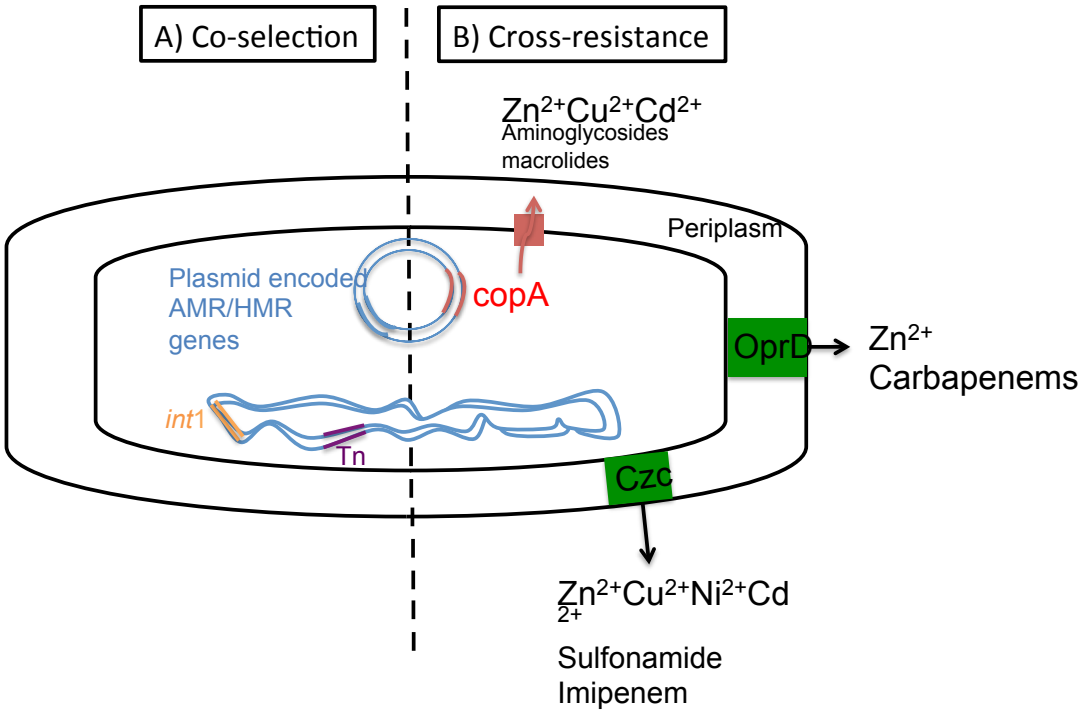

Figure 2.3. Co-selection and cross-resistance mechanisms used by bacteria to resist heavy metal ions and antibiotics. A) Co-selection of HMR and ARGs can be mobilized on a plasmid (blue), on class 1 integrons (int1), or by a transposon (Tn). B) Cross-resistance is mainly facilitated by efflux pumps. Czc is one of the main efflux pumps responsible for the transport of various heavy metal ions and antibiotics and belongs to resistance-nodulation-cell division (RND) and cation diffusion facilitators (CDF) families. OprD is a porin involved in the cross-resistance of zinc and carbapenems. *copA* is a plasmid-encoded gene that uses ATP to transport heavy metal ions and antibiotics from the cytoplasm to the periplasm.

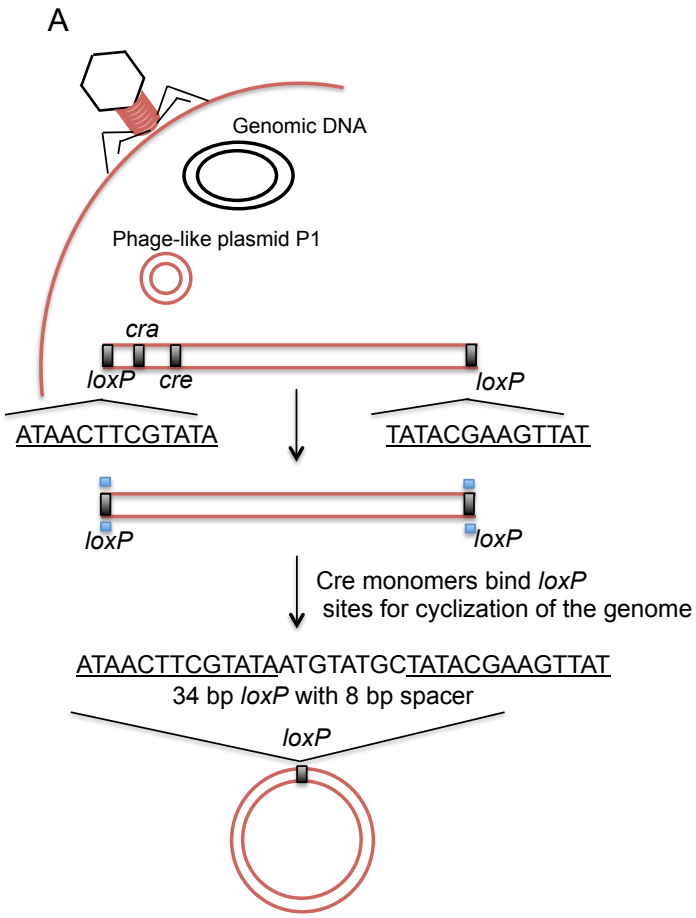

Figure 2.4. The lysogeny mechanism of prototypic phage-like plasmid P1.
On either end of the linear P1 genome are 13-bp *loxP* sites, which the P1 integrase gene, *cre*, will recognition upon P1 host infection. *cre* monomers recognize each loxP sequence and cre initiates a site-specific recombination reaction that cyclizes the P1 genome, which recombines both *loxP* sequence to form a 34-bp *lox* sequence.

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Connecting text

In the previous chapter, a comprehensive review of scientific literature was performed, providing an overview of the bacterial resistance mechanisms to antibiotics, horizontal gene transfer (HGT) mechanisms, the types of mobile genetic elements (MGEs) that carry antibiotic resistance genes (ARGs) and heavy metal resistance (HMR) genes, and the contribution of human medicine, agriculture, animal production, and aquaculture on antibiotic resistance (AR) worldwide. This review also revealed the importance of phage-like plasmids (PLPs), an emerging MGE, as a potential contributor to the spread of AR. Thus, in the present study, PLPs were identified within different bacterial isolates (from food animals and various food sources) and whole genome sequences (WGS), present in public databases (Genbank), were also analyzed for the presence of PLPs.

Chapter III:

Genomic Characterization of Phage-Like Plasmids and Their Potential Role in Antibiotic Resistance

3.0 Abstract

Due to the misuse of antibiotics in agriculture, the environment, and animal and human medicine, antibiotic resistance (AR) has emerged as a major health concern worldwide, and threatens food security as well as human and animal health. Mobile genetic elements (MGEs) including plasmids, transposons, and phages contribute to the spread of antibiotic resistance genes (ARGs), and have the ability to form hybrid elements within the chromosomes and plasmids of foodborne bacteria. Phage-like plasmids (PLPs) are phage/plasmid hybrids that exist in a circular extrachromosomal form within bacteria. Phage-like plasmids (PLPs) are inducible and harbour antibiotic resistance genes (ARGs) and/or heavy metal resistance (HMR) genes and may contribute to the spread of AR.

This study involved the genomic characterization of 8 PLPs that were identified in bovine isolates as well as a food sample (oysters). Additionally, 49 PLPs, identified in whole genome sequences (WGS) obtained from the scientific literature and public databases (Genbank), were also characterized. The objective in genomically characterizing these PLPs was to determine their characteristics in terms of carrying ARGs, heavy metal resistance (HMR) genes and MGEs, their taxonomy, and ultimately their potential contribution to AR.

Phage-like plasmids (PLPs) were observed to be present in 12 different bacterial species. Seventeen PLPs conferred AR resistance to β-lactams, carbapenems, polymyxins, and aminoglycosides. Six PLPs conferred resistance to either mercury or tellurite, which is significant due to co-selection mechanisms. MGEs including insertion sequences (IS), transposons, and transposases flank the ARGs and HMR genes in 76% of the PLPs harbouring these resistance genes. In addition, 75% of PLPs were typeable with the two major replicon types being IncFIB and IncY. Genomic analysis also suggested that PLPs represent a novel class of temperate phages that could be classified into two phage lineages: SSU5-like and P1-like. Based on their worldwide dissemination, the presence of ARGs flanked by MGEs and co-selection

mechanisms of HMR, these results collectively demonstrate the potential contribution of PLPs to the spread of AR.

3.1 Introduction:

Temperate phages, such as the prototypical phage lambda, are traditionally known to integrate their genome into the bacterial host chromosome (Casjens and Hendrix, 2015). Temperate phages also exist as low copy extrachromosomal plasmids that can be either circular, such as phage P1, or linear, such as N15 (Łobocka et al., 2004; Ravin, 2011). Phage-like plasmids (PLPs) are defined as phage/plasmid hybrids that exist in a circular extrachromosomal form. Linear extrachromosomal phages have been extensively studied in the literature and have been termed as plasmid-like phages (Alton and Vapnek, 1979; Inal and Karunakaran, 1996; Rybchin and Svarchevsky, 1999; Hertwig et al., 2003; Strömsten et al., 2003; Verheust et al., 2003; Casjens et al., 2004; Verheust et al., 2005; Thomas et al., 2007; Mobberley et al., 2008; Sozhamannan et al., 2008; Lan et al., 2009; Zabala et al., 2009; Villa et al., 2012; Kan et al., 2013; Vörös et al., 2013; Dziewit and Radlinska, 2016). There is a knowledge gap in the literature concerning the differences between phage-like plasmids (PLPs) and plasmid-like phages (Table 3.1). For example, while plasmid-like phages have been studied since the 1970's, phage-like plasmids have only been extensively studied since 2011 (Alton and Vapnek, 1979; Octavia et al., 2015b).

Phage-like plasmids (PLPs) are characterized by dsDNA genomes that contain structural phage genes as well as plasmid DNA. There are currently 21 PLPs reported in the literature, isolated from both Gram-negative and Gram-positive bacterial species including *Bacillus sp., Salmonella enterica, Escherichia coli, Acinetobacter sp., Vibrio sp., Helicobacter sp, Staphylococcus sp., Klebsiella sp.,* and *Yersinia sp.* (Alton and Vapnek, 1979; Lindler et al., 1998; Kidgell et al., 2002; Smeesters et al., 2011; Liu et al., 2012; Uchiyama et al., 2013b; Billard-Pomares et al., 2014; Falgenhauer et al., 2014; Hammerl et al., 2014; Huang et al., 2014; Utter et al., 2014; Octavia et al., 2015a; Shin and Ko, 2015; Li et al., 2016; Bai et al., 2017; Colavecchio et al., 2017c; Colavecchio et al., 2017d; Gillis et al., 2017; Yang et al., 2017; Zhang et al., 2017; Liu et al., 2018). Phage-like plasmids (PLPs) differ from plasmid-like phages in their replication and acquisition of AR and HMR genes. In contrast to PLPs that enter their host and circularize their genome, plasmid-like phages will adopt a linear form, either by covalently closed hairpin ends (N15-like) or through terminal inverted repeats (Tectiviral-like) (Savilahti and Bamford, 1993; Ravin, 2003).

A preliminary analysis demonstrates that various PLPs harbour ARGs and HMR genes, while plasmid-like phages do not. Phages are known to contribute to the dissemination of AR given their ubiquity and abundance. Moreover, evidence suggests that they contribute to AR in both Shiga toxin-producing *Escherichia coli* (STEC) and nontyphoidal *Salmonella* (NTS) via transduction (Colavecchio et al., 2017a). Since PLPs harbour ARGs and HMR genes, have various MGEs, are inducible, and can be transferred to other hosts via horizontal gene transfer (HGT) mechanisms, it is hypothesized that they may also contribute to the spread of AR among bacteria. In an effort to better understand the scope of the recent emergence of PLPs and mechanisms to reduce AR, the characterization of PLPs is an important step towards developing strategies to control their dissemination. Hence, the objective of our study was to genomically characterize PLPs to determine the types of resistance genes (AR and HMR) and MGEs they harbour, as well as their taxonomic structure.

3.2 Materials and Methods:

3.2.1 Bacterial isolates and growth conditions

The bacterial isolates used in this work came from the culture collections of Dr. Lawrence Goodridge (McGill University, Montréal, Québec, Canada), Dr. Séamus Fanning (University College Dublin, Dublin, Ireland), and the *Salmonella* Foodborne Syst-OMICS Database (SalFoS), which can be accessed at https://salfos.ibis.ulaval.ca. *E. coli* 243 and *E. coli* 244 were isolated from the feces of wildlife in bovine feedlots in Colorado, USA (Table 3.2). *Salmonella* Kouka was isolated from oysters in Ottawa, ON, Canada. *E. coli* XX-29735, *E. coli* XX-29747, and *E. coli* XX-29728 are bovine mastitis isolates from France, while *E. coli* XX-22725 is a bovine diarrhoea isolate from France. Isolates were maintained at –80°C in glycerol, and were revived by streaking the frozen culture on modified Luria-Bertani agar (LBA), followed by incubation at 37°C in modified Luria-Bertani broth (LBB) with orbital shaking at 225 rpm for 16 hours as follows: *E. coli* 243 and *E. coli* 244 were grown on LBA and in LBB supplemented with 1 mg/ml of cefotaxime sodium salt (Sigma-Aldrich, St-Louis, Missouri, USA). *S.* Kouka was grown on LBA and in LBB supplemented with 50 µg/ml of

mercury chloride (Sigma-Aldrich, USA). For all *E.* coli isolates from France, LBA and LBB were supplemented with 30 µg/ml of potassium tellurite (Sigma-Aldrich, USA).

3.2.2 Whole genome sequencing (WGS) and assembly

Whole genome sequencing of *E. coli* 243, *E. coli* 244, and *S.* Kouka was performed using the EcoGenomics Analysis Platform (Institute of Integrative Biology and Systems (IBIS), Université Laval, Québec City, QC) on an Illumina® MiSeq sequencer using 300-bp paired-end libraries with 40X coverage. The raw reads were assembled using the A5 pipeline (Tritt et al., 2012). WGS of *E. coli* XX-29725, *E. coli* XX-29735, *E. coli* XX-29728, and *E. coli* XX-29747 was performed at the Center for Food Safety at University College Dublin (UCD) (Dublin, Ireland). Libraries were prepared using the Nextera XT DNA Library Prep Kit (Illumina®), sequenced on the MiSeq platform (Illumina®) using 300 bp paired-end libraries, and *de novo* assembly was performed by SPAdes (Bankevich et al., 2012).

3.2.2 Phage-like Plasmid (PLP) detection

Fifty-seven PLPs were analyzed in this study. The WGS sequences of 18 PLPs were retrieved from GenBank based on their accession numbers from publications (Table 3.3). Thirty-one PLPs were identified by performing a BLAST of genes that define a PLP (*repA, parA, parB*, integrase, terminaseA) against 9116 bacterial WGS in GenBank (Table 3.4) (Altschul et al., 1990). In addition, a BLAST of PLP genes mentioned above was also performed to confirm the presence of PLPs within the WGS from bovine and food sources (Table 3.2). Intact phage regions of 39 PLPs (31 PLPs from Genbank and 8 from bovine and food isolates) were confirmed using PHASTER (Arndt et al., 2016). Only prophages identified as “complete” or “intact” were considered for further analysis.

3.2.3 Genomic analysis

Eighteen PLPs (Table 3.3) that were identified from the scientific literature had been previously analyzed and annotated. The remaining 39 PLPs (31 from WGS in Genbank and 8

PLPs from bovine and food isolates) were annotated using PATRIC (Wattam et al., 2018). ResFinder and CARD databases were used to identify ARGs, while BacMet databases were used to identify HMR genes (McArthur et al., 2013; Pal et al., 2013; Kleinheinz et al., 2014). The ACLAME database identified MGEs flanking ARGs and HMR determinants (Leplae et al., 2004). PCR-based replicon typing was performed for 18 replicon types using the Sequence Manipulation Suite's PCR product tool (Stothard P, 2000) (Carattoli et al., 2005). Analysis showed that 52 PLPs in study demonstrated nucleotide sequence identity with two phages, SSU5 and P1. Thus, BLAST Ring Image Generator (BRIG) and EasyFig were used to perform whole genome comparisons of the PLPs to phages SSU5 and P1 (Alikhan et al., 2011; Sullivan et al., 2011).

3.2.4 Phylogenetic tree construction

A maximum-likelihood phylogenetic tree of PLP integrase genes was constructed based on a phylogenetic tree previously described by Balding *et al*., (2005). Briefly, these authors designed a PCR prophage integrase assay that consisted of 11 degenerate primer sets by aligning the conserved regions, designated as "box I" and "box II", of the tyrosine integrases of 32 enteric prophages encoded by members of the *Enterobacteriaceae* family. The two conserved regions are located in the C-terminal of the tyrosine integrase and consist of residues A202–G227 ("box I") and T206–D344 ("box II") in the lambda prophage. Based on this premise, to identify "box I" and "box II" regions in the PLP integrase genes, MUSCLE was used to perform an alignment of the amino acid sequences of the integrase genes of all 57 PLPs in this study against the amino acid sequence of the lambda phage integrase gene (Edgar, 2004; Balding et al., 2005). A maximum-likelihood phylogenetic tree of the conserved regions of the integrase genes of the 57 PLPs analyzed in this study and the 32 enteric prophages from Balding *et al*., (2005) was constructed using MEGA7 software with bootstrap analysis of 500 replicates. The resulting phylogenetic tree was edited using the interactive tool of life (iTOL) (Letunic and Bork, 2006; Kumar et al., 2016).

3.3 Results:

3.3.1 Phage-like plasmids (PLPs) represent their own class of temperate phages

To investigate the nucleotide sequence identity of the PLPs, a nucleotide BLAST was performed for each PLP. Of the 57 PLPs included in this study, 52 demonstrated nucleotide sequence identity to either prototypical phage-like plasmid P1 or phage SSU5. As described in chapter II (Section 2.5), phage SSU5 is a virulent *Salmonella* phage that infects rough strains of *S.* Typhimurium and phage P1 is the prototypical PLP. Using BRIG, WGS comparisons of the PLPs were performed to either phage SSU5 (3.1A) or phage P1 (Figure 3.1B), and determined that 27 PLPs and 26 PLPs showed nucleotide sequence identity to phage SSU5 and phage P1, respectively. Four PLPs (pBtic235, KHP30, PhiBU01, and vB_BceS-IEBH) did not show nucleotide sequence identity to either phage. To further investigate the homology of PLPs to phage SSU5 and phage P1, the integrase gene of the PLPs was studied, as it has previously been described as a signature gene of temperate phages and an indicator of genome diversity (Colavecchio et al., 2017b). Thus, a maximum-likelihood phylogenetic tree was constructed based on the integrase genes of 57 PLPs analyzed in this study and 32 enteric temperate phages previously described by Balding *et al.*, (2005) (Figure 3.2). Of note, the integrase gene of vB_BceS-IEBH could not been identified; therefore, this PLP is not represented in the phylogenetic tree (Smeesters et al., 2011). The resulting phylogenetic tree demonstrated that PLPs cluster separately from the 32 enteric temperate phages, suggesting that they represent their own class of temperate phages. Furthermore, SSU5-like PLPs and P1-like PLPs cluster within their own branches suggesting the existence of two lineages of PLPs.

