Characterization of novel extended spectrum beta-lactamase producing bacteria and their bacteriophages from wastewater

by

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Abstract

Multidrug-resistant bacteria strains possessing extended-spectrum β-lactamases (ESBLs) has become an increasing problem worldwide. Bacteria resistant to antibiotics are mostly enteric, they can contaminate the environment and, through ingestion, enter new hosts to cause infections. Therefore, emphasis was put on isolating ESBL-producing bacteria from treated wastewater. The overall aim of this thesis was to isolate, identify and characterise ESBL-producing bacteria from wastewater followed by isolation and characterisation of bacteriophages specific for these bacteria. Bacterial isolates were recovered after growth on selective media and multiplex PCR was used to amplify SHV, TEM, CTX-M and OXA genes. Biochemical test and whole genome sequencing were applied to identify and characterise the isolated strains. The cell-free supernatants were then used to isolate bacteriophages. A high titer lysate was then used to test the specificity of bacteriophages for different bacterial strains. The nature of phage genetic material was established and morphology of isolated phages was determined by the transmission electron microscopy imaging. Two cold-tolerant bacteria isolated harbouring CTX-M gene were studied by 16S rRNA gene sequence analysis and housekeeping gene sequences analysis which revealed that these isolates showed no close similarity to any known member of the *Enterobacteriaceae* but are related to the members of *Rahnella*, *Rouxiella* and *Ewingella* genera. The phenotypic characteristics of the two isolates were, however, discrete from these 3 genera. Furthermore, two different bacteriophages infected the two newly identified cold tolerant bacterial strains. These bacteriophages were found to be novel viruses, most likely belonging to the *Siphoviridae* family, based on their characteristics, morphology, and genome size. In conclusion, all the analysis showed that they two isolates belong to a novel genus in the *Enterobacteriaceae* family. Given the menacing impact of disease outbreaks caused by contaminated water resources, work presented here identifies novel bacteria with harmful capabilities and potentially offers new tools for environmentally safer treatment to overcome this threat.

Declaration

Whilst registered as a candidate for the above degree, I have not been registered for any other research award. The results and conclusions embodied in this thesis are the work of the named candidate and have not been submitted for any other academic award.

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List of Abbreviations

Abi Abortive Infection
ADH Arginine DiHydrolase
AS Activtaed Sludge
ATCC American type culture collection
BLAST Basic Local Alignment Search Tool
BSAC British Society for Antimicrobial Chemotherapy
CDs Coding Sequences
CFS Cell Free Supernatant
CFU Colony forming unit
CGH Core Genome Hypothesis
CRISPR Clustered Regularly Interspersed Palindromic Repeats
dsDNA Double-Stranded DNA
dsRNA Double-Stranded RNA
EcC1 *E. coli* Clear 1
EIBMV Eliava Institute of Bacteriophage, Microbiology, and Virology
EPS Extracellular Polymeric Substances
EpC1 Ewingella phage Clear 1
EpT2 Ewingella phage Turbid 2
ESBL Extended Spectrum Beta Lactamase
EwS1 *Ewingella* Small 1
EwB2 *Ewingella* Big 2
FDA US Food and Drug Administration
***fus*A** Translation elongation factor G
GEIs Genomic Islands
HIIET Hirszfeld Institute of Immunology and Experimental Therapy
HGT Horizontal Gene Transfer
H2S Sodium ThioSulfate
ICTV International Committee on Taxonomy of Viruses
IF Inoculating Fluid
IMP IMiPenem
kbp kilo base pairs
KPCs *Klebsiella Pneumoniae* Carbapenemases
KpC1 *Klebsiella pneumonia* Clear 1
KpT1 *Klebsiella pneumonia* Turbid 1
LB Lysogeny Broth
LCB Local Colinear Blocks
LDC Lysine DeCarboxylase
LO Lysis from without
LIN Lysis Inhibition
MOI Multiplicity of Infection
MTase DNA Methyltransferase
OD Optical Density
ODC Ornithine DeCarboxylase
PAmpC Plasmid-mediated AmpC
PBP Penicillin-Binding Protein
PCR Polymerase Chain Reaction
PEG Polyethylene glycol
PFU Plaque forming unit
PsB1 *Pseudomonas* Big 1

PsS2	*Pseudomonas* Small 2
***pyr*G**	CTP synthase
REase	Restriction Endonuclease
RM	Restriction Modification
***rpl*B**	50S ribosome protein L2
RPM	Revolutions per minute
***rpo*B**	β subunit RNA polymerase
SD	Serial dilution
Sie	Superinfection Exclusion
ssDNA	Single-Stranded DNA
ssRNA	Single-Stranded RNA
***suc*A**	2-oxoglutarate dehydrogenase E1
TAE	Tris-Acetate EDTA
TBR	Tree-Bisection-Reconnection
TDA	Tryptophan Deaminase
TF	Trickling Filter
TSB	Tryptone Soy Broth
TSA	Tryptone Soy Agar
VIM	Verona Integron-encoded Metallo-βlactamase
°C	degree Celsius

1.0 Introduction

Untreated water has a high health risk of causing waterborne diseases. Most disease-causing pathogens are enteric in origin, that is, they are excreted in faecal matter, contaminate the environment, including drinking water, and through ingestion enter new hosts. Many members of the order *Enterobacterales* have been implicated as pathogens in humans and these include *Vibrio cholerae* causing cholera, *Salmonella typhi* causing typhoid, *Shigella dysinterae* causing dysentery, *Yersinia enterocolitica* causing yersiniosis, among others.