3.3.2 Genomic characterization of 8 phage-like plasmids (PLPs) isolated from farm-to-fork

Of the 57 PLPs, 8 were isolated from bovine feedlots, bovine feces, a clinical sample, and oysters, and their WGS were analyzed genomically. Of these, AnCo1, AnCo2, and AnCo3 were found to be SSU5-like PLPs. The genome size of AnCo1 is 112.2 kb and harbours 134 coding sequences (CDS), while the genome size of AnCo2 is 109 kb and harbours 132 CDS (Colavecchio et al., 2017d). Both PLPs are highly similar, as they share 127 CDS with 100%

nucleotide sequence identity. In comparison to AnCo2, AnCo1 encodes 7 different CDS, mainly hypothetical proteins. Phage-like plasmid AnCo3 shares 84 CDS of 134 CDS and 132 CDS to AnCo1 and AnCo2, respectively, with >68% nucleotide sequence identity. Phage-like plasmid AnCo3 is 105.9 kb and harbors 130 CDS (Colavecchio et al., 2017c).

Genomic analysis revealed that AnCo1, AnCo2, and AnCo3 share 92 CDS, 91 CDS, and 97 CDS of the 130 CDS in SSU5, respectively, with >67% nucleotide sequence identity (Figure 3.3). Phage-like plasmids AnCo1 and AnCo2 also share 122 CDS and 117 CDS of the 128 CDS to PLP pECOH89, respectively, with >91% nucleotide sequence identity (Colavecchio et al., 2017d). While AnCo3 shares 114 of 130 CDS to PLP pSTM_Phi, of which 96 CDS have >95% nucleotide sequence identity and 18 CDS have >75% nucleotide sequence identity (Colavecchio et al., 2017c).

Phage-like plasmids SJ1, MA725, JEC735, SN747, and DH728 were found to be P1-like PLPs. Phage-like plasmid SJ1, with a genome size of 24.5 Kb, has the smallest genome size of a PLP to date. Having 39 CDS, SJ1 shares 20 of 110 CDS in PLP P1, with >90% nucleotide sequence identity; thus making it a mini-P1 PLP (Figure 3.4) (Pal et al., 1986). Phage-like plasmids MA725, JEC735, SN747, and DH728 range from 92.2 Kb to 103 Kb, and all share >82 of 110 CDS in PLP P1, with >90% nucleotide sequence identity (Figure 3.5). Phage-like plasmids MA725, JEC735, and DH728 share the most nucleotide sequence identity to each other with 82 CDS having >80% nucleotide sequence identity). Phage-like plasmid SN747 is significantly different from the other P1-like PLPs, as it contains a unique insertion region of 39 CDS for plasmid-like genes that may be mediated by IS2 or IS911.

Compared to AnCo1 and AnCo2, the P1-like PLPs contain toxin-antitoxin systems and restriction modification systems to ensure PLP maintenance during host cell division and prevention of host killing during P1-like PLP lysogenization, respectively (Łobocka et al., 2004). Phage-like plasmid SJ1 harbours a toxin anti-toxin system, phd/doc, and an incomplete Type-1 restriction modification (R-M) system consisting only of subunit M. The three subunits of a type 1 R-M systems include HsdR (host specificity for DNA, restriction), HsdM (modification or methylation), and HsdS (specificity) and they are dependent on each other, therefore, the SJ1 R-M subunit isn't functional (Bourniquel and Bickle, 2002). However, three of the above PLPs (MA725, DH728 and SN747) carry an identical Type-1 R-M system containing all 3 subunits.

Collectively these results demonstrate that PLPs have high nucleotide sequence identity to phage SSU5 and PLP P1, further strengthening the notion that two lineages of PLPs exist. Furthermore, these results also highlight PLP maintenance systems, thus demonstrating the ability of PLPs to maintain their presence within hosts.

3.3.3. Genomic characterization of 31 phage-like plasmids (PLPs) from public databases

In order to understand the geographical distribution of PLPs, a custom script was developed to BLAST PLP genes (*repA, parA, parB*, integrase, terminaseA) against 9118 bacterial WGS in GenBank. Based on this analysis, 31 PLPs were detected from this dataset, spanning 5 different bacterial genera that are all foodborne pathogens. The majority of these PLPs (25 of 31) were detected within *E. coli* (13 of 31) and *Klebsiella pneumoniae* (12 of 31), an emerging foodborne pathogen associated with retail meats, vegetables, and seafood (Davis et al., 2015; Davis and Price, 2016). Phage-like plasmids were detected within the genomes of bacterial isolates from 11 countries and 4 continents, highlighting their global dissemination (Table 3.5). The majority of these PLPs (16 of 25 PLPs for which a country was denoted) were from clinical samples. PLPs were also isolated in different biomes including the environment (river water and wildlife), at the farm level (bovine carcass, bovine mastitis, milk sampled from a farm), and food (lettuce).

The PLPs detected in GenBank ranged in size from 58.8–225.9 Kb; however, the majority (24 of 31) were between 90 Kb and 120 Kb. The 13 P1-like PLPs share >85 of 110 CDS in PLP P1, with >90% nucleotide sequence identity. In contrast, 15 of 18 SSU5-like PLPs share >92 of 130 CDS in phage SSU5, with >65% nucleotide sequence identity. Three SSU5-like PLPs, pSg_1, GCA_000007885.1, and GCA_000834825.1, demonstrated less nucleotide sequence identity to phage SSU5. Phage-like plasmid pSg1_1 shares only 74 of 130 CDS in phage SSU5, with >65% nucleotide sequence identity. In addition, approximately half of the CDS of GCA_000007885.1 (59 CDS) and GCA_000834825.1 (55 CDS) have >90% nucleotide sequence identity to SSU5.

Of the P1-like PLPs, all encode a toxin/antitoxin system (phd/doc), with the exception of pSJ_98 that only encodes doc, which is still functional alone (Liu et al., 2008). SSU5-like PLP pUCLAOXA232_4X carries YafO, a toxin encoded by a type II toxin/antitoxin system yafN-

yafO, generally found in *E. coli* genomes (Zhang et al., 2009). Furthermore, 13 PLPs encode non-functional type 1 restriction-modification (R-M) systems consisting only of subunit M.

Collectively, these results demonstrate that PLPs demonstrate nucleotide sequence identity to SSU5 and P1, are ubiquitous, and although they are not prevalent in Genbank (31 PLPs in 9116 bacterial WGS), they are present in environmental, agricultural, clinical and food samples.

3.3.4 Plasmid typing

Plasmid classification is an important consideration because of the role plasmids play in horizontal gene transfer (HGT) and AR. Seventy-five percent of PLPs (42 of 57) in this study were typeable based on the plasmid replicon typing scheme developed to identify 18 incompatibility groups within members of the *Enterobacteriaceae* family (Table 3.5) (Carattoli et al., 2005). The most prevalent plasmid type was IncFIB (19 of 57 PLPs), followed by IncY (17 of 57 PLPs). Studies have demonstrated that IncFIB plasmids are most prevalent in *K. pneumoniae* and *E. coli* isolates that carry extended-spectrum β-lactamases (ESBL) (Johnson et al., 2007; Ali et al., 2014; Shin et al., 2015). All P1-like PLPs were classified as IncY plasmid type, and this was in accordance with the replicon type of phage P1. Three PLPs (pMCR_SCKP-LL83, p_0111_2, and plasmid_unamed_2) were replicon type pO111, and pKP12226 and p1002_1 were replicon type IncFII. Finally, 2 PLPs (plasmid_2 and pSLK172-1) had multiple replicon types.

Twenty-five percent (15 of 57) of PLPs in this study were untypeable, as per the plasmid replicon-typing scheme by Carattoli *et al.,* (2005). Phage-like plasmids isolated from wildlife feces samples (AnCo1 and AnCo2), slaughterhouse samples (SJ46 and pSAN1_1735), the Baltic sea (pVv01), food samples (SJ1 and pCS1), and clinical samples (pECOH89, pABTJ2, KHP30, phiBU01, and pECAZ155_2) were untypeable. In addition, two PLPs (pBtic235 and vB_BceS-IEBH) that did not demonstrate nucleotide sequence identity to SSU5 or P1 were also untypeable.

3.3.5 Phage-like plasmids (PLPs) encode ARGs and HMR genes

Twenty-six percent (15 of 57) of PLPs encode ARGs and 10% (6 of 57) PLPs encode HMR genes. The majority of PLPs that harbour ARGs (11 of 15) encode ESBL determinants. For example, AnCo1, AnCo2, p1002_1, pU2501_39, pECOH89, and pKP12226 encode CTX-M-15, and pKPHS1 and pSLK172-1 encode CTX-M-14. These ARGs are the most predominant ESBL enzymes disseminated worldwide (Table 3.6) (Cantón et al., 2012). Moreover, AnCo1, AnCo2, and pECOH89 all share the CTX-M-15 resistance cassette consisting of a hypothetical protein and IS*Ecp1* transposase gene, commonly associated with CTX-M variants, with 100% nucleotide sequence identity (Cantón et al., 2012). Phage-like plasmids SJ46 and p11219_CTXM encode ESBL enzymes CTX-M-27 and CTX-M-28, respectively. Genomic analysis revealed CTX-M-27 is flanked by IS5 and CTX-M-28 is located on transposon Tn1721. These MGEs are not known to be specifically associated with these CTX-M variants, however, their presence suggests a possible mechanism by which these resistance genes may have mobilized to these PLPs. Phage-like plasmid RCS47 encodes SHV-2, a β-lactamase, that is flanked on either side by IS5. Furthermore, pCRKP_59_KPC encodes β-lactamase TEM-1 and also carbapenamase, KPC-2, which provides resistance to carbapenem treatment reserved for multidrug-resistant (MDR) bacterial infections.

Four PLPs (pMCR_SCKP-LL83, pHYEC7-*mcr1*, p-MCR-1-P3, and pSLK172-1) encode colistin resistance gene *mcr-1*, which confers resistance to polymyxin, a last resort antibiotic for Gram-negative infections. *ISAPI1,* a composite transposon demonstrated to play a pivotal role in the mobilization of *mcr-1*, flanks *mcr-1* on one side in pHYEC7-*mcr1* and pMCR_SCKP-LL83, and flanks both sides in pMCR-1-P3 and pSLK172-1. Furthermore, pKP12226 and pSLK172-1 encode 11 and 12 ARGs, respectively. In PLP pKP12226, the ARGs are located on transposon Tn3 flanked by insertion sequences IS5075 and IS1. Two PLPs, p1002_1 and pCRKP_59_KPC, also encode the aminoglycoside resistance genes *aadA5* and *aac(3)-lia*, respectively.

In addition to AR, 6 PLPs harbour either mercury or tellurite resistance genes. Phage-like plasmid SJ1 encodes a mercury resistance operon that is flanked by a MGE identified as transposon Tn21, which is commonly associated with broad-spectrum mercury resistance operons (Liebert et al., 1999). These operons are comprised of 3 mercury transport genes (*merC*, *merE*, and *merT*), a mercury ion reductase, a periplasmic mercury binding protein, and a mercury

resistance coregulator. The PLPs MA725, JEC735, SN747, and DH728 harbour a tellurite resistance gene, *terB*, which is flanked by tRNA-Met, while pBTIC235 encodes *terD, terC*, and *terE.*

3.4 Discussion:

The objective of this study was to genomically characterize PLPs to determine their taxonomic structure, the type of resistance genes (AR and HMR) and MGEs they harbour, and their potential contribution to AR. To investigate their taxonomic structure, a maximum-likelihood phylogenetic tree using the integrase gene of PLPs was constructed, and demonstrated that the majority of PLPs (52 of 57 PLPs) clustered into two lineages, either SSU5-like or P1-like (Figure 3.2). The integrase genes of 5 PLPs (pBtic235, phiBU01, KHP30, vB_BceS-IEBH, and pABTJ2) reported in the scientific literature did not cluster within these two lineages. Currently, there is a literature gap concerning the characteristics that define a PLP. The phage/plasmid hybrid characteristics of the PLPs are achieved by encoding various phage genes that are essential for PLPs to form functional virions, as well as essential plasmid genes for maintenance and incorporation into daughter cells (Łobocka et al., 2004). Genomic analysis demonstrated that 56 PLPs in this study, with the exception of vB_Bces-IEBH, encode an integrase gene for the cyclization of the PLP genome upon entry to its host, and all 57 PLPs in this study encode the terminase A gene for genome packaging into the procapsid head. As for the plasmid genes, all PLPs with the exception of 4 PLPs (pBtic235, phiBU01, KHP30, and vB_BceS-IEBH), encode essential plasmid genes *parA*, *parB*, and *repA*. The *repA* gene is the origin and initiator of PLP genome replication, while *parA* and *parB* genes facilitate partitioning of the PLP genome during bacterial cell division. These findings suggest that the presence of the integrase, terminase A, *repA, parA* and *parB* genes define a PLP.

Although the scientific literature described PLPs pBtic235, phiBU01, KHP30, and vB_BceS-IEBH as extrachromosomal phage/plasmid hybrids, they do not contain any coding sequences (CDS) with nucleotide sequence identity to essential PLP genes *repA*, *parA*, and *parB*. This, therefore, suggests that pBtic235, phiBU01, KHP30, and vB_BceS-IEBH are not PLPs. On the other hand, pABTJ2 contains essential PLP genes, and its phage portion (11 CDS) shares >66% nucleotide sequence identity to phage SSU5, yet it did not cluster with the SSU5-like

PLPs in the integrase phylogenetic tree (Huang et al., 2014; Octavia et al., 2015b). pABTJ2 was the only PLP in the scientific literature demonstrated to be uninducible (Huang et al., 2014). The authors suggested that it is a cryptic prophage, which is a prophage that has suffered mutations or partial deletions in prophage genes essential for the lytic cycle (Campbell, 1998; Huang et al., 2014). Thus, mutations may have occurred in the pABJT2 integrase gene, which could explain why this PLP does not cluster with the SSU5-like PLPs.

In further support of the results demonstrated by the integrase maximum-likelihood tree phylogenetic tree, WGS alignments by BRIG (Figure 3.1) also revealed the presence of two PLP lineages, SSU5-like and P1-like. These results also correlate with those of Octavia et al. (2015b), who performed genomic analysis on 5 of the PLPs previously reported in the scientific literature (pSTM_Phi, pECHOH89, pHCM2, pMT1, and pKPHS1), which are included in the integrase phylogenetic tree and BRIG WGS alignments. As demonstrated in this study, the authors observed that these 5 PLPs have nucleotide sequence identity to phage SSU5.

Based on the presence of the two PLP lineages, an amendment to the phage classification scheme of the International Committee on Taxonomy of Viruses (ICTV) should be proposed to include P1-like and SSU5-like PLPs as two new genera (ICTV, 2017). ICTV classifies tailed phages into 3 families, Myoviridae, Siphoviridae, and Podoviridae, based on virion morphology (Adriaenssens and Brister, 2017). Within each family, tailed phages are further classified into genus and sub-genus by considering criteria such as genome configuration, host range, and genome size (Nelson, 2004). Phage-like plasmid P1 is currently classified under the Myoviridae family as its own phage genus, P1virus. The P1virus genus should be renamed to the P1 phage-like plasmid genus since P1 represents the prototypical PLP (Łobocka et al., 2004). In addition, the P1-like PLPs could be classified under the newly named genus because, per ICTV phage classification regulations, they have >50% nucleotide sequence similarity to each other (Adriaenssens and Brister, 2017). Genomic analysis revealed that P1-like PLPs and SSU5-like PLPs have >80% and >73% nucleotide sequence identity to each other, respectively.

Based on electron microscopy, phage SSU5 was observed to have a non-contractile tail characteristic of the Siphoviridae family of phages (Kim et al., 2014). The SSU5-like PLPs harbour tail genes with nucleotide sequence identity to phage SSU5, therefore, a novel SSU5 phage-like plasmid genus should be created under the Siphoviridae family.

Due to the continual emergence of AR and the presence of ARGs in phages isolated from various environments throughout the food chain, the types of ARGs present in PLPs were investigated (Muniesa et al., 2013; Ross and Topp, 2015; Colavecchio et al., 2017a). Results from our analysis demonstrated the presence of ARGs of clinical importance in 26% (15 of 57) of the PLPs that were analyzed. The majority of ARGs in the PLPs were ESBLs, which is significant given that β-lactam antibiotics are the most common treatment for bacterial infections and continue to promote the emergence of ESBL resistance in Gram-negative bacteria worldwide (Shaikh et al., 2015). Among the various ESBL enzymes, the rapid emergence of CTX-M variants worldwide has been referred to as a pandemic (Cantón et al., 2012). This notion is further supported by the presence of CTX-M variants isolated globally from countries including China, South Korea, Germany, and the US (Liu et al., 2012; Falgenhauer et al., 2014; Shin and Ko, 2015; Colavecchio et al., 2017d). The global dissemination of CTX-M-15 is further demonstrated by the presence of a CTX-M-15 resistance cassette sharing 100% nucleotide sequence identity in AnCo1, AnCo2, and pECOH89, with pECOH89 being isolated in Germany, and AnCo1 and AnCo2 isolated in the US. Moreover, AnCo1 and AnCo2 were isolated from the feces of wildlife in bovine feedlots while pECOH89 was isolated from a clinical sample highlighting the dissemination of CTX-M-15 throughout the food chain. In addition to ESBLs, the presence of *Klebsiella pneumoniae* carbapenem (KPC) determinants in PLP pCRKP_59_KPC is also significant because KPCs confer resistance to carbapenems, which is the most reliable treatment for last-resort Gram-positive and Gram-negative bacterial infections. *Klebsiella pneumoniae* carbapenem resistant strains first emerged in North Carolina in 2001, and 4 years later in 2005 were isolated for the first time outside the USA, in France (Arnold et al., 2011). Since then, KPC-resistant strains have been isolated in 9 countries including China, which is where pCRKP_59_KPC was isolated.

In addition to the presence of ESBLs, 4 of 57 PLPs investigated in this study encoded *mcr-1,* which confers resistance to the last resort antibiotic colistin for treatment of multi-drug resistant (MDR) bacterial infections. *Mcr-1* was first reported in 2015 and has since been reported in over 40 countries and 5 continents (Wang et al., 2017). Shen et al. (2018) investigated the anthropogenic and environmental factors associated with clinical *E. coli* isolates encoding *mcr-1* genes in different provinces in China. The authors observed a significant correlation of clinical *mcr-1* with the consumption of meat, pork, and sheep and unexpectedly,

consumption of aquaculture. In China, colistin is not added to fish feed; however, it is added to livestock feed as a growth promoter. Colistin is poorly absorbed in livestock and can be excreted at high levels in animal feces. Thus, the authors attributed the correlation of *mcr-1* to aquaculturally produced fish through the contamination of runoffs from livestock farming, manure application, and agriculture (Shen et al., 2018). Hence, it is hypothesized that *mcr-1* resistance emerged in the *Enterobacteriaceae* family from the selective pressure conferred by the addition of colistin to livestock feed. In accordance with this hypothesis, 2 PLPs (pMCR-1-P3 and pHYEC7-*mcr1*) in this study encoding *mcr-1* were observed in an *E. coli* isolate from a pig farm in China (Zhang et al., 2017; Li et al., 2016). The other 2 PLPs (pMCR-SCKP-LL83 and pSLK172-1) encoding *mcr-1* were isolated from a clinical *K. penumoniae* isolate and from an atypical enteropathogenic *Escherichia coli* (aEPEC) isolate in China, respectively (Bai et al., 2017; Zhou et al., 2018). Furthermore, *mcr-1* has not previously been associated with any specific plasmid replicon type, and this observation is supported by this study, as the 4 PLPs harbouring *mcr-1* are of replicon type IncY, pO111 or of multi-replicon types (HI2, HI2A, Y, and N) (Zurfluh et al., 2016). The presence of *mcr-1* in 2 PLPs isolated from livestock samples and 2 PLPs isolated from clinical samples suggests that PLPs may contribute to the dissemination of colistin resistance in the food chain.