Although advances have been made in water source protection and water treatment, sewage treatment plants receive wastewater from a variety of sources and have great microbial diversity, including microorganisms that are carbon degraders, nitrifiers, denitrifiers, as well as phosphate- and glycogen-accumulating bacteria (Cydzik-Kwiatkowska and Zielinska, 2016). It is unsurprising that these treatment plants are also rich in diverse pathogens. However, it is concerning that treated water can be enriched for pathogens and antibiotic resistant bacteria which has become a global public health threat causing serious illness and even death. (Gomez *et al* 2010; Gouliouris *et al* 2019; Amos *et al* 2014). As a result of this and similar observations, waste-water treatment plants are regarded as hotspots for microbial pathogens and the spread of antibiotic resistances into the environment, although the processes governing this dissemination remain poorly understood (Ju *et al* 2019).

One important mechanism in treating wastewater involves bacterial viruses (bacteriophages). While an appropriate wastewater treatment is efficient in removing most bacterial species, it is evident that bacteriophages survive the process. Through a phenomenon known as transduction bacteriophages can transfer resistance genes between bacteria during infection and thus establish a reservoir of antibiotic resistance genes (Lood *et al*., 2017).

1.1 Wastewater treatment

Wastewater treatment is the process of producing a final effluent appropriate for either disposal or reuse, by removing physical, chemical, and microbiological contaminants from any kind of wastewater coming from community, commercial or industrial waste. Wastewater treatment plant consist of different treatment levels of the wastewater which are employed depending on the main purpose of the treatment. The different treatment levels include (i) preliminary treatment, to remove coarse solids. (ii) primary treatment, to remove suspended solids and organic matter; (iii) secondary treatment, to remove

biodegradable organic matter in solution or suspension and suspended solids; (iv) tertiary treatment, to remove specific compounds, such as nutrients, pathogens, etc. (Tchobanoglous *et al*., 2014).

Community wastewater treatment development started in the early 20th century in the United Kingdom in response to a 1908 report by the Commission on Sewage Disposal (OFWAT/DEFRA, 2006). The knowledge of waterborne diseases was limited at that time, therefore, the biological or secondary wastewater treatment systems, including the two most commonly used, namely activated sludge (AS) and trickling filter (TF), were developed with the purpose of removing organic matter and suspended solids, but pathogen levels are reduced by these processes. In practice, all wastewater effluent is reused either in agricultural irrigation, aquaculture, water toilet flushing or in industrial activities. In some cases, it is indirectly reused when treated wastewater is discharged directly into the large bodies of water and such are used for recreational activities like fishing and swimming. As reported by Angelakis and Durham (2008), wastewater reuse is potentially an economically attractive and sustainable option for water resource management. They reported that in 2004 in Europe about 700 million cubic metres of treated wastewater were reused. In the UK, only 3 million cubic metres of treated wastewater were reused in 2005, but this number is expected to increase ten-fold by 2025 (Angelakis and Durham, 2008).

Although reusing wastewater poses many economic and environmental advantages, a potential risk to human health related with waterborne pathogenic microorganisms in the wastewater is of great concern to the health sector. One of the leading worldwide causes of human morbidity and mortality documented by the World Health Organisation (WHO) is acute gastroenteritis, which is associated with ingestion of contaminated water (WHO, 2011b). Waterborne pathogens have led to over 10 million deaths yearly, with Diarrhoeal disease alone being responsible for the deaths of 1.5 million people every year (WHO, 2012). Most deaths related with diarrhoea globally are thought to be caused by unsafe drinking water or inadequate sanitation or hygiene: more than 99% of these deaths occur in low-income countries, and around 84% of those are children (WHO, 2009). Waterborne bacterial pathogens in wastewater remains an important public health concern due to the high cost of disinfecting wastewater by using physical and chemical methods in treatment plants. Bacteriophages, which have been used as biocontrol agents in phage therapy treatment of people, are in phage treatment of food, are now proposed for treatment of wastewater. Bacteriophages have been used as alternative treatments for drug resistant pathogens and some of the characteristics that make it unique include: high effectiveness in killing their target bacteria, high target specificity, adaptivity, natural residence in the environment and the fact that they are self-replicating and self-limiting (Jassim *et al*., 2016).

1.2 Enteric microorganisms present in wastewater

A large range of microbial species are found in natural waters and wastewaters such as of bacteria, archaea, protozoa, helminths, fungi, rotifers, algae, and viruses. Most of these microorganisms are harmless to humans and are needed to maintain the ecological niche in aquatic environments. However, some of these microorganisms are defined as 'pathogens' because the can trigger a variety of illnesses in humans. These pathogens have been divided into 4 major taxonomic groups: Bacteria, viruses, protozoa, and helminths (von Sperling, 2007; Tchobanoglous *et al*., 2014). These pathogens commonly found in wastewaters normally would have been previously egested by humans (and other animals) and can cause a wide range of diseases in humans.