In addition to AR, the PLPs were analyzed for the presence of HMR genes. Six of fifty-seven PLPs in this study encoded mercury or tellurite resistance genes, which is significant because heavy metal ions contribute to the dissemination of AR via co-selection mechanisms on plasmids (Seiler and Berendonk, 2012). For instance, co-selection of mercury resistance operons has been documented on conjugable plasmids encoding ARGs for resistance to chloramphenicol, tetracycline, sulphonamide and streptomycin (Davis et al., 2005; McIntosh et al., 2008). Co-selection of tellurite resistance genes has also been observed on IncHI2 plasmids conferring resistance to β-lactams and quinolones as well as a conjugative IncP-1α plasmid harbouring a class 1 integron encoding ARGs for resistance to β-lactams and aminoglycosides (Tennstedt et al., 2005; Fang et al., 2016).

Tellurite is added to copper to improve its machinability without decreasing conductivity. Tellurite is also a secondary vulcanizing agent for rubber, allowing it to retain flexibility at high temperatures, and is also used in the electronic industry as an alloy in selenium photoreceptors for photocopiers, laser printers, and solar cells (Taylor, 1999). Due to its applications across

various industries, this has led to an increase in environmental tellurite contamination and the emergence of bacteria resistant to tellurite (Ram and Shanker, 2005; Chien et al., 2011; Grudén, 2013; Akhtar and Rehman, 2017).

Mechanisms of tellurite resistance are not well understood; however, it has been proposed that tellurite (TeO_3^{2-}) enters cells through a phosphate transporter and is reduced to a less toxic form, Te^0, through the action of a range of different enzymes such as nitrate reductases, thiols, catalases, and dihydrolipoamide dehydrogenases (Chasteen et al., 2009). To date, 5 genetic mechanisms of microbial tellurite resistance have been characterized (Taylor, 1999). One such genetic mechanism is a plasmid-mediated 7 gene operon consisting of *terZ, terA, terB, terC, terD, terE*, and *terF* (Taylor, 1999; Kormutakova et al., 2000). The phage-like plasmids MA725, JEC735, SN747, and DH728 encode *terB*, while PLP pBtic235 encodes *terC, terD*, and *terE* (Gillis et al., 2017). Tellurite resistance requires only the presence *of terB, terC, terD,* and *terE* and these genes rarely occur independently from each other; in such instances, there are generally *ter* genes elsewhere in the genome (Kormutakova et al., 2000; Taylor et al., 2002; Anantharaman et al., 2012; Gillis et al., 2017). Interestingly, our results showed that PLPs MA725, SN747, and DH728 harboured *terB* alone, and only PLP JEC735 encoded additional *ter* operon genes (*terZ, terB, terC, terD, terE*) on its chromosome. To the best of our knowledge, this is the first observation in the scientific literature of *terB* alone in a bacterial strain. Chapter IV section 4.3.3 will investigate whether this resistance determinant can confer resistance to tellurite in the absence of the other *ter* operon genes. If found to be so, PLPs harbouring *terB* could be important vectors of HMR and potentially contribute to AR through co-selection, since tellurite resistance mainly occurs on plasmids (Hou and Taylor, 1994). For example, Hou and Taylor, (1994), observed the co-selection of various AR determinants and tellurite resistance determinants on 32 different plasmids spanning 4 different incompatibility groups (IncP, IncHII, IncH2, and IncP2) from bacterial strains of the *Enterobacteriaceae* family. Tennstedt et al. (2005), observed the co-selection of genes encoding aminoglycoside, tetracycline, and β-lactamase resistance, as well as a tellurite resistance operon on an IncP plasmid isolated from a wastewater treatment plant. Additionally, 25 IncHI2 plasmids, harbouring the quinolone efflux pump oqxAB and/or CTX-M genes, as well as a *ter* operon, were transferred to recipient hosts by conjugation (Fang et al., 2016). All 25 transconjugants were found to carry a tellurite-resistance system (Fang et al., 2016). If *terB* alone confers resistance to tellurite, the results by

Fang *et al.,* (2006) suggest the potential for any PLPs harbouring *terB* to transfer this gene to bacteria of foodborne importance via HGT. Moreover, these PLPs could potentially confer resistance to AR via co-selection mechanisms if ARGs are transferred by MGEs into the chromosomes of PLPs. Finally, while IncHI2 is the most prevalent plasmid replicon type associated with tellurite resistance, our results demonstrate that PLPs analyzed in this study (MA725, JEC735, SN747, and DH728) are replicon type IncY, thus, potentially demonstrating a novel plasmid type associated with tellurite resistance (Hou and Taylor, 1994).

A mercury resistance operon responsible for the enzymatic reduction of Hg^{2+} to Hg^{0} was observed in PLP SJ1. Transport genes *merC, merD,* and *merT,* which are contained within the operon, carry Hg^{2+} into the cytoplasm for reduction into the less volatile form, Hg^{0}, by the mercury reductase gene, *merA* (Osborn et al., 1997). The operon is also flanked by transposon Tn21, which is a transposon family often associated with mercury resistance (Liebert et al., 1999). Although PLP SJ1 does not contain any ARGs, it could potentially acquire ARGs through co-selection mechanisms since Tn21 is known to be flanked by class 1 integrons harbouring AR determinants (Liebert et al., 1999). Example of mercury co-selection mechanisms has been observed in bacterial isolates from different environments such as a fish gut microbiome, two sphagnum bogs in Maine that date back 2000 years, floc samples from freshwater systems in Canada, and Atlantic salmon (McIntosh et al., 2008; Wardwell et al., 2009; Drudge et al., 2012; Lloyd et al., 2016). Furthermore, Skurnik *et al*. (2010) explored commensal *E. coli* isolates from 3 human populations exposed to different levels of mercury and AR. They observed that mercury-resistant *E. coli* was found to be significantly more frequent in human populations exposed to the highest levels of AR. Correspondingly, AR *E. coli* levels were highest in human populations living in an environment with high exposure to mercury, suggesting co-selection mechanisms as the cause (Skurnik et al., 2010). Rodríguez-Rojas et al. (2016) also demonstrated that mercury resistance could confer cross-resistance to tellurite. The exposure to heavy metal ions such as mercury and tellurite may be a driving force in the emergence and maintenance of AR in the absence of antibiotic selective pressure (Skurnik et al., 2010; Rodríguez-Rojas et al., 2016).

Mobile genetic elements play a pivotal role in the dissemination of AR (Partridge et al., 2018). Genomic analysis demonstrated that each AR and HMR determinant within PLPs, with the exception of PLPs harbouring tellurite resistance, was associated with a MGE. ARGs with

low fitness costs are preferentially recruited by MGEs and sub-inhibitory concentrations of antibiotics are sufficient to maintain resistance genes on MGEs (Bengtsson-Palme et al., 2017). Plasmid-like phages do not carry any ARGs or HMR genes or any MGEs, which suggests how the latter may play a role in resistant gene recruitment and dissemination. Moreover, the large genome sizes of PLPs compared to plasmid-like phages could be attributed to the presence of various MGEs, which in turn allows them to further recruit foreign DNA into their MGEs.

In summary, 57 PLPs were characterized by investigating their integrase genes and performing whole genome alignments, which demonstrated that two PLP lineages (SSU5-like and P1-like) exist. Our findings suggest that the presence of phage genes (integrase and terminase A) and plasmid genes (*repA, parA,* and *parB*) define a PLP. Moreover, the results from our analysis suggest that PLPs potentially contribute to the dissemination of AR and HMR because PLPs are inducible and harbour ARGs (ESBLs, KPCs and *mcr-1*), HMR genes (*ter* and *mer* resistance operon) and MGEs. Future studies should be performed to investigate the potential of PLPs to horizontally transfer ARGs and HMR genes via transduction, transformation, and conjugation. Additional studies should also be conducted to determine if the tellurite resistance gene *terB* can confer phenotypic resistance in the absence of other *ter* operon genes.

Table 3.1: Comparison of phage-like plasmid (PLPs) and plasmid-like phage characteristics.

Characteristics	Phage-like plasmids (PLPs)	Plasmid-like phages
Genome	dsDNA circular 26–369 kb	dsDNA linear 14–51 kb
Plasmid maintenance system	*parA, parB, repA*	*sopA, sopB*
Integration system	Integrase	Prototelomerase
Host range	Gram-positive and Gram-negative	Gram-positive and Gram-negative
Encode antibiotic or heavy metal resistance genes	Yes	No
Encode mobile genetic elements	Yes	No
Horizontal gene transfer	– Inducible – Transformation and conjugation demonstrated	– Inducible – Horizontal gene transfer (HGT) not demonstrated

Table 3.2: Summary of 8 phage-like plasmids (PLPs) isolated during this study.

Phage-like plasmid	Bacterial host	Origin	Source	Publication
AnCo1	*Escherichia coli* 243	Colorado, USA	Wildlife feces in bovine feedlots	(Colavecchio et al., 2017d)
AnCo2	*Escherichia coli* 244	Colorado, USA	Wildlife feces in bovine feedlots	(Colavecchio et al., 2017d)
AnCo3	*Salmonella* Derby S701	Quebec, Canada	Clinical	(Colavecchio et al., 2017c)
SJ1	*Salmonella* Kouka S42	Ottawa, Canada	Oysters	n/a
MA725	*Escherichia coli* XX-29735	France	Bovine mastitis	n/a
JEC735	*Escherichia coli* XX-22725	France	Bovine diarrhoea	n/a
SN747	*Escherichia coli* XX-29747	France	Bovine mastitis	n/a
DH728	*Escherichia coli* XX-29728	France	Bovine mastitis	n/a

Table 3.3: Summary of 18 phage-like plasmids (PLPs) reported in the scientific literature and analyzed in this study.

Phage-like plasmid	Bacterial host	Accession number	Publication
pBtic235	*Bacillus thurigiensis*	CP003765	(Gillis et al., 2017)
pSTM_Φ	*Salmonella* Typhi	KP763470	(Octavia et al., 2015a)
SJ46	*Salmonella* Derby,	KU760857	(Yang et al., 2017)
pHYEC7-*mcr1*	*Escherichia coli* HYEC7	KX518745	(Li et al., 2016)
p-MCR-1-P3	*Escherichia coli* EMP163	KX880944	(Zhang et al., 2017)
RCS47	*Escherichia coli*	FO818745	(Billard-Pomares et al., 2014)
pECOH89	*Escherichia coli* H89	HG530657	(Falgenhauer et al., 2014)
pABTJ2	*Acinetobacter baumanii*	NC_020524	(Huang et al., 2014)
vB_BceS-IEBH	*Bacillus cereus* CD555	NC_011167	(Smeesters et al., 2011)
pHCM2	*Salmonella* Typhi	AL513384	(Kidgell et al., 2002)
pSLK172-1	*Escherichia coli*	CP017632.1	(Bai et al., 2017)
PVv01 (p48/10)	*Vibrio vulnificus*	HG803186	(Hammerl et al., 2014)
pKP12226	*Klebsiella pneumoniae*	KP453775.1	(Shin and Ko, 2015)
KHP30	*Helicobacter pylori*	AB647160.1	(Uchiyama et al., 2013b)
PhiBU01	*Staphylococcus aureus*	KF831354.1	(Utter et al., 2014)
pKPHS1	*Klebsiella pneumoniae*	CP003223.1	(Liu et al., 2012)
pMT1	*Yersinia pestis*	AF074611	(Lindler et al., 1998)
pMCR_SCKP-LL83	*Klebsiella pneumoniae*	MF510496.1	(Liu et al., 2018)

Table 3.4: Summary of 31 phage-like plasmids (PLPs) identified in this study.

Phage-like plasmid	Bacterial host	Accession number
pSAN1_1735	*Salmonella* Anatum	NZ_CP014707.1
p1002_1	*Escherichia coli*	CP021203.1
H8_plasmid_A	*Escherichia coli*	CP010173.1
pCFSAN004176P_03	*Escherichia coli*	CP012491.1
pCRKP_59_KPC	*Klebsiella pneumoniae*	KX928752.1
p91	*Escherichia coli*	CP023381.1
plasmid unamed 3	*Escherichia coli*	CP023896.1
p0111_2	*Escherichia coli*	AP010962.1
pCREC_532_2	*Escherichia coli*	CP024832.1
pSJ_98	*Escherichia coli*	CP011063.1
pU2501_39	*Escherichia coli*	KU980950.1
plasmid_unamed_2	*Escherichia coli*	CP013030.1
ptig00000008	*Klebsiella pneumoniae*	CP021962.1
pKO_JKo3_2	*Klebsiella oxytoca*	AP014953.1
pUCLAOXA232_4X	*Klebsiella pneumoniae*	CP012570.1
pKP301b	*Klebsiella pneumoniae*	KY354306.1
pPMK1_B	*Klebsiella pneumoniae*	CP008931.1
pKPN_04f	*Klebsiella pneumoniae*	CP014756.1
p205880_NR1	*Klebsiella pneumoniae*	MF144193.1
AR_0117_plasmid_unitig_2	*Klebsiella pneumoniae*	CP020063.1
pMRSN480738	*Klebsiella pneumoniae*	CP024460.1
pSg1_1	*Klebsiella pneumoniae*	CP012427.1
FDAARGOS_442_unamed_2	*Klebsiella pneumoniae*	CP023929.1
pECAZ155_2	*Escherichia coli*	CP019002.1
p11219_CTXM	*Klebsiella pneumoniae*	MF133442.1
pCS1	*Cronobacter sakazakii*	CP012254.1
p1303_95	*Escherichia coli*	CP009168.1
p15648_1	*Escherichia coli*	CP009051.1
GCA_000007885.1	*Yersinia pestis*	CP011061.1
GCA_000834825.1	*Yersinia pestis*	CP009904.1
plasmid_2	*Klebsiella pneumoniae*	CP017387.1

Table 3.5: Genomic characteristics of 57 PLPs in this study including their genome size (Kb), plasmid replicon type, source, country of isolation, and lineage (SSU5 or P1)

PLP	Bacterial host	Genome Size (Kb)	Plasmid Typing	Source	Country	Lineage
AnCo1	*Escherichia coli* 243	112.2	Untypeable	Wildlife feces in bovine feedlots	USA	SSU5
AnCo2	*Escherichia coli* 244	109	Untypeable	Wildlife feces in bovine feedlots	USA	SSU5
AnCo3	*Salmonella* Derby S701	106	Untypeable	Clinical	Canada	SSU5
pSTM_Φ	*Salmonella* Typhi	107.7	IncFIB	Avian	Australia	SSU5
pECOH89	*Escherichia coli* H89	111.7	Untypeable	Wound swab, Clinical	Germany	SSU5
pABTJ2	*Acinetobacter baumanii*	110.9	Untypeable	Clinical	China	SSU5
pHCM2	*Salmonella* Typhi	106.5	IncFIB	Clinical	Vietnam	SSU5
pKPHS1	*Klebsiella pneumoniae*	122.7	IncFIB	Sputum sample, clinical	China	SSU5
pMT1	*Yersinia pestis* KIM10+	100.9	IncFIB	Laboratory	Unknown	SSU5
pSAN1_1735	*Salmonella* Anatum	101.1	Untypeable	Bovine pre-evisceration carcass	USA	SSU5
ptig00000008	*Klebsiella pneumoniae*	132.2	IncFIB	Unknown	Unknown	SSU5
pKO_JKo3_2	*Klebsiella oxytoca*	104.2	IncFIB	Unknown	Unknown	SSU5
pUCLAOXA 232_4X	*Klebsiella pneumoniae*	111.2	IncFIB	Clinical, rectal	USA	SSU5
pKP301b	*Klebsiella pneumoniae*	110.2	IncFIB	Lettuce	Brazil	SSU5
pPMK1_B	*Klebsiella pneumoniae*	111.6	IncFIB	Clinical	Nepal	SSU5
pKPN_04f	*Klebsiella pneumoniae*	121.0	IncFIB	Clinical	USA	SSU5
p205880_NR 1	*Klebsiella pneumoniae*	108.0	IncFIB	Unknown	China	SSU5
AR_0117_pla smid_unitig_2	*Klebsiella pneumoniae*	109.0	IncFIB	Unknown	Unknown	SSU5
pMRSN4807 38	*Klebsiella pneumoniae*	112.7	IncFIB	Clinical, sputum	Thailand	SSU5

Table 3.5 continued: Genomic characteristics of 57 PLPs in this study including their genome size (Kb), plasmid replicon type, source, country of isolation, and lineage (SSU5 or P1)

PLP	Bacterial host	Genome Size (Kb)	Plasmid Typing	Source	Country	Lineage
pSg1_1	*Klebsiella pneumoniae*	126.4	IncFIB	Clinical	Signapore	SSU5
FDAARGOS_442_unamed_2	*Klebsiella pneumoniae*	112.2	IncFIB	Clinical, blood	Canada	SSU5
pECAZ155_2	*Escherichia coli*	112.9	Untypeable	Clinical	China	SSU5
p11219_CTXM	*Klebsiella pneumoniae*	122.0	IncFIB	Unknown	Unknown	SSU5
pCS1	*Cronobacter sakazakii*	110.0	Untypeable	Milk at farm	UK	SSU5
GCA_000007885.1	*Yersinia pestis*	72.5	IncFIB	Unknown	Unknown	SSu5
GCA_000834825.1	*Yersinia pestis*	58.8	IncFIB	Unknown	Unknown	SSU5
plasmid_2	*Klebsiella pneumoniae*	225.9	IncA/C2	Clinical, urine	Taiwan	SSU5
SJ1	*Salmonella* Kouka	24.5	Untypeable	Oysters	Canada	P1
MA725	*Escherichia coli* XX-29735	103.8	IncY	Bovine mastitis	France	P1
JEC735	*Escherichia coli* XX-22725	93.5	IncY	Bovine diarrhoea	France	P1
SN747	*Escherichia coli* XX-29747	92.8	IncY	Bovine mastitis	France	P1
DH728	*Escherichia coli* XX-29728	92.8	IncY	Bovine mastitis	France	P1
SJ46	*Salmonella* Derby, *Salmonella* Indiana	103.4	Untypeable	Slaughterhouse	China	P1
pHYEC7-*mcr1*	*Escherichia coli* HYEC7	97.5	IncY	Fecal sample, pig farm	China	P1
p-MCR-1-P3	*Escherichia coli* EMP163	97.3	IncY	Pig anal swabs	China	P1
RCS47	*Escherichia coli*	115	IncY	Clinical	France	P1
pSLK172-1	atypical enteropathogenic *Escherichia coli* (aEPEC)	369.2	Multi-replicon types: HI2, HI2A, Y and N	Clinical	China	P1
PVv01 (p48/10)	*Vibrio vulnificus*	79.2	Untypeable	Baltic sea	Baltics	P1

Table 3.5 continued: Genomic characteristics of 57 PLPs in this study including their genome size (Kb), plasmid replicon type, source, country of isolation, and lineage (SSU5 or P1)

PLP	Bacterial host	Genome Size (Kb)	Plasmid Typing	Source	Country	Lineage
pKP12226	*Klebsiella pneumoniae*	267.6	IncF	Clinical	South Korea	P1
pMCR_SCKP-LL83	*Klebsiella pneumoniae* SCKP83	94.7	pO111	Sputum sample, clinical	China	P1
p1002_1	*Escherichia coli*	183.5	IncFII	Clinical, blood	China	P1
H8_plasmid_A	*Escherichia coli*	94.3	IncY	River water	Unknown	P1
pCFSAN004176P_03	*Escherichia coli*	95.7	IncY	Clinical	USA	P1
pCRKP_59_KPC	*Klebsiella pneumoniae*	216.9	IncFII	Clinical	China	P1
p91	*Escherichia coli*	91.1	IncY	Canine	UK	P1
plasmid unamed 3	*Escherichia coli*	83.9	IncY	Clinical, rectal	Canada	P1
p0111_2	*Escherichia coli*	97.8	p0111	Clinical_diarhea	Japan	P1
pCREC_532_2	*Escherichia coli*	96.9	IncY	Clinical_urine	South Korea	P1
pSJ_98	*Escherichia coli*	98.4	IncY	Pheasant duodenum	China	P1
pU2501_39	*Escherichia coli*	98.8	IncY	Clinical	Unknown	P1
plasmid_unamed_2	*Escherichia coli*	97.7	p0111	Clinical	USA	P1
p1303_95	*Escherichia coli*	94.9	IncY	Bovine mastitis	Unknown	P1
p15648_1	*Escherichia coli*	97.2	IncY	Clinical, stool	South Korea	P1
pBtic235	*Bacillus thurigiensis* subsp. israelensis	235	Untypeable	Unknown	Unknown	Unknown
vB_BceS-IEBH	*Bacillus cereus* CD555	53.1	Untypeable	Unknown	Unknown	Unknown
KHP30	*Helicobacter pylori* NY43	26.2	Untypeable	Clinical	Japan	Unknown
PhiBU01	*Staphylococcus aureus* NRS19	43.8	Untypeable	Clinical	USA	Unknown

Table 3.6: The antibiotic resistance genes (ARGs), heavy metal resistance (HMR) genes and mobile genetic elements (MGEs) harboured by phage-like plasmids (PLPs)

Phage-like plasmid	Mobile genetic elements	Resistance genes (ARGs and HMR)
AnCo1	*ICEcp1*	CTX-M-15
AnCo2	*ICEcp1*	CTX-M-15
SJ1	Tn21	*merC*, *merE* and *merT*, mercury ion reductase, *merP*, and a mercury resistance coregulator
MA725	None	*terB*
JEC735	None	*terB*
SN747	None	*terB*
DH728	None	*terB*
p1002_1	Transposase *InsB* flanking Tn3	CTX-M-15, aadA5
pCRKP_59_KPC	*InsE1* IS5 family transposase	KPC-2, TEM-1, Aac(3)-lia
pU2501_39	*ISEcp*1	CTX-M-15
p11219_CTXM	IS26, IS1380	CTX-M-28
pBtic235	None	Tellurite resistance (*terC*, *terD*, *terE*)
SJ46	Tn1721 containing *ISEcpIB*	CTX-M-27
pHYEC7-*mcr1*	Tn6330 containing *ISAplI*	*mcr-1*
p-MCR-1-P3	*ISApl*1 (2 copies)	*mcr-1*
RCS47	IS5 (2 copies), IS1	SHV-2
pECOH89	*ICEcp1*	CTX-M-15
pSLK172-1	*ISApl1* (2 copies), Tn3, Tn21	*mcr-1*, sul1, aphA1, sul3, aadA1, aadA2, dfrA12, floR, sul2, aac(3)-IV, blaCTX-M-14, fosA3
pKP12226	IS26-*ISEcp*1, tnpA6100, IS5075 Tn3, IS1	CTX-M-15 *mph*(A), *mrx*, *mph*(R), *chrA, sul1, qacEdelta1, aadA4, dfrA17,* TEM-1
pKPHS1	Yes	CTX-M-14
pMCR_SCKP-LL83	*ISApl1*	*mcr-1*

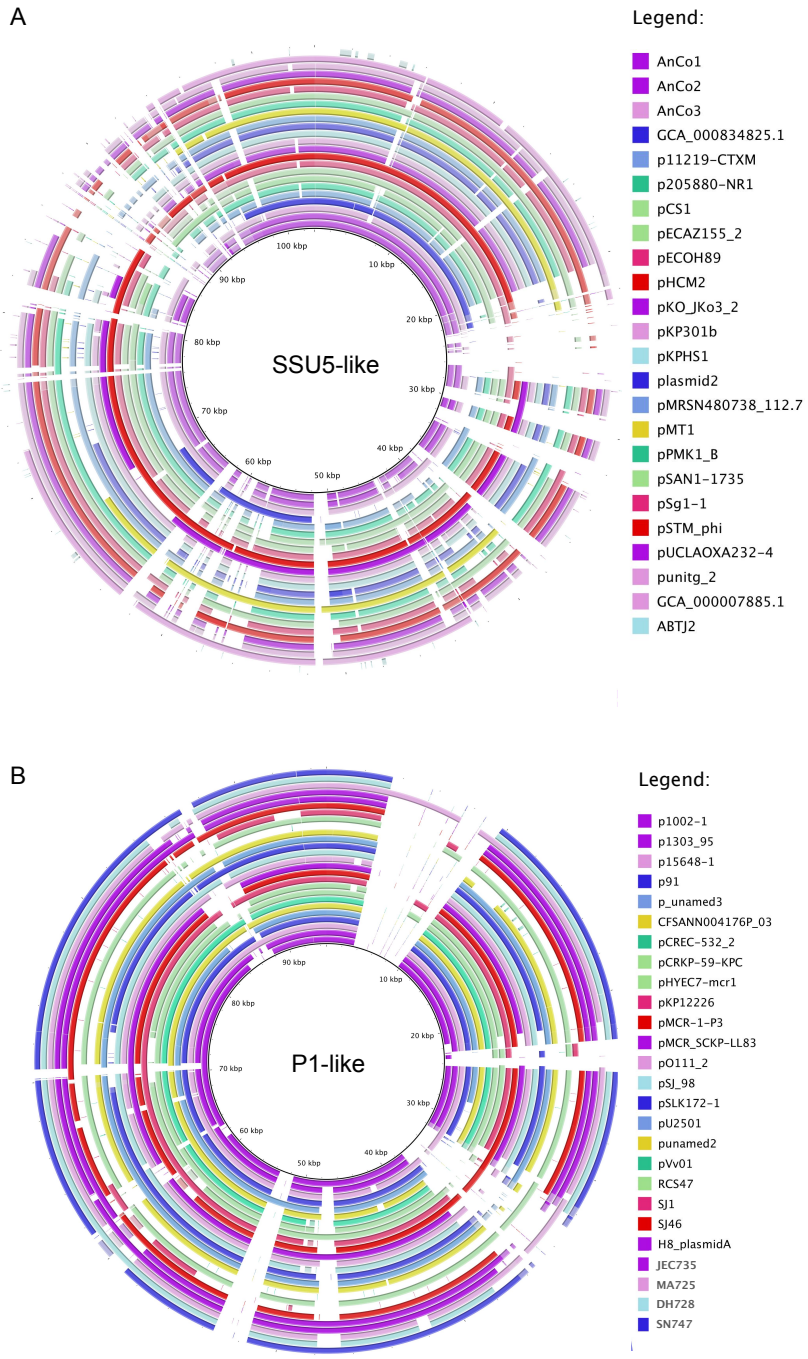

Figure 3.1. Whole genome alignments of PLPs compared to either phage SSU5 (A) or the phage-like plasmid P1 (B) performed by the BLAST Ring Image Generator (BRIG). The solid regions of color represent nucleotide sequence identity to phage SSU5 (A) or phage-like plasmid P1 (B) and the gaps represent genes that are absent in comparison to PLP P1 or phage SSU5.

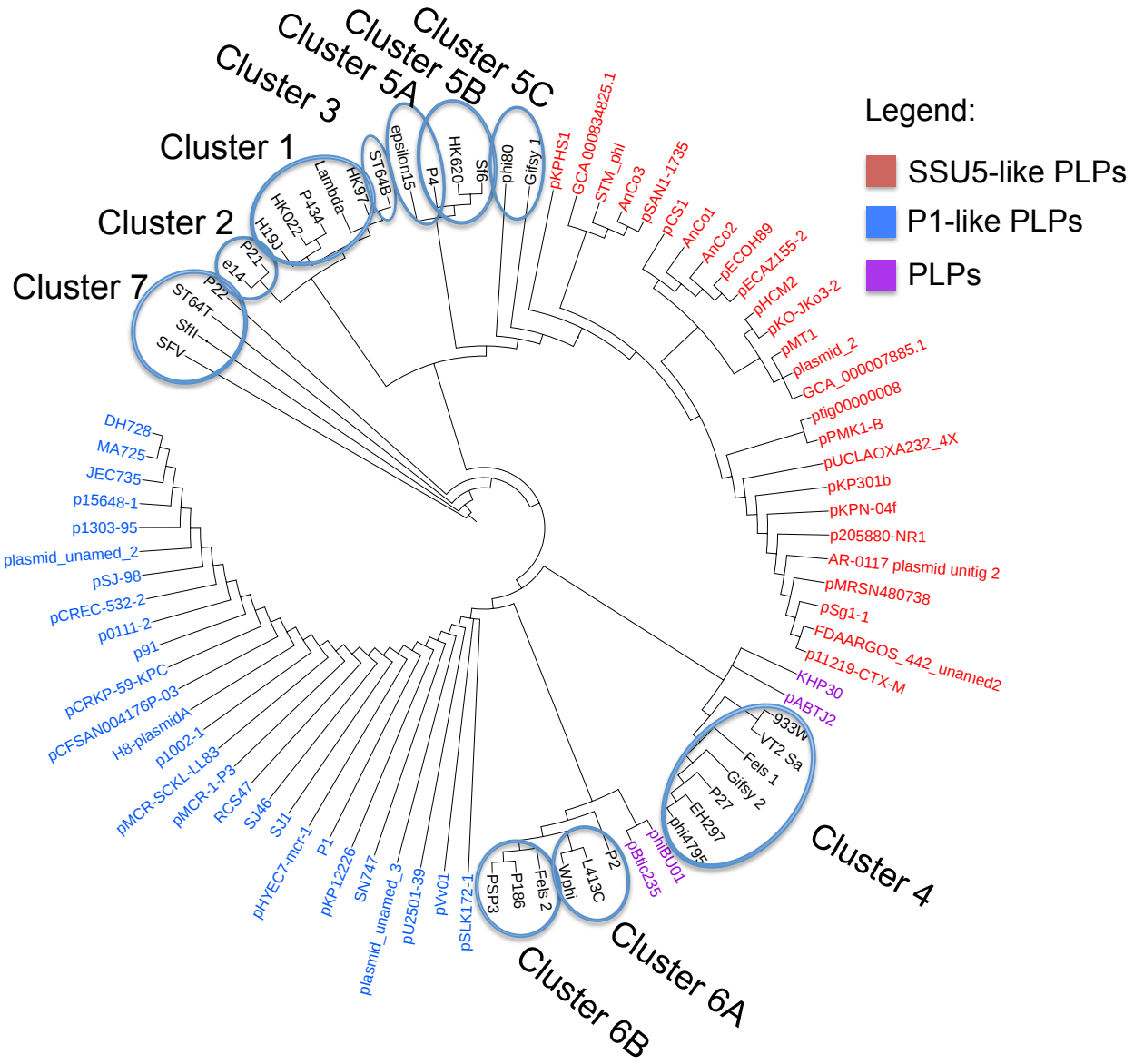

Figure 3.2. Maximum-likelihood phylogenetic tree of the conserved region of the integrase genes of 56 PLPs and 32 enteric prophages. SSU5-like PLPs (orange) and P1-like PLPs (green) cluster within their own branches. Four non P1-like and SSU5-like PLPs (purple) cluster elsewhere in the phylogenetic tree. Groups of enteric temperate prophages cluster together (circled in blue), as per Balding *et al*. (2005).

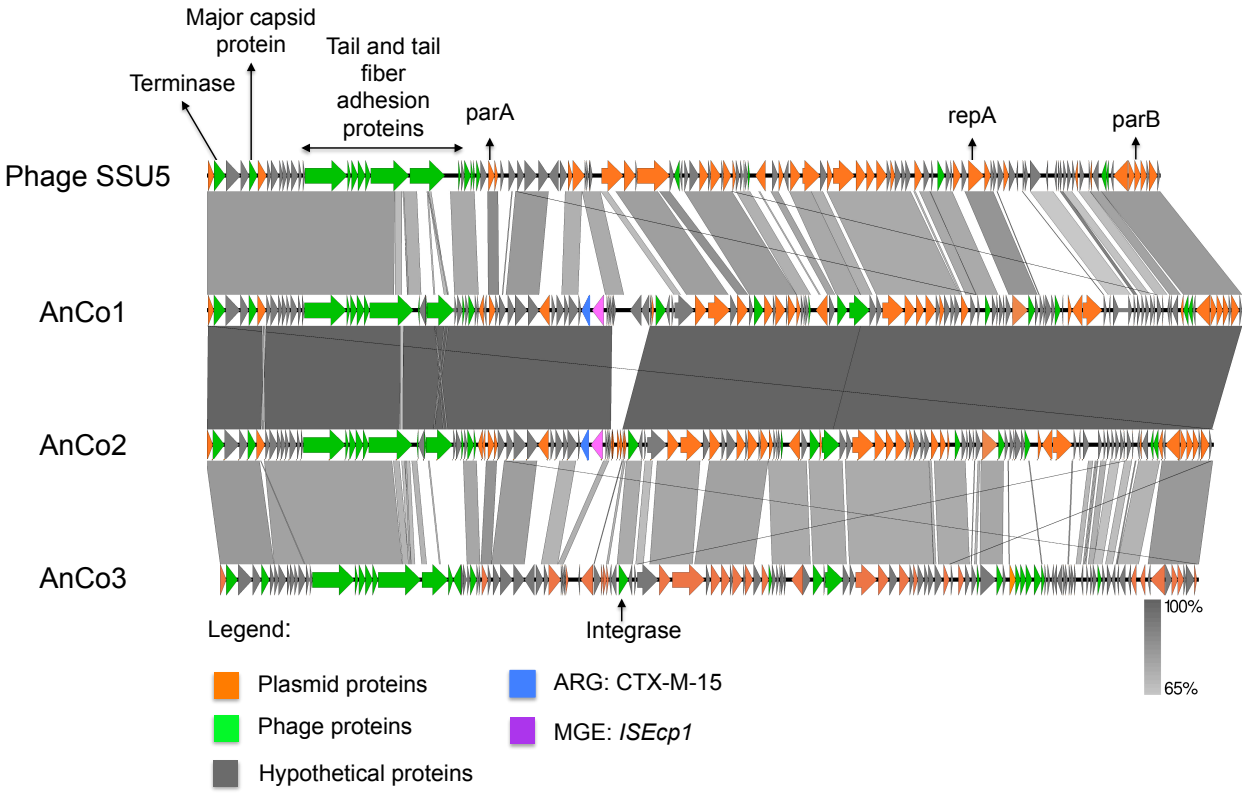

Figure 3.3. Whole genome sequence comparison of AnCo1, AnCo2 and AnCo3 to phage SSU5. Arrows represent CDS and dark grey vertical blocks in between each genome represents regions of nucleotide sequence identity between 65–100%.

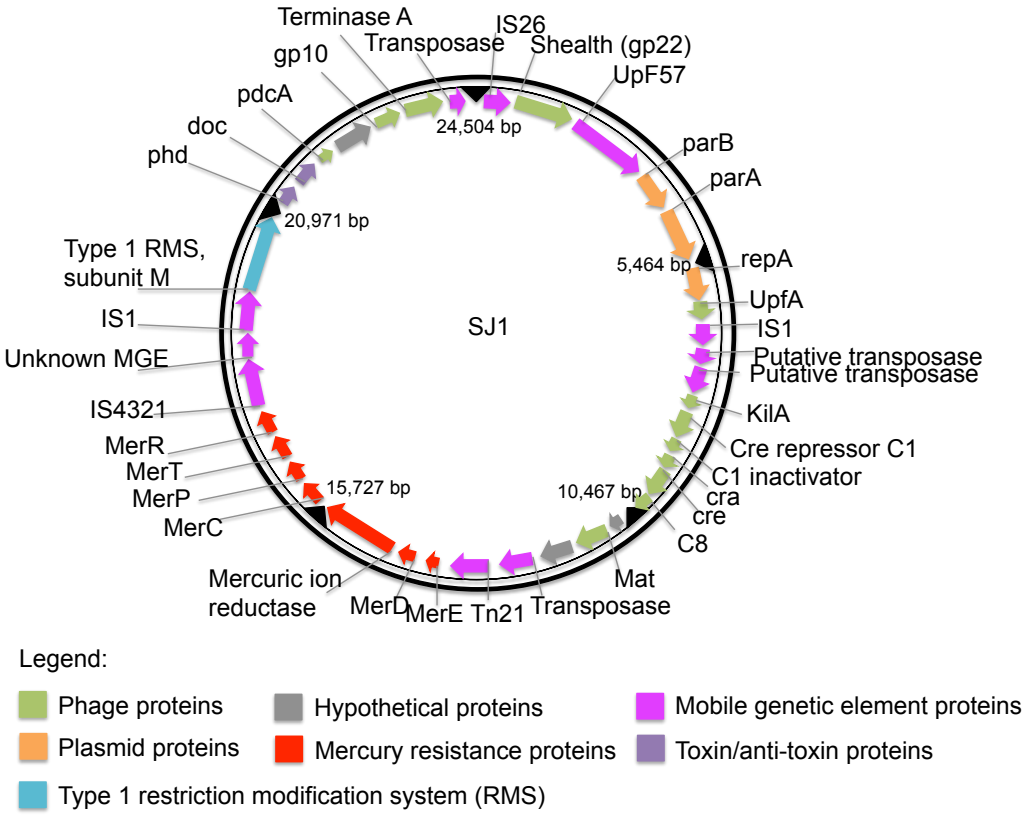

Figure 3.4. Circular genomic map of SJ1 harbouring a mercury resistance operon. CDS are represented by arrows.

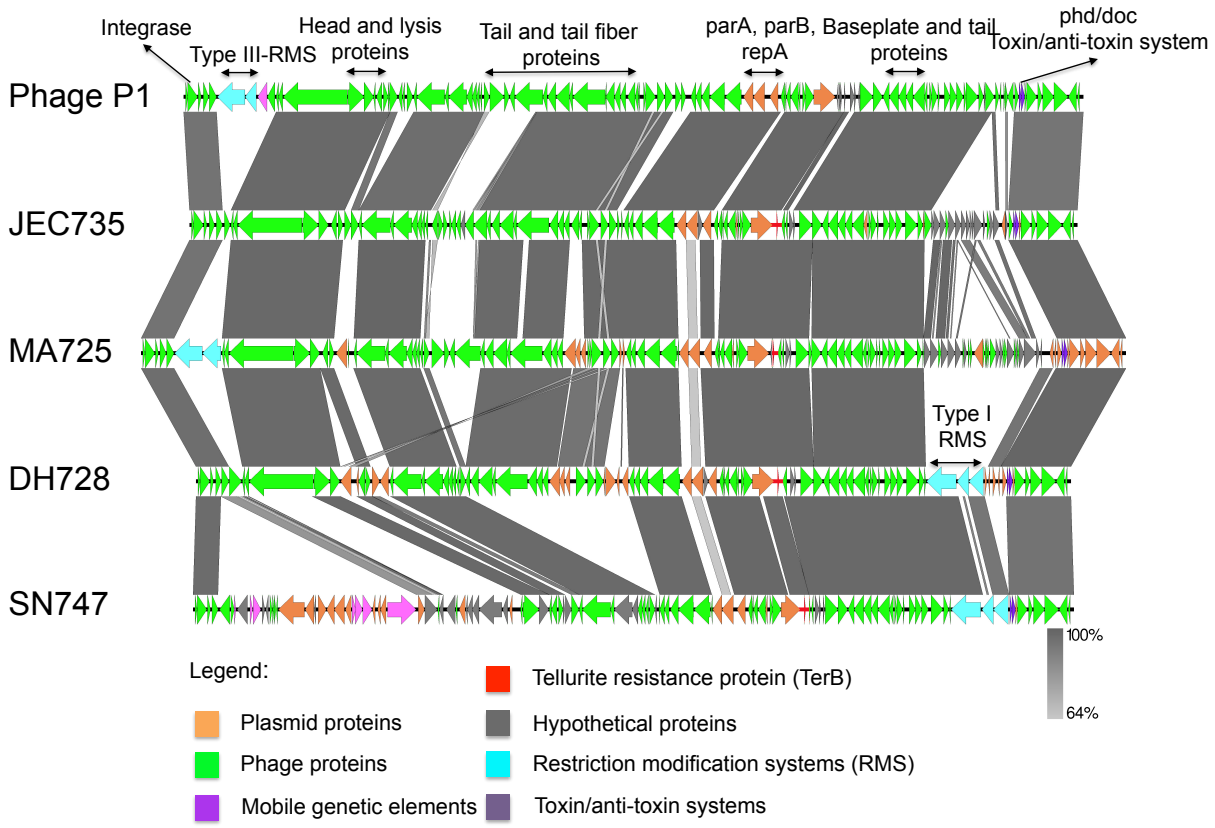

Figure 3.5. Whole genome sequence comparison of P1-like PLPs JEC735, MA725, DH728, and SN747 harbouring tellurite resistance gene (*terB*) to PLP P1. Arrows represent CDS and dark grey blocks in between each genome represents regions of nucleotide sequence identity between 64–100%.