1.2.1 The *Enterobacterales*

The order *Enterobacterales* is a large and diverse group of gram-negative, facultatively anaerobic, non-spore-forming, rod-shaped bacteria within the class *Gammaproteobacteria*. Members of this order are found predominantly in soil, water in association with living organisms including plants, insects, animals and humans (Brenner & Farmer, 2005). In prokaryotic taxonomy, there are 8 families in the order of *Enterobacterales* (Figure 1). The most diverse bacterial families currently recognized are the *Enterobacteriaceae* family. Which constitutes the family with the most genera within the order E*nterobacterales* encompassing over 250 species. The number of genera and species of the *Enterobacteriaceae* has increased from 12 genera and 36 species in 1974, to 34 genera and 149 species with 21 subspecies in 2006 (Baylis, 2006), to 48 genera and 219 species with 41 subspecies in 2011 (Baylis *et al*, 2011). Currently there are over 60 genera representing some 250 species that are taxonomically diverse, ranging from the coliforms, important in monitoring health and safety standards in the water and food industries (Figure 1), the insect symbionts of the genera *Arsenophonus (*belongs to *Morganellaceae* family*), Buchnera (*belongs to *Erwiniaceae* family*)*, to the plant pathogens of the genera *Erwinia*, *Brenneria* or *Pectobacterium (*both belong to the *Pectobacteriaceae* family*).*

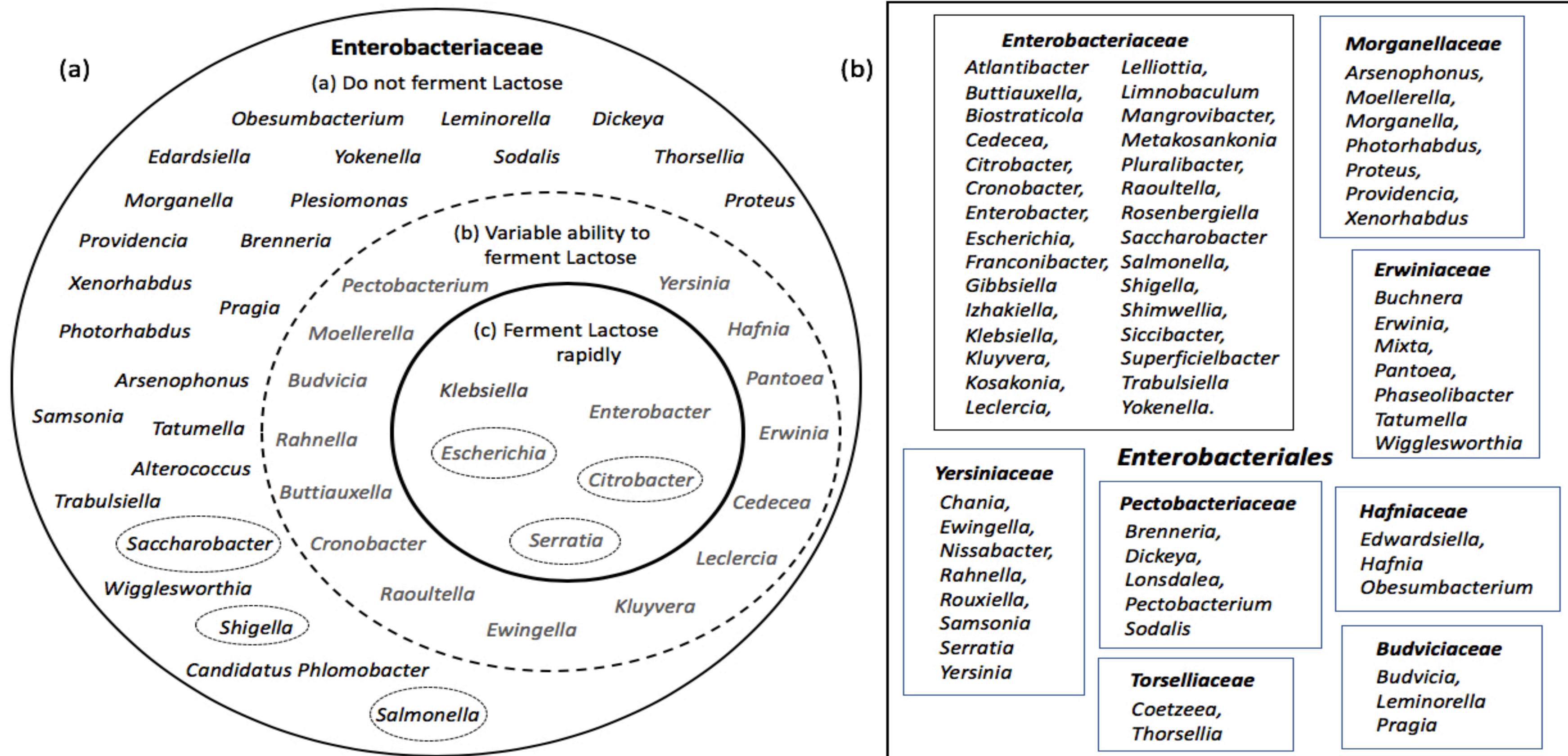

Figure 1. The *Enterobacterales*. (a) Variability in the ability to ferment lactose within genera of the *Enterobacterales* – redrawn from Baylis *et al* 2011. The genera encircled with a dotted line represent those species or strains that are variable between the different categories. (b) The eight families of the *Enterobacterales* (Alnajar and Gupta, 2017).