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Connecting text

In previous chapters, the importance of phage-like plasmids (PLPs) as a potential contributor to antibiotic resistance (AR) and heavy metal resistance was discussed (Chapter II), and the presence of clinically relevant resistance genes in PLPs was revealed (Chapter III), where 57 PLPs from various sources (the scientific literature, Genbank as well as bovine and food isolates) were genomically characterized. Results demonstrated that these PLPs were capable of infecting 12 different bacterial species, are dispersed worldwide, and can be classified into two lineages, SSU5-like and P1-like. Of the 57 PLPs, 15 harboured ARGs conferring resistance to β-lactams, carbapenems, colistin, and aminoglycosides; and 6 harboured HMR genes conferring resistance to either mercury chloride or potassium tellurite.

Building on the previous chapters, the study described in this chapter had two main objectives. First, since PLPs are phage/plasmid hybrids, work was conducted to determine if the antibiotic and heavy metal resistance genes could be horizontally transferred by phage-mediated transduction and/or plasmid-mediated transformation and conjugation. The second was to determine whether horizontally transferred resistance genes could confer resistance to antibiotics and heavy metals. Accomplishing these objectives will help determine the contribution of PLPs in the dissemination of AR and HMR worldwide.

Chapter IV:

Phage-like plasmids transfer antibiotic resistance genes, and mercury and tellurite resistance genes by transduction, transformation and conjugation

4.0 Abstract:

Phage-like plasmids (PLPs) encode antibiotic resistance genes (ARGs) and heavy metal resistance (HMR) genes and belong to two lineages, SSU5-like and P1-like. Phages and plasmids contribute to the spread of antibiotic resistance (AR) through three horizontal gene transfer (HGT) mechanisms, including transduction (phages) and conjugation and transformation (plasmids). Since PLPs are hybrids of both phage and plasmid DNA, the objective of this study was to investigate the potential of PLPs to horizontally transfer ARGs and HMR via all 3 mechanisms of HGT, and determine if the transferred genes confer resistance to antibiotics, mercury and tellurite.

For this study, 3 representative PLPs described in Chapter III, including an SSU5-like PLP (AnCo1) encoding CTX-M-15, and two P1-like PLPs, one encoding a *mer* operon (SJ1) and the other encoding *terB* (MA725), were selected for HGT experiments. All 3 PLPs were transduced to various *E. coli* and *Salmonella enterica* hosts; however, SJ1 had the largest host range by infecting 18 different strains. These PLPs were also transduced to *E. coli* strains containing deletions for glycosyltransferase enzymes present in the lipopolysaccharide (LPS) (AnCo1 to CWG310::*waaW* deletion, SJ1 to CWG297::*waaQ* deletion, and MA725 to CWG309::*waaT* deletion). *waaW* and *waaT* are located in the outer core while *waaQ* is located in the inner core of the LPS. These results suggested that PLPs AnCo1, SJ1 and MA725 may use carbohydrate residues in the core LPS, D-galactose I, L-glycero-D-manno-heptose II and D-glucose II, respectively, as receptors. Results also demonstrated that PLPs AnCo1 and SJ1 were conjugated to *E. coli* J53 and all 3 PLP were transformed to *E. coli* DH10B.

Bacterial hosts that carried successfully transferred PLPs were tested for resistance to antibiotics, mercury and tellurite.. Minimum inhibitory concentration (MIC) results revealed that the CTX-M-15 gene harboured by AnCo1 conferred 3 mg/ml of resistance to cefotaxime, and the mercury operon harbored by SJ1 conferred 50 μg/ml to mercury chloride. This study also revealed that *terB*, harboured by PLP MA725, conferred 40 μg/ml of resistance to potassium

tellurite as well as 10 μg/ml to colistin sulphate. These results are significant because they demonstrate that *terB* alone conferred resistance to tellurite and that *terB* confers cross-resistance to colistin.

Collectively, these results demonstrate that PLPs are important and emerging mobile genetic elements (MGEs), as they can be horizontally transferred by transduction, conjugation and transformation mechanisms, and confer resistance to antibiotics, mercury and tellurite.

4.1 Introduction:

In view of the hundreds of thousands of deaths annually worldwide caused by antibiotic-resistant infections, the World Health Organization (WHO) has established antibiotic resistance (AR) as a threat to global public health (WHO, 2018). Antibiotic resistance development is caused by the use and misuse of antibiotics in clinical and community settings, in animal husbandry as well as in agriculture. Their use and misuse in livestock and aquaculture, as well as run offs from slaughterhouses and manure-treated fields, increase the transmission of ARGs from commensal bacteria to bacteria of foodborne importance and the selective pressure maintains their presence (Muniesa et al., 2013; Colavecchio et al., 2017a). The application of heavy metals contributes to AR via co-selection and cross-resistance mechanisms (Seiler and Berendonk, 2012). In Europe, copper and zinc are used as growth promoters in animal farming and aquaculture and copper is used as a pesticide in agriculture (Monteiro et al., 2010; Seiler and Berendonk, 2012). In Canada and the USA, copper is approved for as an anti-fouling agent on aquaculture netting and as a pesticide in agriculture (US Environmental Protectio Agency (US EPA), 2008; Government of Canada, 2016).

The selective pressure caused by antibiotics increases the potential for point mutations and dissemination of mobile genetic elements (MGEs) through HGT (Stokes and Gillings, 2011). Horizontal gene transfer mechanisms responsible for the increased spread of AR to bacterial foodborne pathogens have been well studied. Conjugation, transformation, and transduction are the mechanisms by which dissemination of ARGs occurs (von Wintersdorff et al., 2016). The notion that phage-mediated transduction is a major driver of HGT of ARGs between foodborne pathogens, as well as from the environment to animals and humans, is increasingly becoming recognized. Phages are the most abundant organism in the biosphere, and are found in diverse environments including oceans, lakes, soil, urban sewage, potable and well water, and plant microbial communities (Clokie et al., 2011). Antibiotic resistance genes are found on MGEs, including phages, plasmids, integrative and conjugative elements (ICE), and transposons, and, as such, can be horizontally transferred. Phage-like plasmids (Chapter III), being phage/plasmid hybrids, allows them to be horizontally transferred by transformation and conjugation, in addition to transduction. For instance, despite their large genome sizes, PLPs RCS47 (115 kb) and SJ46 (103.4 kb) were transformed into a host cell, providing their hosts with resistance to cefotaxime (Billard-Pomares et al., 2014; Yang et al., 2017). Phage-like plasmids pBtic235,

pKP12226, and pSLK172-1 were observed to be conjugative; however, pBtic235 required the presence of conjugative helper plasmid pXO16 in the donor cell to facilitate conjugation (Shin and Ko, 2015; Bai et al., 2017; Gillis et al., 2017).

Although PLPs reported in the scientific literature have been demonstrated to be inducible (Smeesters et al., 2011; Uchiyama et al., 2013a; Billard-Pomares et al., 2014; Hammerl et al., 2014; Utter et al., 2014; Colavecchio et al., 2017c; Colavecchio et al., 2017d; Gillis et al., 2017; Liu et al., 2018), none have been investigated for their potential to transfer ARGs via transduction. Moreover, to the best of our knowledge, no studies have demonstrated the transduction of HMR genes by either phages or PLPs. In Chapter III, it was observed that 37% of PLPs harboured either ARGs or HMR genes. Thus, the objective of this study was to investigate the potential of PLPs to transfer ARGs and HMR genes by the HGT mechanisms transduction, conjugation, and transformation, to commensal non-pathogenic *E. coli* and pathogenic *Salmonella enterica* bacteria.

4.2 Materials and Methods:

4.2.1 Bacterial strains:

The bacterial isolates used in this study (Table 4.1) are from the culture collections of Dr. Lawrence Goodridge (McGill University, Montréal, Québec, Canada), Dr. Séamus Fanning (University College Dublin, Dublin, Ireland), Dr. Chris Whitfield (University of Guleph, Guelph, Ontario, Canada) and the *Salmonella* Foodborne Syst-OMICS Database (SalFoS), which can be accessed at https://salfos.ibis.ulaval.ca/. *Escherichia coli* DH10B was purchased from ThermoFisher Scientific (ThermoFisher Scientific, Waltham, MA, USA) and *E. coli* J53 was purchased from Cedarlane (Cedarlane, Burlington, Ontario, Canada). All strains were grown from frozen stocks on modified Luria-Bertani agar (LBA) (Sigma-Aldrich, St Louis, Missouri, USA) supplemented with appropriate antibiotics or heavy metal solutions followed by subculture in modified LB broth (LBB) and incubation at 37°C with orbital shaking at 225 rpm.

4.2.2 Induction of phage-like plasmids (PLPs):

The PLPs AnCo1, SJ1, and MA725 were induced from their hosts in order to obtain PLP lysates for transduction experiments. A subculture was prepared for *E. coli* 243 (harbors AnCo1), *Salmonella* Kouka (harbors SJ1), and *E. coli* XX-29725 (harbors MA725), by inoculating a colony of each into a 10 ml aliquot of LBB, that was modified as follows: 1 mg/ml of cefotaxime (Sigma-Aldrich, USA) for growth of *E. coli* 243, 50 μg/ml of mercury chloride (Sigma-Aldrich, USA) for growth of *S.* Kouka, and 40 μg/ml potassium tellurite (Sigma-Aldrich, USA) for growth of *E. coli* XX-29725. Each subculture was incubated at 37°C with orbital shaking at 225 rpm for 16 hours. The following day, subcultures were added to 1 L of LLB in a 2 L Erlenmeyer flask, and incubated at 37°C with orbital shaking at 225 rpm. When cells reached mid-logarithmic phase (OD_{600} 0.5), Mitomycin C (Sigma-Aldrich, USA) was added at a concentration of 2 μg/ml to induce the PLPs and incubated at 37°C with orbital shaking at 50 rpm for 20 hours. The following day, an OD_{600} reading <1.5 indicated that induction of PLPs had occurred. Cultures were centrifuged at 4°C at a speed of 16,000 x g for 30 minutes to remove bacterial debris, and the supernatant containing the PLP lysate was recovered. Phage-like plasmid lysates were filter-sterilized using Acrodisc Syringe Filters (0.8/0.2 μm) (Pall Laboratory, New York, USA) and PLP lysates were stored at 4°C for up to a week.

4.2.3 Transduction:

The host range of the PLPs via transduction was investigated using several bacterial strains (Table 4.3) as recipients. For each recipient bacterial strain, one colony was inoculated into 5 ml of LBB and incubated at 37°C with orbital shaking at 225 rpm, until cells reached mid-logarithmic phase (OD_{600} 0.5). Meanwhile, 2 g of Amberlite ion-exchange resin IRA-900 (Sigma-Aldrich, USA) and 100 ml of PLP lysate were added to a conical Falcon™ tube and orbitally shaken at top speed for 1.5 hours at room temperature to bind the PLPs to the Amberlite resin. The supernatant was then decanted and transduction performed by adding the liquid culture (OD_{600} 0.5) to the conical tube containing PLP Amberlite resin complex. To promote PLP infection of bacterial cells, this mixture was incubated at 37°C with orbital shaking at 225 rpm for 1 hour. Next, the supernatant was transferred to a 15 ml culture tube, and incubated at

37°C with orbital shaking at 225 rpm for 16 hours. The cultures were then centrifuged at 4,300 x g for 10 minutes, the supernatant was discarded, and the bacterial pellet was resuspended in 200 µl of fresh LBB before being plated onto modified LBA plates supplemented with the corresponding antibiotic or heavy metal concentration (30 µg/ml of cefotaxime for AnCo1, 20 µg/ml of mercury chloride for SJ1, and 30 µg/ml of potassium tellurite for MA725). Plates were incubated 37°C for 24 hours. Transduction was confirmed by PCR as per section 4.2.7.

4.2.4 Competent cell preparation for transformation:

MAX Efficiency DH10B Competent Cells (ThermoFisher Scientific, USA), were prepared for transformation by inoculating a colony into 5 ml of LBB and incubating overnight at 37°C with orbital shaking at 225 rpm for 16 hours. The overnight culture was transferred to 1 L of LBB in a 2 L Erlenmeyer flask and incubated at 37°C with orbital shaking at 225 rpm until the culture reached an OD_{600} of 0.7. The cells were incubated on ice for 20 minutes and then centrifuged in cold 250 ml containers at 1000 x g for 20 minutes at 4°C. The supernatant was discarded, and the pellets were resuspended in cold 10% glycerol at half the original volume (500 ml) before being centrifuged at 1000 x g for 20 minutes at 4°C. This step was repeated twice. Cells were then pooled and resuspended in 20 ml of cold 10% glycerol and centrifuged at 1000 x g for 20 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 1 ml of cold 10% glycerol. Cells (40 µl) were aliquoted into pre-chilled (–80°C) Eppendorf tubes and then stored at –80°C.

4.2.5 Transformation by electroporation:

For transformation experiments, the PLPs AnCo1, SJ1, and MA725 were isolated as plasmids using the Qiagen Plasmid Maxi Kit (Qiagen, Hilden, Germany), as per the manufacturer's instructions. Eppendorf tubes (one for each PLP) containing 40 µl competent cells maintained at –80°C, were thawed on ice for 20 minutes. Phage-like plasmids AnCo1, SJ1, and MA725 DNA (1–2 µl at a concentration of 330 ng/ml) was added to an individual Eppendorf tube and incubated on ice for 10 minutes. The mixture was then transferred to a pre-chilled electroporation cuvette (0.1 cm gap) (Bio-Rad, Hercules, California, USA) and incubated for

another 10 minutes. Electroporation was performed on the Gene Pulser (Bio-Rad, Hercules, California, USA) with the following conditions: 200 Ω, 25 μF, field strength of 1.7 kV/cm, and a 5 second pulse. Electroporated cells were recovered in 1 ml of Super Optimal broth with Catabolite repression (SOC) medium (Sigma-Aldrich, USA) within 30 seconds, and incubated at 37°C with orbital shaking at 225 rpm for 2.5 hours for AnCo1 and MA725, and 70 minutes for SJ1. Cells (300 μl) were plated onto modified LBA plates supplemented with appropriate antibiotics or heavy metals as describe above and incubated at 37°C for 24 hours. In addition, plasmids pBR322 (New England BioLabs (NEB), Ipswich, Massachusetts, USA) and pRK2013 (Cedarlane, Canada) were transformed into *E.* coli DH10B using the same protocol as above with the exception of a recovery incubation time of 70 minutes and the addition of 50 ng/ml of DNA to the competent cells (Ditta et al., 1980). Transformation was confirmed by PCR as per section 4.2.7.

4.2.6 Conjugation:

Conjugation was performed using a broth method developed in this study. The helper plasmid pRK2013 was transformed into *E. coli* DH10B cells that contained AnCo1 and *E. coli* DH10B that contained SJ1 using the protocol described above. Since donor strains were resistant to the same concentration of sodium azide (Sigma-Aldrich, USA) as the recipient strain *E. coli* J53 (Cedarlane, Canada), pBR322, conferring tetracycline resistance, was transformed into the recipient strain in order to use tetracycline chloride (Sigma-Aldrich, USA) as the selective agent for transconjugants.

Overnight cultures of donor *E. coli* DH10B containing AnCo1 and pRK2013, donor *E. coli* DH10B containing SJ1 and pRK2013, and recipient *E. coli* J53 containing pBR322 were prepared by inoculating a well isolated colony of the respective bacteria into a culture tube containing 5 ml of modified LBB with appropriate antibiotics or heavy metals. The following day, conjugation was performed at ratio of 2:1 (50 μl of the donor strain to 25 μl of the recipient strain) in 3 ml of LBB without any antibiotics or heavy metals, followed by incubation at 37°C with orbital shaking at 225 rpm for 16 hours. The following day, cultures were centrifuged at 4,300 x g for 10 minutes, and the pellet was resuspended in 200 μl of fresh LBB before being plated onto modified LBA plates supplemented with antibiotics or heavy metal concentrations

(50 µg/ml of cefotaxime and tetracycline chloride for AnCo1, 50 µg/ml of mercury chloride and tetracycline chloride for SJ1). Plates were incubated at 37°C for 24 hours. Conjugation was confirmed by PCR as per section 4.2.7.

4.2.7 PCR confirmation of phage-like plasmids (PLPs) after transduction, conjugation, and transformation

Colonies that grew on plates after HGT experiments were selected and grown in 5 ml of modified LBB supplemented with their antibiotics or heavy metals as described, at 37°C with orbital shaking at 225 rpm for 16 hours. Cultures were centrifuged at 4,300 x g for 10 minutes, the supernatant discarded and the pellet recovered. A plasmid extraction was performed using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany), as per the manufacturer's instructions. To confirm the presence of the respective PLP, a PCR was performed to detect the integrase gene of the PLPs. PCR amplification was performed in a Peltier Thermal Cycler (PTC-100, Bio-Rad Laboratories, Inc., Mississauga, ON), and commenced with DNA denaturation for 5 min at 94°C, followed by 32 cycles consisting of DNA denaturation at 94°C for 30 s, annealing (55°C for AnCo1, 53°C for SJ1, 60°C for MA725) for 30 s, extension at 72°C for 90s, and a final extension step at 72°C for 10 min. Primer set sequences (Integrated DNA technologies (IDT), Coralville, Iowa, USA) were as follows: AnCo1: forward: AGGCCGTTATGATCTCGCAG reverse: GCTCCATCAGTTCTGGTCGT; SJ1: forward: CTAGGATCAGAGTTAAAGATATC reverse: GCATGATCTCCGGTAGTG; MA725: forward: GCAGAACGAAAACGCTGGTT reverse: TTGCCCCCGTTTCACTATCC . PCR amplicons were 728 bp for AnCo1, 366 bp for SJ1, and 411 bp for MA725, and were resolved by electrophoresis in 1X Tris/Borate/EDTA (TBE) buffer on 1% (w/v) agarose gels that contained 1x SYBR Safe stain (Thermo Fisher Scientific, USA). Following gel electrophoresis, amplicons were visualized under UV illumination.

4.2.8 Minimum inhibitory concentrations (MICs) for mercury chloride, potassium tellurite, cefotaxime salt and colistin sulphate salt:

Minimum inhibitory concentrations (MICs) were determined by the broth microdilution method (Microbiology and Diseases, 2003) in a 96-well plate containing 200 µl of an antibiotic or heavy metal solution in LBB. The concentration range was 5–80 µg/ml for mercury chloride

and potassium tellurite, 0.5–5 mg/ml for cefotaxime, and 2–30 μg/ml for colistin sulphate salt (Sigma-Aldrich, USA). Overnight cultures of *E. coli* DH10B cells containing PLPs AnCo1, SJ1, and MA725 introduced by transduction, transformation and conjugation, were prepared and 5 μl were added to each well. The plates were incubated statically at 37°C for 24 hours. The highest concentration that inhibited bacterial growth, as determined visually, was recorded as the MIC.

4.3 Results:

4.3.1 Phage-like plasmids (PLPs) transfer antibiotic resistance genes (ARGs), and mercury and tellurite resistance genes by transduction

The host range of 3 PLPs (AnCo1, SJ1 and MA725) was investigated via transduction. SJ1 demonstrated the largest host range, infecting 18 different bacterial strains by transduction (Table 4.3), including 2 *E. coli* strains, 4 *E. coli* O157 strains, and 12 *Salmonella* strains, of which 11 are known to be in the top 14 serovars causing illness in Canada (PHAC, 2016). In contrast, AnCo1 and MA725 had limited host ranges, as they each infected 1 *E. coli* strain each (Table 4.3).

Many phages use components of the lipopolysaccharide as receptors for bacterial attachment. For example, the prototypical PLP P1 used uses the terminal glucose of the LPS core as a receptor and other well known phages such as T4, T7 and P22 use components of the LPS as receptors (Sandulache et al., 1984)(Rakhuba et al., 2010). There exists 5 core types in *E. coli* (R1-R5) with *E. coli* R1 core type being the most prevalent among clinical isolates of *E. coli* (Heinrichs et al., 1998). To precisely define the chemical structure of the LPS, Heinrichs *et al.,* (1998) specifically inserted a nonpolar gentamicin resistance cassette (*aaaC1* from transposon TN1696) by in vitro mutagenesis into specific *waa* genes, which encode glycosyltransferase enzymes, of *E. coli* F470 creating a set of 8 *E. coli* LPS derivative strains. These strains lack specific glycosyltransferase enzymes that catalyze the linkage of carbohydrate residues in the core LPS (Figure 4.1A).

To investigate the possibility that the PLPs infect their hosts via components of the LPS, 3 PLPs were investigated for their potential to infect the panel of 8 *E. coli* derivative strains developed by Heinrichs et al. (1998) to provide insight into the possible LPS carbohydrate

residues PLPs use as receptors. PCR analyses for the integrase genes of the 3 PLPs revealed that they were each transduced to 1 *E. coli* LPS derivative strain: MA725 to CWG309::*waaT,* AnCo1 to CWG310::*waaW* and SJ1 to CWG297::*waaQ* (Figure 4.2). Derivative strains CWG309 and CWG310 have deletions for *waaT* and *waaW*, respectively, in the outer core of the LPS, while CWG297 has a deletion for *waaQ* in the inner core (Yethon et al., 2000). *waaT* encodes an α-1,2-galactosyltransferase involved in the synthesis of the D-galactose I residue in the outer core OS, which suggests that MA725 may target the D-glucose II residue as its receptor in the LPS (4.1B) (Heinrichs et al., 1998). *waaW* encodes an α-1,2-galactosyltransferase enzyme that adds the side branch D-galactose II in the outer core OS, which suggests that AnCo1 may target the D-galactose I residue as its receptor (Figure 4.1C) (Leipold et al., 2007). Finally, *waaQ* encodes the L-glycero-D-manno-heptose transferase for the L-glycero-D-manno-heptose III residue in the inner core OS, which suggests that SJ1 may target the L-glycero-D-manno-heptose II residue as its receptor in the LPS (Figure 4.1D) (Yethon et al., 1998).

Collectively these results demonstrate for the first time that PLPs can transfer ARGs and HMR resistance genes to non-pathogenic *E. coli* and *Salmonella enterica*. Moreover, AnCo1, SJ1 and MA725 may use specific LPS residues D-galactose II, L-glycero-D-manno-heptose II and D-glucose II, respectively, as receptors.

4.3.2 Phage-like plasmids (PLPs) transfer antibiotic resistance genes (ARGs) and mercury and tellurite resistance genes by transformation and conjugation

Transformation and conjugation experiments were performed to determine whether PLPs could transfer ARGs and HMR genes via plasmid-mediated mechanisms. Transformation of AnCo1, SJ1, and MA725 was performed by electroporation to *E. coli* DH10B. Despite the large genome sizes of AnCo1 and MA725, PCR targeting the integrase genes of each PLP confirmed that all 3 representative PLPs were transformed to *E. coli* DH10B (Figure 4.3).

Genomic analysis (Chapter III, section 4.2) showed that PLPs do not harbour the genes necessary to facilitate conjugation of their genome, rendering them non self-transmissible. However, it known that non-self transmissible plasmids can take advantage of conjugation genes (*tra, rlx, mob* and *oriT*) harboured by a co-resident conjugative plasmids to facilitate conjugation (Bellanger et al., 2014). For example, Gillis et al. (2017) demonstrated the conjugative transfer

of PLP pBtiC235 in the presence of a co-resident conjugative plasmid, pXO16. Thus, in order to investigate the potential transfer of PLPs by conjugation, helper plasmid pRK2013 was transformed into *E. coli* DH10B containing AnCo1, and *E. coli* DH10B containing SJ1. With pRK2013 as a co-resident conjugative plasmid, PCR targeting the integrase genes of the PLPs confirmed that AnCo1 and SJ1 were conjugated to recipient strain *E. coli* J53 (Figure 4.4).

For reasons unknown, MA725 and pRK2013, were unable to co-reside within *E. coli* DH10B, thus, conjugation could not be attempted. MA725 (IncY) and pRK2013 (IncPα) are of different incompatibility groups, thus, they should be able to co-reside within the same host cell (Pansegrau et al., 1994). pRK2013 is a RK2 plasmid derivative and harbours a plasmid maintenance system called the *kil* operon, which consisting of 4 genes, *kilA, kilB, kilC and kilE* (Pansegrau et al., 1994*)*. The upregulation of the *kil* operon causes host cell lethality. It could be hypothesized that for an unknown reason, the presence of MA725 induces the expression of the *kil* operon of pRK2013 (Goncharoff et al., 1991).

Collectively, these results demonstrate that SSU5-like and P1-like PLPs can be horizontally transferred as plasmids by transformation and conjugation, in addition to phage-mediated mechanisms.

4.3.3 Minimum inhibitory concentrations (MICs) of PLPs to antibiotics, mercury and tellurite

In this study, PLPs AnCo1 and SJ1 were successfully horizontally transferred via all 3 HGT mechanisms while PLP MA725 was horizontally transferred by transduction and transformation. To investigate whether these PLPs confer resistance to antibiotics and heavy metals after HGT, minimum inhibitory concentrations (MICs) of appropriate antibiotics and heavy metals were measured. After being horizontally transferred by transduction (*E. coli* CWG310), transformation (*E. coli* DH10B), and conjugation (*E. coli* J53), the MIC of AnCo1 was 3 mg/ml of resistance to cefotaxime (Figure 4.5A). After HGT of SJ1 via transduction (*E. coli* CWG297), transformation (*E. coli* DH10B), and conjugation (*E. coli* J53), the MIC of the PLP was 50 μg/ml to mercury chloride (Figure 4.5B). Finally, after HGT of MA725 via transduction (*E. coli* CWG309) and transformation (*E. coli* DH10B), the MIC of MA725 was 40 μg/ml to potassium tellurite (Figure 4.5C). The level of resistance of the individual PLPs to

cefotaxime, mercury chloride and potassium tellurite remained the same regardless of the HGT mechanism. Resistance to potassium tellurite conferred by the *terB* gene of PLP MA725 is significant because the scientific literature indicates that multiple *ter* genes (*terB, terC, terD, terE*) are necessary to confer resistance (Taylor et al., 2002).

Furthermore, the *terB* gene of MA725 was investigated for its potential to confer cross-resistance to colistin sulphate salt because several studies previously demonstrated that the *ter* operon also confers resistance to channel-forming colicins (Jobling and Ritchie, 1987; Whelan et al., 1997; Alonso et al., 2000a), which have a similar mode of action to colistin (Figure 4.6). Channel-forming colicins first bind to a specific receptor on the outer membrane of the bacteria (Figure 4.6A) (Alonso et al., 2000b). After receptor recognition, they are translocated through the outer membrane and their C-terminus attaches to the cytoplasmic membrane where they insert themselves into it, which forms a channel through the membrane (Alonso et al., 2000b). Colistin disrupts the integrity of the outer membrane by displacing divalent cations that stabilize the lipid A component of the LPS, which facilitates active uptake of the antibiotic, pore formation and bacterial cell death (Figure 4.6B) (Mohamed et al., 2016). Thus, it was hypothesized that *terB,* harboured by PLP MA725 may confer resistance to colistin sulphate salt and interestingly results demonstrated that it conferred 10 μg/ml of resistance to this antibiotic (Figure 4.7).

Collectively, these results demonstrated that AnCo1, SJ1, and MA725 conferred resistance to cefotaxime, mercury chloride, and potassium tellurite, respectively, regardless of the mechanisms of HGT. Results also demonstrated for the first time that MA725 conferred cross-resistance to colistin sulphate, which is of major clinical significance because colistin is a last resort antibiotic for treatment of Gram-negative infections.

4.4 Discussion:

The objective of this study was to investigate the potential of PLPs to disseminate ARGs (CTX-M-15) and HMR determinants (*mer* operon and *terB*) to bacteria of foodborne importance by the three HGT (transduction, transformation and conjugation) mechanisms to determine if the

transfer of ARGs and HMR determinants confers resistance to antibiotics and heavy metals in host bacteria and finally to determine if HGT mechanism influences the MICs of the antibiotic and heavy metals. In Chapter III, PLPs AnCo1 and AnCo2 were found to harbour CTX-M-15, an extended-spectrum β-lactamase (ESBL) enzyme that has invaded environments, animals, and humans worldwide (Cantón et al., 2012). Phage-like-plasmid SJ1 was found to harbour a mercury resistance operon, and PLPs MA725, JEC735, SN747, and DH728 were found to harbour tellurite resistance gene, *terB*. These analyses also demonstrated that AnCo1 and AnCo2 were SSU5-like PLPs, while the remaining PLPs were P1-like. Since no PLPs have been investigated for their potential to disseminate ARGs or HMR genes via transduction, one PLP from each lineage (AnCo1 for SSU5 and MA725 for P1) was selected for transduction. Phage-like-plasmid SJ1 was also included in this analysis since no studies thus far have demonstrated the transduction of a mercury resistance operon. One key finding from this study was the demonstration, for the first time, that PLPs could be horizontally transferred by transduction and of the PLPs analyzed, PLP SJ1 was observed to have the largest host range, with the infection of 18 different *E. coli* and *Salmonella enterica* strains. Although MA725 did not have a wide host range, to the best of our knowledge, the transduction of MA725 and SJ1 represents the first observation of transduction of HMR genes. The ability of PLPs to transduce ARGs and HMR genes also emphasizes the contribution PLPs to the spread of AR worldwide. More importantly, given the cross-resistance to colistin sulphate conferred by the *terB* gene of MA725, the transduction of HMR genes by PLPs in this study is clinically significant.

In order to investigate the LPS as a potential receptor used by the PLPs, a panel of 8 *E. coli* LPS derivative strains with specific LPS glycosyltransferase enzyme deletions were used as transduction recipients. In *E. coli* and *Salmonella enterica*, the LPS consists of 3 components: the hydrophobic membrane anchor lipid A, a phosphorylated oligosaccharide chain composed of 10–15 sugar molecules called the core oligosaccharide (core OS), and the O-antigen consisting of a variable polysaccharide (Yethon et al., 2000). There exists 5 distinct core OS types (R1–R5) in *E. coli* and the panel of 8 LPS derivatives were constructed based on *E. coli* F470 core type R1 and were constructed to be sequentially different in the *waa* gene contained within the *rfa* operon (Heinrichs et al., 1998; Yethon et al., 1998). Phage-like plasmids AnCo1, MA725, and SJ1 were horizontally transferred by transduction to 3 different *E. coli* LPS derivative strains, including *E. coli* CWG310 (absence of *waaW*), *E. coli* CWG309 (absence of *waaT*), and *E. coli*

CWG297 (absence of *waaQ*), respectively. *waaW* encodes an α-1,2-galactosyltransferase enzyme that adds the side branch D-galactose II onto the in the outer core OS. The activity of *waaW* is essential for *waaV* to encode a β-1,3-glucosyltransferase that will ligate β-glucose, which is the final core OS residue before ligation of the O-antigen component (Leipold et al., 2007). *waaT* encodes an α-1,2-galactosyltransferase involved in the synthesis of the D-galactose I residue in the outer core OS (Heinrichs et al., 1998). *waaQ* encodes the L-glycero-D-manno-heptose transferase for the L-glycero-D-manno-heptose III residue in the inner core OS (Yethon et al., 1998). The activity of *waaQ* is a prerequisite for *waaY* to phosphorylate the L-glycero-D-manno-heptose II residue (Yethon et al., 1998). Clinical *E. coli* isolates have been mainly found to be of core type R1 and Heinrichs et al., (1998) demonstrated that *Salmonella enterica* and *Shigella sp*., have similar R1 core types, thus they could be part of the host range of PLPs. The core OS of the LPS of *Pseudomonas aeruginosa*, *Vibrio cholerae, Burkholderia sp.,* and *Neisseria sp*., have also been determined but their structures are very different from the *E. coli* R1 core type (Holst and Brade, 1999). However, the core type of *P. aeruginosa* does contain L-glycero-D-manno-heptose II residue, which this study hypothesizes as being a potential receptor for PLP SJ1 (Holst and Brade, 1999). Thus, in addition to *E. coli* and *Salmonella enterica*, SJ1 may potentially infect *P. aeruginosa*.

In addition to transduction, AnCo1, SJ1, and MA725 were also successfully transformed to *E. coli* DH10B. Despite the large genome sizes of AnCo1 (112.2 kb) and MA725 (103.8 kb), these PLPs have the ability to be transferred by transformation. Other instances of large plasmids (>240 Kb genomes) being transformed in the laboratory setting have also been observed (Sheng et al., 1995). In the environment, *E. coli* is not known to be naturally competent; however, recent studies suggest that environmental factors can render *E. coli* competent and influence transformation. In food, Bauer et al. (1999) observed that 12 different foods at temperatures below 5°C facilitated plasmid transformation to *E. coli*. Milk, soy drink, tomato and orange juice demonstrated the highest frequencies of transformation. Maeda et al. (2003), observed that 10 of the 42 food samples tested showed an ability to induce competency as well. Among them, tofu, had the highest activity, which corresponded to approximately half of the activity of the concentration of calcium chloride required to make bacterial cells competent in the laboratory. Ishimoto *et al.,* (2008), observed that freeze-thawing of natural water samples (sea and river water), and food extracts including tofu, tomato, egg white, radishes, and chicken, induced the

transfer of non-conjugative plasmids to *E. coli* strains that were spiked in the food and water samples. These results suggested that the freeze-thawing conditions induced competence in these *E. coli* strains (Ishimoto et al., 2008). Hasegawa et al. (2018), also argued that *E. coli* can develop competence under certain conditions that can occur in the environment. These authors observed that standard *E. coli* strains could be transformed in commercially available bottled water between temperatures of 0°C and 35°C (Matsumoto et al., 2016; Hasegawa et al., 2018). In addition to the environmental factors that can induce competence in *E. coli*, there are specific phages termed "superspreaders" that promote the release of substantial amounts of intact plasmid DNA upon bacterial cell lysis, which promotes HGT by transformation and efficiently disperses ARGs (Keen et al., 2017). Keen et al., (2017) observed that superspreader phage SUSP2 could promote plasmid transformation and the transfer of ARGs to a heterogeneous population of soil bacteria in the absence of artificial laboratory protocols. Thus, the potential of PLP transformation, and spread of ARGs and HMR genes in *E. coli* in the environment is significant (Keen et al., 2017). Moreover, Sugiura *et al.,* (2008), demonstrated that a P1vir phage particle or a derivative can promote transformation of plasmids between *E. coli* cells. P1vir phage is a mutant virulent strain of PLP P1 that may induce lysis of *E. coli* by infection, or spontaneous awakening of a lysogenized phage in plasmid-harbouring bacterial cells (Sugiura et al., 2017). Upon lysis of the host, plasmid DNA that was present in the host is released and can be taken up by other hosts in the environment (Sugiura et al., 2017). These studies suggest that superspreader phages or phages with similar activity to P1vir could help promote the release of PLP DNA and transformation in the environment.

Phage-like plasmids AnCo1 and SJ1 were successfully transferred to *E. coli* J53 via conjugation. Conjugative transfer of ARGs and HMR genes has been extensively documented in the laboratory, environment and gastrointestinal tract of animals and wildlife (Grohmann et al., 2003; Bañuelos-Vazquez et al., 2017). The genomic characterization of PLPs in Chapter III determined that PLPs do not encode conjugation machinery, however since non self-transmissible plasmids can use conjugation genes on other plasmids and integrative conjugative elements (ICEs) within a host cell (Bellanger et al., 2014), a helper plasmid pRK2013 was used in this study to facilitate conjugation. In the presence of pRK2013, PLPs AnCo1 and SJ1 were conjugated to *E. coli* J53.

Several studies have demonstrated the prevalence of conjugative plasmids in environmental and laboratory strains as well as in plasmid sequences in Genbank. For example, Hughes and Datta (1983) investigated 433 different strains that included the genera *Salmonella sp., Shigella sp., E. coli*, *Klebsiella sp*., and *Proteus sp*., and determined that 24% of these strains contained conjugative plasmids. In accordance with these results, Smillie et al. (2010) investigated the presence of conjugation and mobilization genes in 1,730 plasmid sequences in Genbank and determined that 25% of these plasmids encoded genes for conjugation. This percentage has probably increased since 2010 due to the reduced cost of whole genome sequencing in recent years. Alvarez et al. (2004) identified even higher percentages of conjugative plasmids in bacteria. The authors investigated the number of conjugative plasmids involved in β-lactamase transfer in 752 resistant isolates of *Klebsiella pneumoniae, Klebsiella oxytoca* and *E. coli* from 70 different sites in 25 U.S. states. They demonstrated that conjugative plasmids conferring β-lactamase genes were present in 59% of *Klebsiella pneumoniae,* 24% of *Klebsiella oxytoca* and 44% of *E. coli.* In addition to plasmids, the presence of integrative and conjugative elements (ICEs) in bacteria can also help facilitate the conjugation of PLPs. Due to the novelty of ICE, their prevalence in bacteria and in the environment has not been described in the literature, however, Wozniak and Waldor (2010) summarized experimentally described ICE within 8 different species of the bacteria within the *Enterobacteriaceae* family.

These studies suggest that conjugative plasmids may be present in approximately 25% of isolates studied in these publications and in Genbank and demonstrates the potential for PLPs to be horizontally transferred by conjugation to different hosts in the environment.

After investigating the HGT ability of PLPs, MIC experiments were performed to determine whether PLPs could confer resistance to antibiotics, and heavy metals. Results determined that the CTX-M-15 resistance gene harboured by AnCo1 conferred 3 mg/ml resistance to third-generation cephalosporin, cefotaxime regardless of the HGT method. This finding is significant because studies define strains as cefotaxime-resistant when MICs >2 μg/ml are observed (Jacobs et al., 1996; Amaya et al., 2012; Mir et al., 2016). However, it is also important to consider the relationship of MICs to clinical cefotaxime treatment. Although data is unavailable for adults and children, it is available for neonates, where a 25 mg/kg intravenous dose is recommended for neonates every 12 h for less than 7 days, producing a serum concentration of 60–80 μg/ml. After 1 week of life, the recommended dose is 50 mg/kg dose

every 8–12 h, producing 45–186 μg/ml in the serum (Finch et al., 2010; Wade and Benjamin, 2011). Thus, infection by bacteria harbouring AnCo1 encoding CTX-M-15, conferring resistance at 3 mg/ml, would be clinically untreatable with cefotaxime.