Although the 16S rRNA molecule is a standard phylogenetic marker for most bacterial species, 16S rRNA sequence analysis is poorly informative within the family of *Enterobacteriaceae* because it reaches its limit at the order level. It is better used for kingdom/class/order. The classification of the *Enterobacteriaceae* within the *Enterobacterales* has undergone revision recently, based on the comparison of whole genomes including multilocus sequence analysis (MLSA) based on genes such as *sucA, pyrG, rplB, rpoB* among others (Adeolu *et al* 2016; Alnajar and Gupta, 2017). Comprehensive phylogenomics and comparative genomic analyses has resulted in the division of the *Enterobacterales* into seven families as described by clades: The *Escherichia*-*Enterobacter* clade; the *Erwinia*-*Pantoea* clade; the *Pectobacterium*-*Dickeya* clade: the *Yersinia*-*Serratia* clade; the *Hafnia*-*Edwardsiella* clade; the *Proteus*-*Xenorhabdus* clade and the *Budivicia* clade. These clades are recognised molecularly by the presence of conserved signature indels (CSIs).

One of the most important clades relevant to this study is the *Yersinia*-*Serratia* one. This clade includes genera such as *Yersinia*, *Rahnella, Ewingella*, *Rouxiella*, *Serratia*, among others.

Yersinia is a genus in the family *Yersiniaceae*. *Yersinia* species are gram-negative straight rods, oxidase-negative, catalase-positive, coccobacilli bacteria, and are facultative anaerobes. *Yersinia pestis* (the 'plague bacillus') was first identified by Alexandre Yersin in 1894 and classified as *Pasteurella pestis* soon after. Von Logham reclassified *Pasteurella pestis* and *Pasteurella rodentium* into the new genus of *Yersinia* in 1944. The genus *Yersinia* contains 17 species, of which only 3 are known to be pathogenic to humans. These include *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis (*Rogers *et al., 2015). Yersinia* spp. have a number of virulence factors that contribute to their ability to establish an infection in their mammalian host such as ability to evade and inhibit the immune and inflammatory responses of the host. *Yersinia enterocolitica* causes gastroenteritis and is the most significant *Yersinia* species related to water transmission. *Yersinia enterocolitica* mainly causes acute enteritis, but systemic infections, such as bacteraemia, joint pain, and rashes have occasionally resulted. The most common symptoms include fever, diarrhoea, and abdominal pain (Percival and Williams, 2013). *Yersinia pestis* is nonmotile. Other species are nonmotile at 37°C but motile at temperatures less than 30°C by means of peritrichous flagella. The optimal temperature for growth is 27.8–30°C. They do not form spores or capsules, but *Y. pestis* produces a capsule-like envelope (Slack, 2010).

The genus *Rahnella* is also a member of the family *Yersiniaceae.* It originally consisted of only one species, *Rahnella aquatilis*, and two genomospecies. However, 5 additional species have now been identified: *Rahnella victoriana, Rahnella variigena, Rahnella inusitata, Rahnella bruchi, Rahnella woolbedingensis* (Brady *et al*., 2017). The name *Rahnella* was coined in honour of the German American bacteriologist Otto Rahn, and the species was termed *aquatilis* because it was isolated from water.

Rahnella aquatilis is a rare gram-negative rod which is 2-3 microns in length, although usually found in fresh water. It has been isolated from blood, surgical wounds, and urine, among others (Oh & Tay, 1995; Tash, 2005). *Rahnella aquatilis* can cause a wide variety of abnormalities such as bacteraemia, sepsis, respiratory infection, urinary tract infection, and wound infection (Chang *et al*., 1999). No single biochemical feature differentiates *Rahnella* from the other *Yersiniaceae.* However, *Rahnella* does have a few distinguishing traits. Characteristically, it is motile at 22°C but nonmotile at 36°C, and it can grow at 4 to 10°C. It is also negative for lysine and ornithine decarboxylases and arginine dehydrolase (Tash, 2005).

Ewingella is a new group in the *Yersiniaceae* family, consisting of only one species, *Ewingella americana*. It is a rare gram-negative rod first described from clinical specimens in 1983 by Grimont *et al*. *Ewingella* is phenotypically distinct from all other groups of *Yersiniaceae*. The members of this genus are lactose fermenting, oxidase negative, catalase positive, indole negative, facultative anaerobes, lipase- and deoxyribonuclease-negative; Voges-Proskauer-positive; lysine-, orni- thine- and arginine-decarboxylase-negative; they produce acid from glucose but fail to produce acid from L-arabinose, melibiose, raffinose, D-sorbitol or sucrose (Grimont *et al*., 1983). It rarely causes human infections and has been identified from various clinical samples including sputum, conjunctiva, blood, wound.

Ewingella americana isolates have been reported to cause peritonitis, conjunctivitis, bacteraemia, and pneumonia. It can be treated with aminoglycosides, fluoroquinolones, and sulfamethoxazole/trimethoprim. These bacteria can survive in water and grow preferentially at 4 °C and this isolate is not known to be the normal flora of respiratory tract (Hassan *et al*., 2012; Esposito *et al*., 2019). However, it is not known whether *Ewingella americana* is a true pathogen or simply an opportunistic infectious agent, as most of the cases have been described in old patients and/or suffering from severe underlying conditions, such as complicated surgeries, injuries from accidents, drug abuse, and renal failure (Esposito *et al*., 2019).