Minimum inhibitory concentration experiments also determined that the *mer* operon, carried within PLP SJ1, conferred 50 μg/ml of resistance to mercury chloride to hosts *E. coli* DH10B, *E. coli* J53, and *E. coli* CWG297. This is significant because as discussed in chapter III (section 3.4) mercury resistance and antibiotic resistance have been demonstrated to be associated through co-selection mechanisms in the environment (McIntosh et al., 2008; Wardwell et al., 2009; Drudge et al., 2012; Lloyd et al., 2016). Skurnik et al. (2010) suggested that exposure to mercury could be a driving force in the co-resistance of mercury and antibiotics when antibiotic selective pressure is absent. There have been few studies that have characterized mercury resistance in bacteria. Essa et al. (2003), investigated mercury resistance in *E. coli*. The authors of this study observed that *E. coli* plasmid M634, harbouring a *mer* operon, conferred 20 μg/ml of resistance to mercury chloride after conjugation to recipient strain E. *coli* TG2. Genomic analysis demonstrated that the *mer* operon of plasmid M634 shares 99% nucleotide sequence identity to the *mer* operon and transposon Tn21 of PLP SJ1. Thus, this further confirms suggest that the *mer* operon of SJ1 provides, through infection, *E. coli* and *Salmonella enterica* hosts with significant resistance to mercury chloride. Rahman and Singh (2016) demonstrated that the MICs of Gram-negative and Gram-positive bacteria (genus and species unspecified) to mercury chloride ranged from 50–100 mg/L (or 50–100 μg/ml). Mercury resistance in *Salmonella* Typhi ranges from 60–250 μg/ml (Jevanand et al., 1997). *Arthrobacter sp.*, *Bacillus sp.*, *Citrobacter sp.*, *Enterobacter sp., Pseudomonas sp.,* and *Vibrio* sp. isolates have been observed to be resistant to between 5 and 25 μg/ml to mercury chloride, and demonstrate to morphological changes in their growth at these concentrations (Vaituzis et al., 1975). Collectively, these studies suggest that the resistance to mercury chloride conferred by PLP SJ1 may be on the low end compared to the concentrations observed in the literature.

Minimum inhibitory concentration experiments also determined that the *terB* gene, carried within PLP MA725, conferred 40 μg/ml of resistance to potassium tellurite to hosts *E. coli* DH10B, and *E. coli* CWG309. There are 5 genetic determinants of tellurite resistance, of which 3 are chromosomally encoded including the *tmp* gene, *trgA, trgB, crsK* operon and *tehA* and *tehB* operon, while 2 mechanisms are plasmid-mediated, including the *kilA, telA, telB* operon

and the *terABCDEFZ* operon (Chasteen et al., 2009). Although there are different tellurite resistance operons, only one study has investigated the MIC of potassium tellurite conferred by the *ter* operon in *E. coli*. Taylor *et al*., (2002), observed that *E. coli* O157:H7 EDL933 containing a complete *ter* operon conferred between 64 and 128 μg/ml of resistance to potassium tellurite compared to 1 μg/ml conferred by the control strain *E. coli* DH5 alpha. The authors observed that genes *terB, terC, terD* and *terE* are essential for resistance to potassium tellurite; however, results from this study (chapter IV, section 4.3.3) demonstrate for the first time that *terB*, harboured by MA725, conferred resistance of 40 μg/ml without other *ter* genes. The level of resistance conferred by *terB* in MA725 is significant because the complete *ter* operon in *E. coli* O157:H7 EDL933 confers between 64 and 128 μg/ml of resistance to potassium tellurite. Of note, although Taylor et al., (2002), used Shiga-toxin producing *E. coli* (STEC) strains in their study, the authors observed no correlation between the production of *stx* and potassium tellurite resistance levels; thus, the resistance conferred by MA725 can be compared to *E. coli* O157:H7 EDL933.

Although this study now demonstrates that *terB* alone confers resistance to potassium tellurite, as mentioned above it was originally believed that the complete *ter* operon (*terABCDEFZ*) was required to confer resistance to potassium tellurite. Whelan et al., (1997) described an IncHI2 conjugable plasmid, R478, which has a resistance region harbouring a complete tellurite resistance operon (*terABCDEFZ*) and genes that mediate resistance to phages as well as channel-forming colicins (PacB). The authors observed that when *terZ, terC* or *terD* were removed, resistance to phage T5, colicins A, B and K and potassium tellurite were abolished (Whelan et al., 1997). The resistance phenotype to tellurite and colicins was also demonstrated on plasmid pMER610 and pMip233 (Jobling and Ritchie, 1987; Alonso et al., 2000a). Łobocka et al. (2004) reported that another gene, *tciA*, has homology to *terB* and the authors proposed that this gene confers resistance to channel-forming colicins as well as potassium tellurite and some bacteriophages.

Based on these studies and on the fact that PLP MA725 harboured *terB*, it was hypothesized that *terB* would confer resistance to channel-forming colicins as well as potassium tellurite. Furthermore, it was hypothesized that *terB* would also confer resistance to colistin through two potential mechanisms. First, due to the similarities in the mode of action of channel-forming colicins and colistin (Figure 4.6) and due to the fact that *terB* has been shown to attach

to the periplasmic membrane (Alekhina et al., 2011) and may even form a periplasmic transmembrane domain (Anantharaman et al., 2012), it was hypothesized that in addition to conferring resistance against channel forming colicins, *terB* would also confer resistance to colistin. Second, both tellurite and colistin have been proposed to affect bacterial viability through superoxide formation. For example, Pérez et al. (2007) showed that tellurite toxicity in *E. coli* involves an increase of cytoplasmic reactive oxygen species (ROS) and an increase in *sodA, sodB ,soxS* and mRNA transcription leading to the generation of superoxide radicals during in vitro enzymatic reduction of potassium tellurite. Several research groups suggested that colistin inhibits bacterial survival through oxidative stress and ROS generation (Brochmann et al., 2014; Dong et al., 2015); Dong et al. 2015). In particular, Dong *et al.,* (2015) demonstrated that colistin induced transcription in *E. coli* of the oxidative stress gene soxS, caused an increase in ROS. The similarities between the mode of action of tellurite and colistin suggested that *terB* may confer resistance to colistin. Valková et al. (2007) showed that the *ter* operon increased the level of potassium tellurite resistance as well as the level of resistance to oxidative stress mediated by hydrogen peroxide, which led to the prolonged ability of uropathogenic *E. coli* to survive in macrophages. Finally, Malhotra-Kumar et al. (2016), isolated a plasmid, pKH-457-3-BE, that carried both *mcr-1,* conferring resistance to colistin, and a tellurite resistance operon (*terABCDEZ*). Due to the significance of colistin, being a last resort antibiotic for Gram-negative infections, the implication of *mcr-1* and *terB* co-selected on a PLP, that has the ability for HGT , has major public health implications.

Collectively, these results demonstrate that PLPs represent perhaps the most versatile MGE with respect as to how they can be transferred between bacteria. Further studies should focus on investigating if PLPs can infect strains through other cell surface receptors and whether the *mer* operon of SJ1 and *terB* of MA725 can confer cross-resistance to other antibiotics of clinical importance.

Table 4.1: List of bacterial strains used in this study.

Taxon	Strain	Source	Origin	Antibiotic or heavy metal resistance
Escherichia coli	243 (Harbouring PLP AnCo1)	Feces of wildlife from bovine feedlots, Colorado, USA	Dr. Lawrence Goodridge, McGill University	Cefotaxime
Escherichia coli	XX-29725 (Harbouring PLP MA725)	Bovine mastitis, France	Dr. Séamus Fanning, University College Dublin (UCD)	Potassium Tellurite
Salmonella Kouka	S42 (Harbouring PLP SJ1)	Oysters, Canada	SalFos Database	Mercury Chloride
Escherichia coli	DH10B	Laboratory strain	ThermoFisher	none
Escherichia coli	J53	Laboratory strain	Cedarlane	Sodium azide
Escherichia coli	CWG297 (LPS derivative *waaQ*::*aacC1)*	Laboratory	Dr. Chris Whitfield, University of Guelph	Gentamicin
Escherichia coli	CWG303 (LPS derivative *waaG*::*aacC1)*	Laboratory	Dr. Chris Whitfield, University of Guelph	Gentamicin
Escherichia coli	CWG308 (LPS derivative *waaO*::*aacC1)*	Laboratory	Dr. Chris Whitfield, University of Guelph	Gentamicin
Escherichia coli	CWG309 (LPS derivative *waaT*::*aacC1)*	Laboratory	Dr. Chris Whitfield, University of Guelph	Gentamicin
Escherichia coli	CWG310 (LPS derivative *waaW*::*aacC1)*	Laboratory	Dr. Chris Whitfield, University of Guelph	Gentamicin
Escherichia coli	CWG311 (LPS derivative *waaV*::*aacC1)*	Laboratory	Dr. Chris Whitfield, University of Guelph	Gentamicin
Escherichia coli	CWG346 (LPS derivative *waaF*::*aacC1)*	Laboratory	Dr. Chris Whitfield, University of Guelph	Gentamicin
Escherichia coli	CWG350 (LPS derivative *waaJ*::*aacC1)*	Laboratory	Dr. Chris Whitfield, University of Guelph	Gentamicin
Escherichia coli O157:H7	EC2079	Bovine feces	Dr. Lawrence Goodridge, McGill University	none
Escherichia coli O157:H7	EC2080	Bovine feces	Dr. Lawrence Goodridge, McGill University	none

Table 4.1 continued: List of bacterial strains used in this study.

Taxon	Strain	Source	Origin	Antibiotic or heavy metal resistance
Escherichia coli O157:H7	EC2081	Bovine-soil	Dr. Lawrence Goodridge, McGill University	none
Escherichia coli O157 toxin negative	ATCC 43888	unknown	Dr. Lawrence Goodridge, McGill University	none
Salmonella Typhimurium	S415	Animal, Québec, Canada	SalFos Database	none
Salmonella Heidelberg	S838	Chicken, Pennsylvania, USA	SalFos Database	none
Salmonella I 4, [5], 12:i:-	S377	Feces-Clinical, Québec, Canada	SalFos Database	none
Salmonella Newport	S1650	River surface water, Guelph, Canada	SalFos Database	none
Salmonella Infantis	S198	Pasta, Ottawa, Canada	SalFos Database	none
Salmonella Javiana	S200	Clinical, Vancouver, Canada	SalFos Database	none
Salmonella Typhi	S165	Clinical, Chili	SalFos Database	Rifampicin
Salmonella Saintpaul	S205	Shrimp, Toronto, Canada	SalFos Database	none
Salmonella bovismorvicans	S256	Beef, Vancouver, Canada	SalFos Database	none
Salmonella Agona	S1552	Cecal content of pork, Québec, Canada	SalFos Database	none
Salmonella Agona	S1869	Almonds, California, USA	SalFos Database	none
Salmonella Thompson	S1885	Almonds, California, USA	SalFos Database	none

Table 4.2: List of phage-like plasmids (PLPs) used in this study.

Phage-like plasmid	Bacterial host	Origin	Source	AMR or HMR	Publication
AnCo1	*Escherichia coli* 243	Colorado, USA	Wildlife feces in bovine feedlots	CTX-M-15	(Colavecchio et al., 2017d)
AnCo2	*Escherichia coli* 244	Colorado, USA	Wildlife feces in bovine feedlots	CTX-M-15	(Colavecchio et al., 2017d)
SJ1	*Salmonella* Kouka S42	Ottawa, Canada	Oysters	*merC*, *merE* and *merT*, mercury ion reductase, *merP* and a mercury resistance coregulator	n/a
MA725	*Escherichia coli* XX-29735	France	Bovine mastitis	*terB*	n/a
JEC735	*Escherichia coli* XX-22725	France	Bovine diarrhoea	*terB*	n/a
SN747	*Escherichia coli* XX-29747	France	Bovine mastitis	*terB*	n/a
DH728	*Escherichia coli* XX-29728	France	Bovine mastitis	*terB*	n/a

Table 4.3: Host range of phage-like plasmids AnCo1, SJ1 and MA725.

PLP	Taxon	Strain
AnCo1	*Escherichia coli*	CWG310
MA725	*Escherichia coli*	CWG309
SJ1	*Escherichia coli*	CWG297
SJ1	*Escherichia coli*	DH10B
SJ1	*Escherichia coli* O157:H7	EC2079
SJ1	*Escherichia coli* O157:H7	EC2080
SJ1	*Escherichia coli* O157:H7	EC2081
SJ1	*Escherichia coli* O157 toxin negative	ATCC 43888
SJ1	*Salmonella* Typhimurium	S415
SJ1	*Salmonella* Heidelberg	S838
SJ1	*Salmonella* I 4, [5], 12:i:-	S377
SJ1	*Salmonella* Newport	S1650
SJ1	*Salmonella* Infantis	S198
SJ1	*Salmonella* Javiana	S200
SJ1	*Salmonella* Typhi	S165
SJ1	*Salmonella* Saintpaul	S205
SJ1	*Salmonella* bovismorvicans	S256
SJ1	*Salmonella* Agona	S1552
SJ1	*Salmonella* Agona	S1869
SJ1	*Salmonella* Thompson	S1885

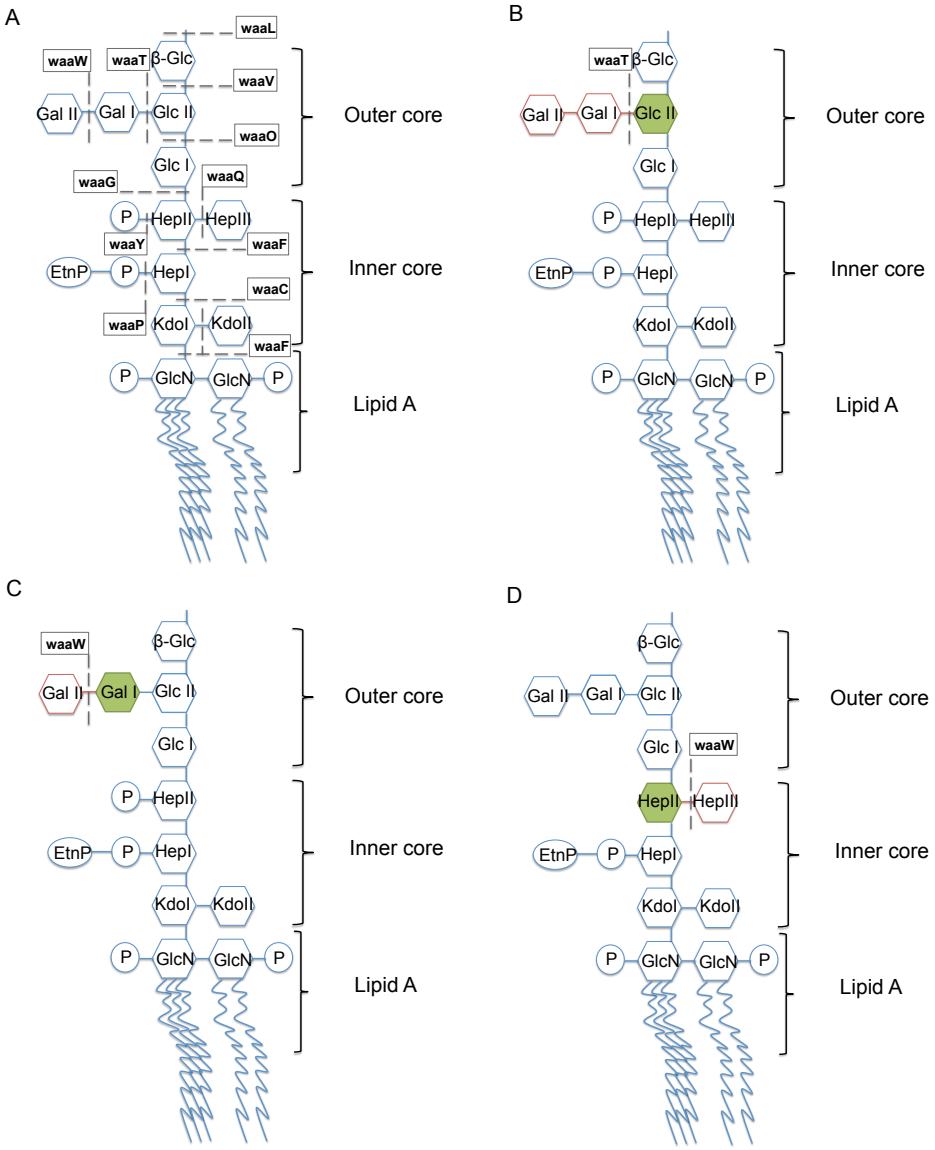

Figure 4.1. Structures of *E. coli* F470 lipopolysaccharide (LPS) R1 core type and *E. coli* F470 derivatives CWG309, CWG310 and CWG297. A) Structure of the *E. coli* F470 lipopolysaccharide (LPS) R1 core type (adapted from Yethon *et al*., 2000). B) Structure of the *E. coli* F470 derivative strain CWG309 with a deletion for glycosyltransferase enzyme waaT, which encodes the Gal I residue. C) Structure of the *E. coli* F470 derivative strain CWG310 with a deletion for glycosyltransferase enzyme waaW, which encodes the Gal II residue. D) Structure of the *E. coli* F470 derivative strain CWG297 with a deletion for glycosyltransferase enzyme waaQ, which encodes the Hep III residue.

Core residues are designated by sugar abbreviation and numbers to facilitate identification. Blue hexagon shapes demonstrate carbohydrate residues that are present within the derivative strain. Red hexagon shapes demonstrate carbohydrate residues that are absent because of the deletion of the glycosyltrasnferase that normally encodes it. Green hexagon shapes demonstrate the carbohydrate residue hypothesized to be the receptor of PLPs A) MA725, B) AnCO1 and C) SJ1. Abbreviations: GlcN: D-glucosamine; Hep: L-glycero-D-manno-heptose; P: phosphate; EtNP: 2-aminoethyl phosphate; Glc: D-glucose; and Gal: D-galactose.