1.2.2 *Pseudomonadales*

Pseudomonas species are gram-negative bacteria belonging to the family *Pseudomonadaceae*. They are psychrophilic catalase-positive obligate aerobic (they require presence of oxygen to survive) They are usually single curved or in the shape of straight rods within the class *Gammaproteobacteria* (Feiner, 2006). The genus *Pseudomonas* contains more than 140 species, most of which are saprophytic. More than 25 species are associated with humans. The most pseudomonads known to cause disease in humans are associated with opportunistic infections and include *P. aeruginosa* and *P. maltophilia* (Iglewski, 1996).

P. aeruginosa is a gram negative, rod-shaped bacterium that is well adapted to live in moist environments. This versatile bacterium grows in water and can infect plants and animals as well as humans (Selezska *et al*., 2012). *P. aeruginosa,* are commonly found as part of the normal flora in the oral cavity and intestinal tracts and in the skin of some healthy persons (Iglewski, 1996). They have been described as nosocomial opportunistic infectious agent, as most of the described cases were in immunocompromised patients, ventilated patients in the intensive care unit and patients with burn wounds. *P. aeruginosa* can cause a wide variety of infections including pneumonia, urinary tract, and gastro-intestinal infections (Stover *et al*., 2000).

Its ubiquitous growth capacity combined with a high intrinsic resistance against antibiotics and disinfectants and the ability to readily acquire resistance mechanisms makes *P. aeruginosa* an important pathogen for humans. As it is intrinsically resistant to various classes of antibiotics including some beta-lactam antibiotics, resistance to carbapenems and aminoglycosides can be acquired (Oliver *et al*., 2008; Strateva and Yordanov, 2009)

1.3 Antibiotics, β-lactam antibiotics, and mode of resistance

In trying to maintain an ecological niche, microorganisms have resulted in competitive interactions and battle for survival. This competition has been seen as the base for evolution of antibiotics millions of years ago (Medeiros,1997). Alexander Fleming, a Scottish physician-scientist was the first to be recognised for discovering Penicillin as early as 1928. He noticed an antibacterial effect from a mould isolate he later identified as belonging to *Penicillium* genus and during World War II penicillin G became available for treating infections (Fleming, 1929).

Cell wall active agents, especially beta-lactam antibiotics, play a crucial role in the treatment of bacterial infections. β-lactam antibiotics (penicillins, cephalosporins, monobactams and carbapenems) are very important drugs due to their effective and broad mode of action on

different bacterial pathogens combined with their low toxicity in humans and animals (Abraham, 1981). The ß-lactam antibiotics have bactericidal effects that involves the inhibition of synthesis in the bacteria cell wall peptidoglycan layer. This process occurs through covalent attachment of antibiotics to penicillin-binding protein (PBP) on cell membrane (Figure 2). This enzyme catalyses the final stages in bacterial cell wall formation. (Drawz and Bonomo, 2010).

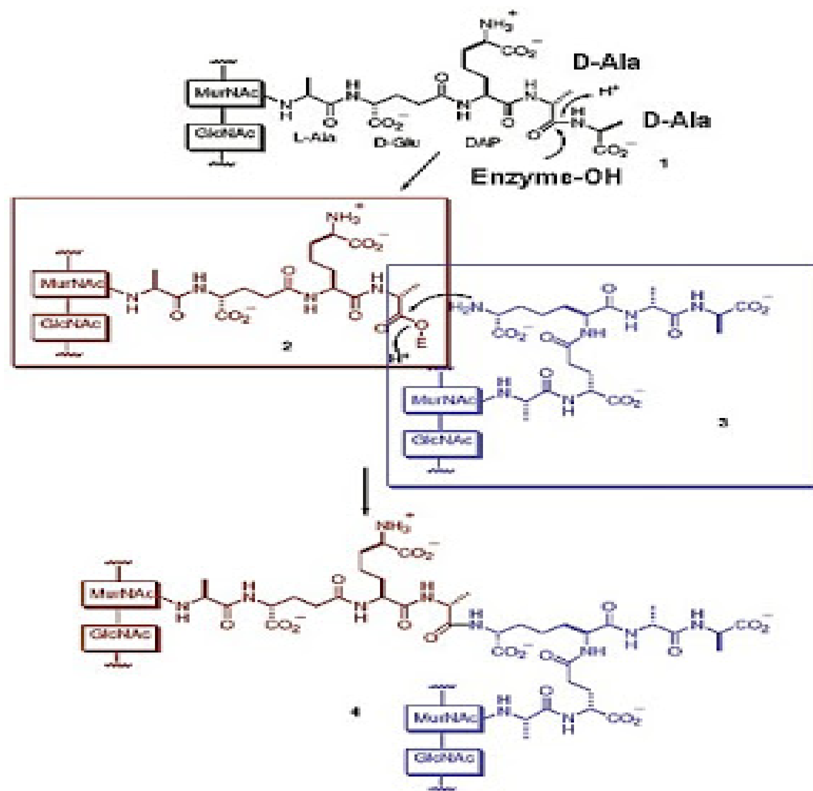

Figure 2. The transpeptidase reaction. In order to form a rigid structure, the polysaccharide chains (glycans) are linked together by peptide crosslinks. The first step in the formation of the crosslinks involves attachment of a short five residue peptide to the MurNAc sugar in the polysaccharide. This peptide ends in two D-Alanine (D-Ala) residues. The top structure (1) is a polysaccharideMurNAc-GlcNAc) with the first peptide already bound. Note that it ends in two D-Ala residues. The first step in the transpeptidase reaction involves binding of the enzyme (Enzyme-OH) to the D-Ala-D-Ala end of the chain. A reaction takes place in which one of the D-Alanine residues is released and the enzyme become attached to the end of the peptide (2). In the second step, an adjacent peptidoglycan (purple) (3) with a branched structure is covalently linked to the first peptidoglycan forming a crosslink between the two polysaccharides. Almost all bacteria have cell walls, and they have transpeptidase enzymes that catalyze this reaction (Lee et al. 2003).

The carbapenems are usually regarded as the last resort treatment against Extended Spectrum β-lactamases (ESBLs) because they have the broadest spectrum and potency among the different β-lactams.

1.3.1 Antibiotic resistance

Antibiotic resistance is defined as the ability of a bacterium to resist an antibiotic that would normally kill them or stop their growth. This ability to resist antibiotics is achieved through four core strategies (Figure 3): i) decreased permeability of bacterial membranes; (ii) antibiotic efflux; (iii) altered target sites; (iv) inactivating enzymes (Baker-Austin *et al*., 2006).

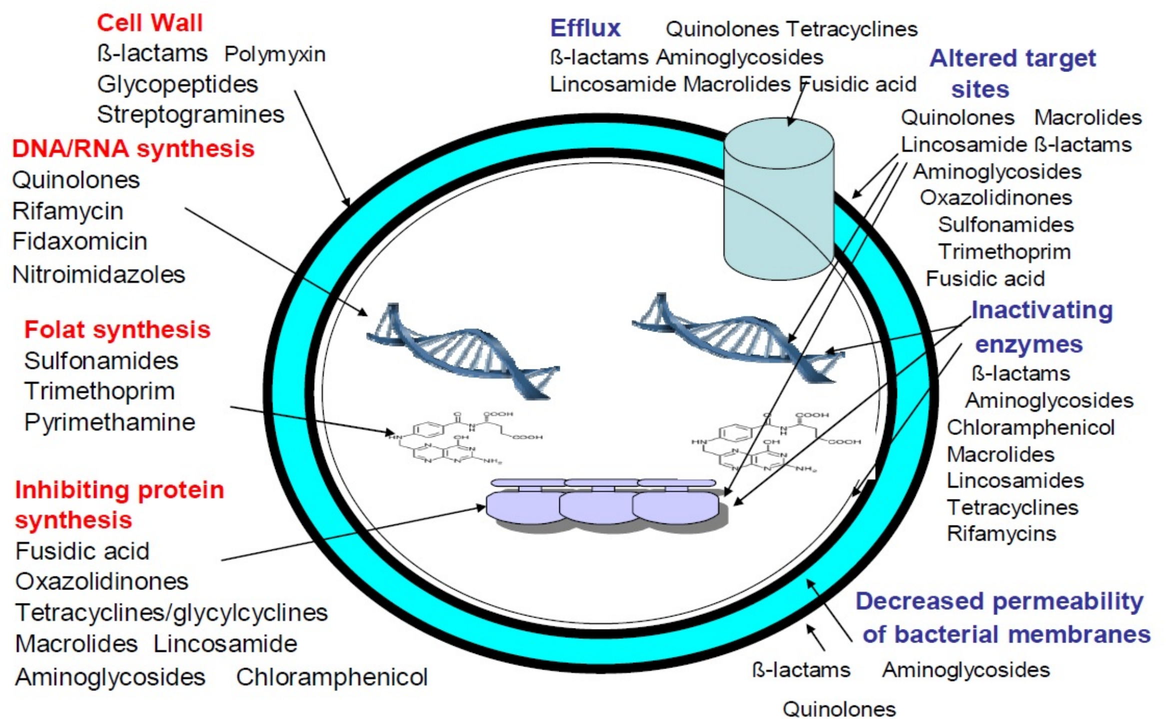

Figure 3. Mechanisms of antimicrobial activity and of antibiotic resistance. (Wright, 2010)

Most antibiotics are a product of nature and in the battle that microbes have to fight in this context, their ability to adapt to changes in the environment is a crucial aspect for survival and as such antibiotic resistance is a naturally occurring phenomenon in microorganisms (D'Costa *et al*., 2011). Horizontal gene transfer (HGT) and mutations have successfully given rise to new bacterial genotypes, which can spread in the larger population through adaptive evolution or random genetic drift (Thomas and Nielsen, 2005). Bacteria have a short generation time, large population size and a high rate of spontaneous mutations, which enables fast and effective adaptive evolution (Davies and Davies., 2010), as was demonstrated by the effectiveness of a wild-type *E. coli* evolving from a susceptible to a highly resistant strain (Baym *et al*., 2016).