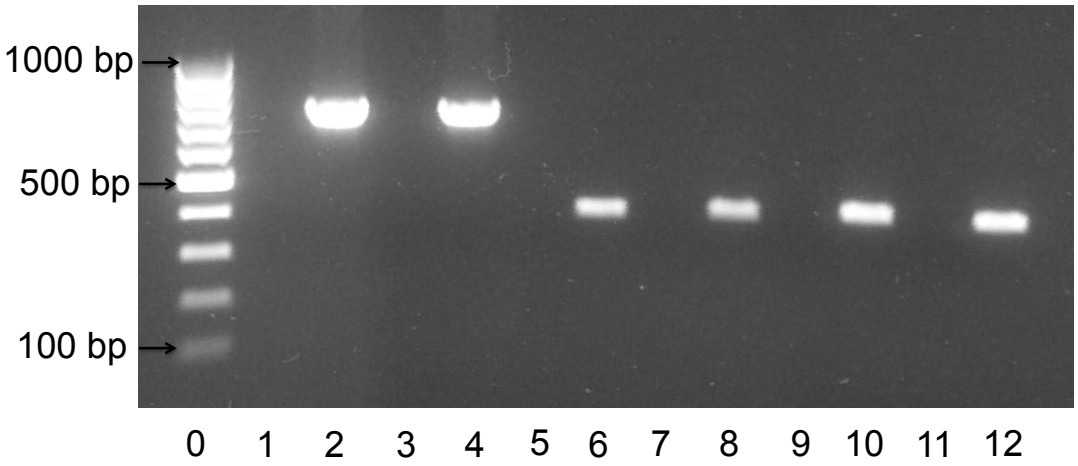

Figure 4.2. Gel electrophoresis of PCR products of the integrase genes from PLPs AnCo1, SJ1, and MA725 transduced to *E. coli* LPS mutants CWG310 (lane 4), CWG297 (lane 8), and CWG309 (lane 12).
0- 1 kb ladder; 1- Negative control (water); 2- Positive control (DNA of AnCo1from plasmid extraction); 3- Negative control (*E. coli* CWG310); 4- AnCo1 transduced to *E. coli* CWG310; 5- Negative control (water); 6- Positive control (DNA of SJ1 from plasmid extraction); 7- Negative control (*E. coli* CWG297); 8- SJ1 transduced to *E. coli* CWG297; 9- Negative control (water); 10- Positive control (DNA of MA725 from plasmid extraction); 11- Negative control (*E. coli* CWG309); 12- MA725 transduced to *E. coli* CWG309

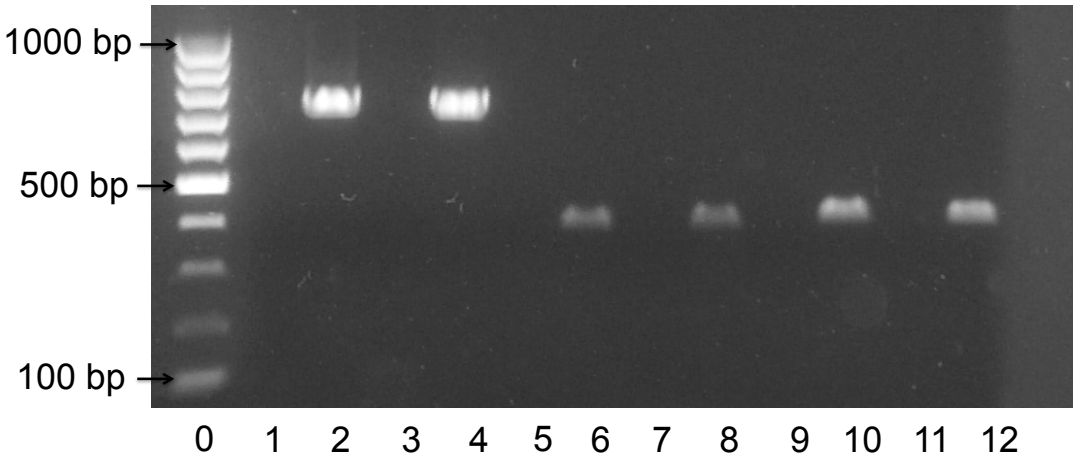

Figure 4.3. Gel electrophoresis of PCR products of the integrase genes from PLPs AnCo1, SJ1, and MA725 transformed to *E. coli* DH10B (lanes 4, 8, and 12).
0- 1 kb ladder; 1- Negative control (water); 2- Positive control (DNA of AnCo1from plasmid extraction); 3- Negative control (*E. coli* DH10B); 4- AnCo1 transformed to *E. coli* DH10B; 5- Negative control (water); 6- Positive control (DNA of SJ1 from plasmid extraction); 7- Negative control (*E. coli* DH10B); 8- SJ1 transformed to *E. coli* DH10B; 9- Negative control (water); 10- Positive control (DNA of MA725 from plasmid extraction); 11- Negative control (*E. coli* DH10B); 12- MA725 transformed to *E. coli* DH10B

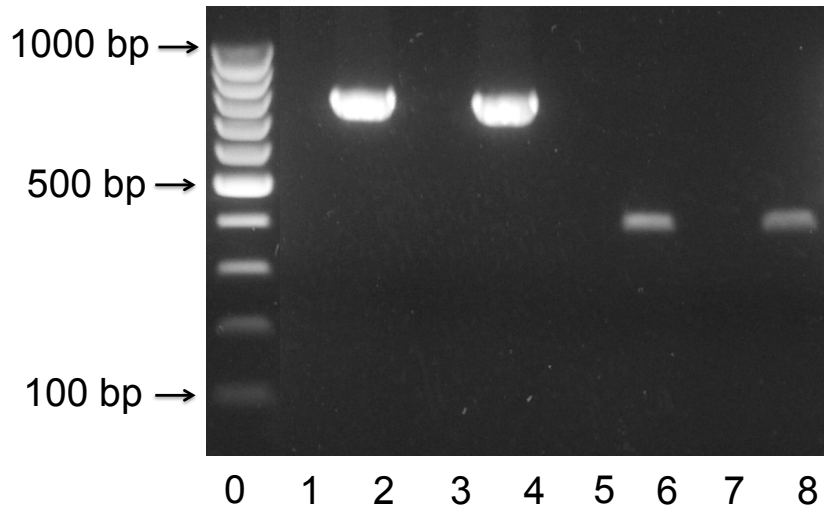

Figure 4.4. Gel electrophoresis of PCR products of the integrase genes from PLPs AnCo1 and SJ1 conjugated to *E. coli* J53 (lanes 4 and 8).
0- 1 kb ladder; 1- Negative control (water); 2- Positive control (DNA of AnCo1from plasmid extraction); 3- Negative control (*E. coli* J53); 4- AnCo1 conjugated to *E. coli* J53; 5- Negative control (water); 6- Positive control (DNA of SJ1 from plasmid extraction); 7- Negative control (*E. coli* J53); 8- SJ1 conjugated to *E. coli* J53

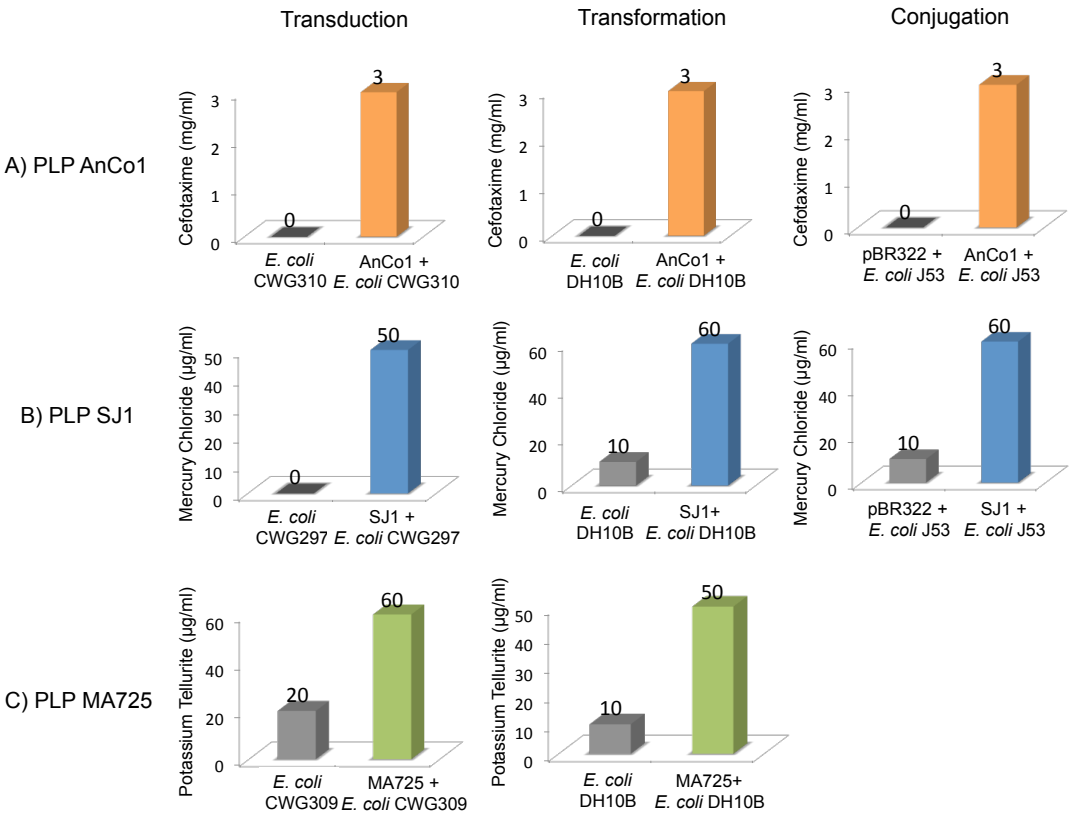

Figure 4.5. Minimum inhibitory concentrations (MICs), performed in triplicate, of (A) AnCo1 (orange colored bars), (B) SJ1 (blue colored bars), and (C) MA725 (green colored bars), horizontally transferred to new hosts through transduction (first column), transformation (second column) and conjugation (third column). AnCo1, SJ1, and MA725 confer resistance to 3 mg/ml of cefotaxime, 50 µg/ml of mercury chloride and 40 µg /ml of potassium tellurite, respectively. Un-transduced, Un-transformed and un-conjugated strains were used as negative controls.

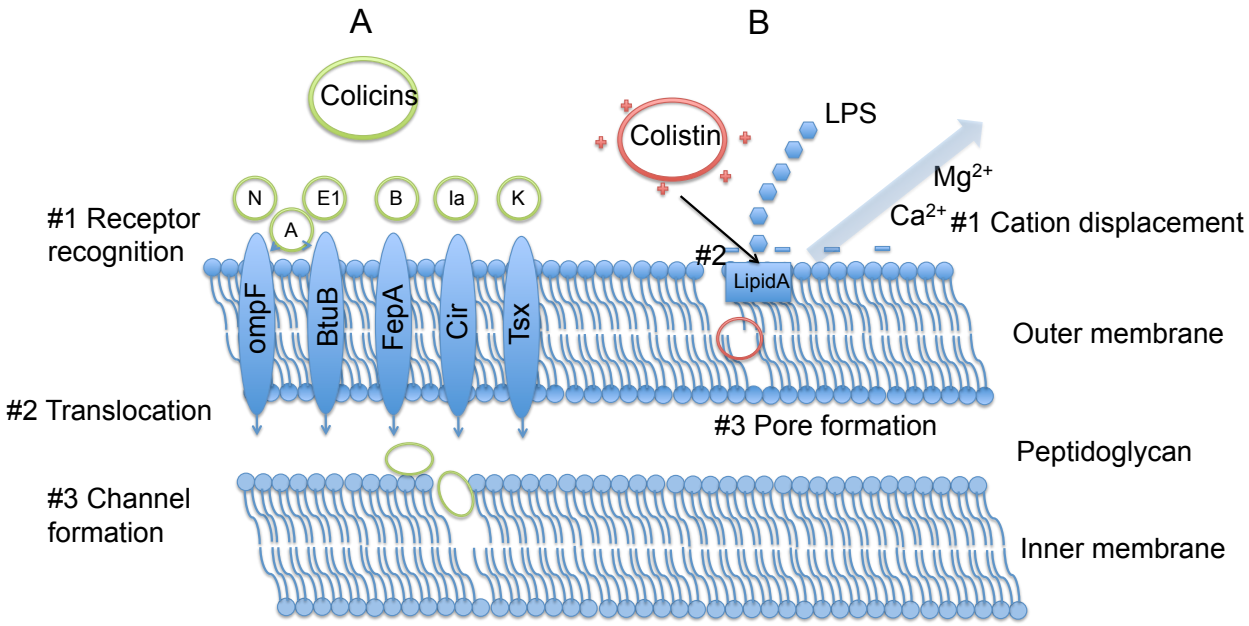

Figure 4.6. Comparison of the mode of action of channel-forming colicins (A) and colistin (B). (A) Channel-forming colicins recognize one specific specific receptor on the outer membrane surface, with the exception of channel-forming colicin A, which recognizes two receptors (ompF and BtuB). Next, they are translocated through the outer membrane, the C-terminus attaches itself electrostatically to the inner membrane surface and inserts its hydrophobic helixes into the membrane to form a channel in the inner membrane. Channel-formation eventually leads to cell death. (B) Colistin interacts electrostatically with the outer membrane of Gram-negative bacteria and displaces cations competitively to destabilize the lipid A component of the lipopolysaccharide (LPS). This disrupts the integrity of the outer membrane creating pores, causing leakage and cell death.

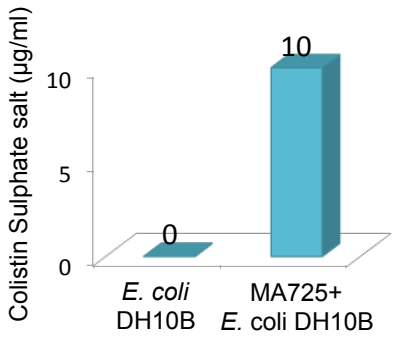

Figure 4.7. Minimum inhibitory concentrations (MICs) of colistin sulphate salt were performed in triplicate on PLP MA725 transformed in *E. coli* DH10B and determined to be 10 µg/ml.

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Chapter V

General Conclusion, Contribution to Knowledge, and Future Work

5.0 General Conclusion

The current study was conducted to investigate a novel mobile genetic element, called phage-like plasmids (PLPs), which harbour antibiotic resistance genes (ARGs) of clinical importance and heavy metal resistance (HMR) genes, including mercury and tellurite resistance genes, that can contribute to antibiotic resistance (AR) through co-selection and cross-resistance mechanisms. This study genomically characterized 57 PLPs consisting of 18 PLP genomes reported in the scientific literature, 31 PLP genomes from Genbank, and 8 PLPs that were detected in bacteria isolated from bovine and food samples. The research conducted during this Thesis has led to several key findings. Based on genomic analyses, the majority of PLPs can be classified into two lineages: SSU5-like and P1-like. Additionally, PLPs harbour restriction modification systems and toxin-antitoxin systems that may play a role in their maintenance within bacteria. Genomic analysis also demonstrated that PLPs harbour mobile genetic elements (MGEs), such as transposons, insertion sequences, and transposases, that flank ARGs, and a mercury resistance operon. These MGEs may be responsible for the mobilization of ARGs and HMR genes from and to PLPs. With respect to contributing to the spread of AR, PLPs were found to harbour ARGs conferring resistance to β-lactams, carbapenems, colistin, and aminoglycosides, as well as HMR genes conferring resistance to mercury and tellurite. Moreover, 75% of PLPs were found to be typeable, with IncFIB (33%) and IncY (30%) being the two major replicon types. This finding is significant because IncFIB is the most prevalent incompatibility group associated with extended-spectrum β-lactamases (ESBL) in *Klebsiella pneumoniae* and *Escherichia coli.*

Functional characterization of the PLPs demonstrated for the first time that they are capable of transferring ARGs and HMR genes via all three horizontal gene transfer (HGT) (transduction, transformation and conjugation) mechanisms to *E. coli* and *Salmonella enterica* isolates. Transduction experiments suggested that PLPs may use specific carbohydrate residues within the inner and outer core lipopolysaccharide (LPS) as receptors, including D-galactose I,

L-glycero-D-manno-heptose II and D-glucose II, respectively. Furthermore, one PLP designated SJ1 demonstrated a wide host range by infecting 18 different bacterial strains by transduction, including 2 *E. coli* strains, 4 *E. coli* O157 strains, and 12 *Salmonella* strains. Building on the previous observation that PLPs harbour ARGs and HMR genes, this study also demonstrated that the resistance genes harboured by these PLPs were of functional significance, conferring resistance to cefotaxime, mercury chloride and potassium tellurite, regardless of the mechanism of HGT. Furthermore, it was demonstrated that PLP MA725 carried the *terB* gene, which not only conferred resistance to potassium tellurite, but also conferred cross-resistance to colistin sulphate, a last resort antibiotic for Gram-negative infections. This latter observation has not been previously reported.

Based on the global dissemination of PLPs, and their ability to horizontally transfer ARGs and HMR genes to bacteria of foodborne importance, the research conducted in this thesis demonstrates the contribution of PLPs to antibiotic and heavy metal resistance. The genomic and phenotypic characterization of PLPs presented here will help facilitate the development of strategies to reduce the spread of PLPs carrying ARGs and HMR genes.

5.1 Contribution to knowledge:

The work presented in this study provided evidence for the first time that:

1. Whole genome sequence (WGS) alignments of PLPs as well as genomic analysis of the integrase gene of PLPs revealed that they have nucleotide sequence identity to phage SSU5, a virulent *Salmonella* Typhimurium phage, or P1, the prototypical PLP. The majority of PLPs identified to date can be classified into two lineages: SSU5-like and P1-like. A recommendation arising from the research conducted in this thesis is the addition of two new genera (SSU5-like and P1-like) to the phage classification scheme of the International Committee on Taxonomy of Viruses (ICTV).
2. Five genes define PLPs, including the phage integrase and terminase A genes, and the plasmid *parA*, *parB* and *repA* genes. The integrase gene facilitates the cyclization of the PLP genome upon entry to its host. Terminase A is required for genome packaging into

the procapsid head. The *repA* gene is the origin and initiator of PLP genome replication and *parA* and *parB* are required for partitioning of the PLP genome during bacterial cell division. Genomic analysis revealed these 5 genes were present in all of the PLPs in this study, with the exception of pBtic235, phiBU01, KHP30, and vB_BceS-IEBH that were proposed in chapter III not to be PLPs since they did not harbour *repA, parA* and *parB*.

3. Phage-like plasmids can horizontally transfer antibiotic resistance genes (ARGs), including CTX-M-15, as well as heavy metal resistance (HMR) including a mercury resistance operon (*mer* operon) and a tellurite resistance gene (*terB*), via transduction. Transduction of CTX-M-15 by PLP AnCo1 is significant as it confers resistance to third-generation cephalosporin, cefotaxime and is the most prevalent CTX-M variant disseminated worldwide. Transduction of these heavy metal resistance genes is significant because their resistance has been demonstrated to be associated with co-selection and cross-resistance mechanisms. Transformation and conjugation of PLPs has been previously shown but this study demonstrates for the first time that PLPs can be transduced and that PLPs have the ability to be transferred by all 3 HGT mechanisms.
4. The *terB* gene confers resistance to potassium tellurite. This is significant because it has been reported in the scientific literature that 4 of the 7 genes in the *ter* operon (*terB. terC, terD* and *terE*) are required to confer resistance to potassium tellurite.
5. The *terB* gene confers cross-resistance to colistin sulphate. This result is of particular significance because colistin is a last-resort antibiotic for treatment of Gram-negative infections. Bacterial resistance to colistin was first reported in 2015 and has been associated with the *mcr-1* gene. This finding demonstrates for the first time, another mechanism of colistin resistance that can be disseminated via transduction and transformation of PLP MA725.

5.2 Future Research:

Several additional experiments and genomic analysis should be conducted to further explore the results of this study. Regarding the two lineages of PLPs, none of the reported phage-like plasmids (PLPs) in the scientific literature thus far have been characterized morphologically. As such, the morphology of P1-like and SSU5-like PLPs should be investigated by electron

microscopy in order to help facilitate the classification of these two lineages of PLPs within the International Committee on Taxonomy of Viruses (ICTV) classification scheme.

Concerning PLP receptors, this study suggested that PLPs potentially target specific carbohydrate residues of the lipopolysaccharide (LPS) as their receptor for host infection. Random transposon mutagenesis could be performed in order to identify if other components of the outer membrane can be receptors of PLPs in order to better understand the scope of their host range. Once the nature of PLP receptors is elucidated, a broad host range study should be conducted to complement the small number of strains tested in this study.

The ability of PLPs to transfer ARGs and HMR via all 3 mechanisms of horizontal gene transfer (HGT) (transduction, transformation and conjugation) was demonstrated in this study. The frequency of each transfer mechanism should be assessed in order to determine which method most efficiently introduces PLPs to the bacterial cell. Moreover, broth mediated conjugation was attempted in this study; however, solid media conjugation should also be investigated as it is the most efficient method for conjugation. Such studies would represent a first step in developing approaches to block HGT of PLPs. Additionally, conjugation of PLPs required a helper plasmid as none of the 57 PLPs analyzed in this study harboured conjugation genes. Studies should be performed to investigate the prevalence of helper plasmids in bacteria in order to determine the potential for PLPs to be horizontally transferred by conjugation in the environment.

Finally, in regard to antibiotic and heavy metal resistance, this study demonstrated that the *terB* gene confers cross-resistance to colistin sulphate, a clinically important antibiotic. Further minimum inhibitory concentration (MIC) experiments should be performed to determine if *terB* confers resistance to any other antibiotics and whether the mercury resistance operon of PLP SJ1 is also capable of conferring cross-resistance to antibiotics of clinical importance.

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