The development path of antibiotic resistance is complex and reliant on several factors including the level of resistance conferred by the resistance mechanism, the mutation rate of bacteria, the fitness of the resistant mutant and the strength of the selective pressure

(Hughes and Andersson, 2017). The strong burden exerted by centuries of misuse and overuse of commercial antibiotics in humans, animals and agriculture have augmented the development of antibiotic resistance in pathogenic bacteria (Levy, 1998). Plasmids which are replicons of extrachromosomal DNA in bacterial cells, play a significant part in the development of these microorganisms. They are found in most bacteria and because the vary in size and usually circular in form, they are very adaptable in spreading multiple traits such as drug resistance and virulence factors. Some are spread by plasmids through HGT (Stokes and Gillings, 2011). HGT is the movement of genetic information between organisms and is crucial for the spread of antibiotic resistance (Burmeister, 2015). Through the mechanism of HGT, antibiotic resistance genes can be spread throughout an entire population of bacterial pathogens and spread to new bacterial species (Davies and Davies, 2010). HGT occurs by three genetic mechanisms (Figure 4); Transformation (direct uptake of naked DNA), Conjugation (transfer of mobile genetic elements by pili structures assembled by two adjacently located bacteria) and Transduction (bacteriophage-mediated transfer of DNA between bacteria) (Thomas and Nielsen., 2005). In Enterobacteriaceae, the conjugation of plasmids plays a major role for the spread of antibiotic resistance genes (Carattoli, 2009).

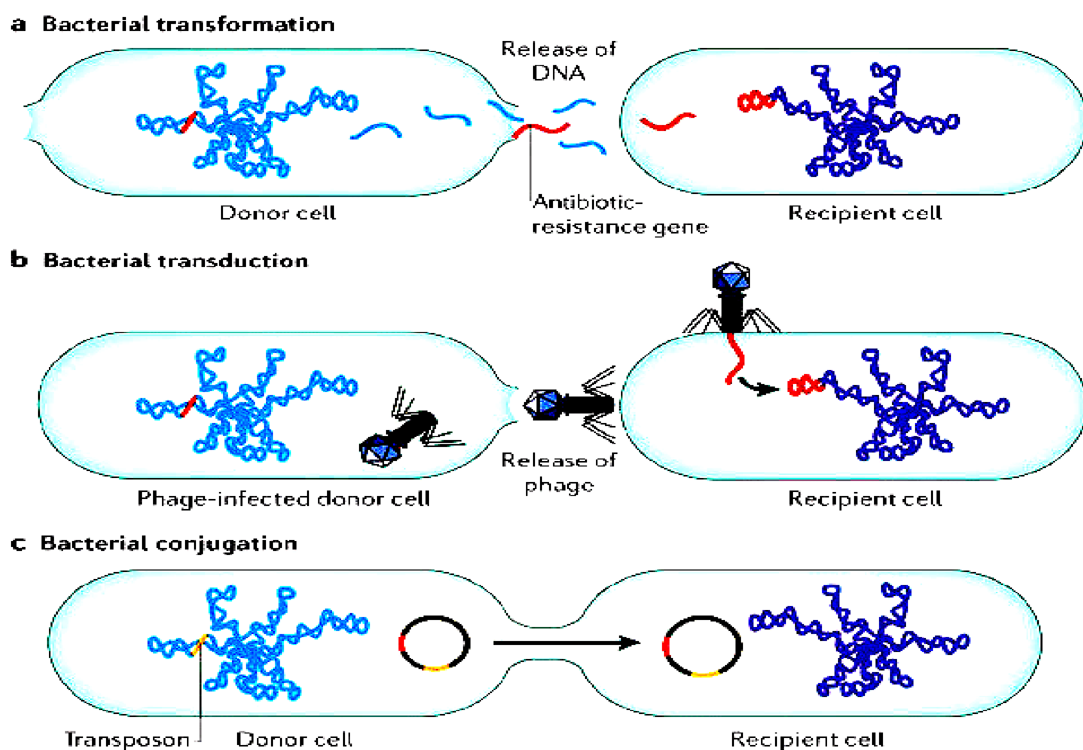

Figure 4. Mechanisms of bacterial horizontal gene transfer a- Bacterial transformation; b- Bacterial transduction; C- Bacterial conjugation (Manage, 2018).

1.3.2 β-lactamases

The most important resistance mechanism in bacteria is the production of β-lactamases that hydrolyses β-lactam ring by adding a water molecule to the common ß-lactam bond, and this inactivates the ß-lactam antibiotic (Figure 5).

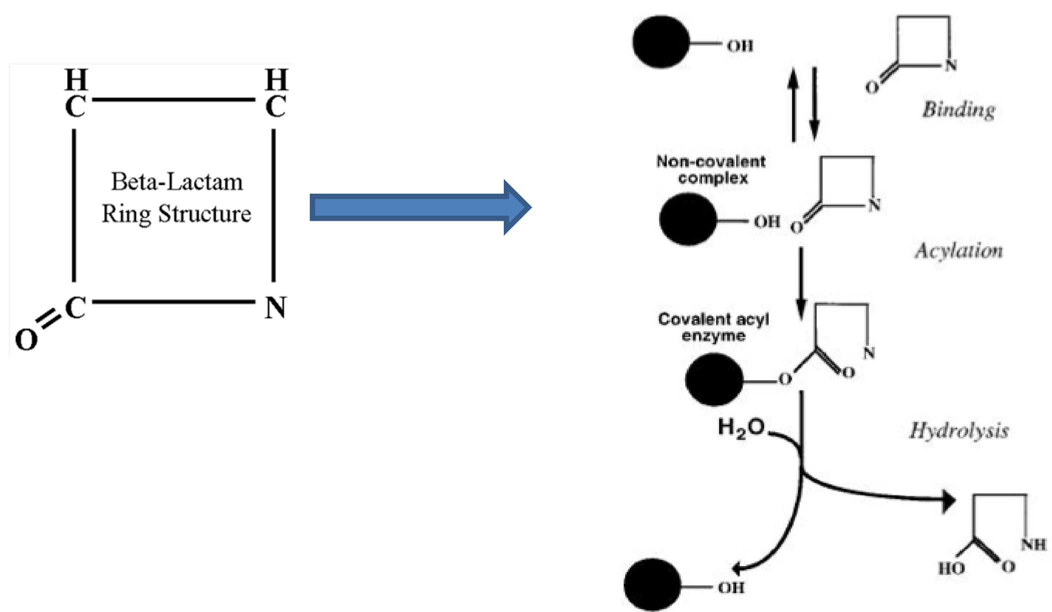

Figure 5. Hydrolysis of β-lactam antibiotics by β-lactamase enzymes

The group β-lactamases consists of around 2800 unique proteins and were evolved in microorganisms to protect them from naturally occurring β-lactams in the environment. Based on the mechanism by which they hydrolyse β-lactam antibiotics, β-lactamases are classified biochemically into two groups: serine- β-lactamases and metallo- β-lactamases. Sequence analyses revealed four major molecular differentiations for key β-lactamases. This led to the establishment of molecular classes A, B, C and D (Table 1). Serine- β-lactamases were subdivided as A, C or D while metallo- β-lactamases were classified as B. The four molecular classes were further subdivided based on functional capability related to substrate and inhibitor profiles into 17 functional groups (Bush, 2018).

Table 1. Classification of ß lactamases.

Functional group	Molecular class	Distinctive substrates	Inhibited by		Representative enzymes
			Clavulanic acid	EDTA	
1	C	Cephalosporins	-	-	AmpC enzymes from gram negative bacteria
2a	A	Pencillins	+	-	Penicillinases from gram positive bacteria
2b	A	Pencillins Early cephalosporins	+	-	TEM-1, TEM-2, SHV-1
2be	A	Extended spectrum cephalosporins Monobactams	+	-	TEM-3 to TEM-26, SHV-2 to SHV-6, *Klebsiella oxytoca* K1
2br	A	Pencillins	±	-	TEM-30 to TEM-36, TRC-1
2c	A	Penicillins, carbenicillin	+	-	PSE-1, PSE-3, PSE-4
2d	A	Penicillins, cloxacillin	±	-	OXA-1 to OXA-11, PSE-2 (OXA-10)
2e	A	Extended spectrum cephalosporins	+	-	CepA
2f	A	Penicillins, cephalosporins, carbapenems	+	-	KPC-2, IMI-1, SME-1
3	B	Most b-lactams, including carbapenems	-	+	IMP-1, VIM-1, CerA, IND-1
4	Not determined	Pencillins	-	?	Penicillinase from *Pseudomonas cepacia*

1.3.3 Extended-spectrum β-lactamases

ESBLs are defined as active site serine Amber's class A or class D ß-lactamases which can hydrolyse broad-spectrum penicillins, cephalosporins and aztreonam. They can be inhibited by clavulanic acid *in vitro*. They are located according to the Bush-Jacoby-Medeiros functional classification system in subgroups 2be and 2D or Amber class A or D structural classification (Table 1). Some ESBL-genes such as TEM-10, OXA-12 and SHV-28 in potential pathogenic bacteria like *E. coli* and *K. pneumoniae* originated through different point mutations that alter the amino acid configuration around the active sites in naturally

occurring β-lactamase genes like TEM-1, OXA-1 and SHV-1. Other ESBL-genes emerged in non-pathogenic bacteria, and were horizontally transferred to pathogenic bacteria, the most important example being the CTX-M ESBLs that are believed to originate from environmental bacteria belonging to the genus Kluyvera (Bush, 2018). Most discovered and identified ESBLs belong to these families: TEM with approximately 200 variants, SHV with over 140 variants and the most found according to published literature CTX-M with about 130 variants.

CTX-M-type ESBLs are the most commonly found ESBL gene in the clinic, the community and the environment and have spread into a global pandemic (Woerther *et al*., 2013). Fifteen AmpC β-lactamases are found in the molecular class C and functional group 1 (Table 1), and they are categorised by their ability to hydrolyse most penicillins, extended-spectrum cephalosporins and show resistance to clavulanic acid (Bush, 2018). AmpC β-lactamases are encoded on the chromosomes of many bacteria belonging to the family *Enterobacteriaceae*. They are inducible in many bacteria and mutations in AmpC enzymes can lead to overexpression, which leads to resistance to broad-spectrum cephalosporins like cefotaxime and ceftazidime. AmpC genes have been transferred to transmissible plasmids and can thus appear in bacteria lacking or poorly expressing chromosomal AmpC, such as *E. coli* and *K. pneumonia*. Plasmid-mediated AmpC (pAmpC) enzymes are generally harder to detect, broader in conferring resistance and less common compared to plasmid-mediated ESBL enzymes, but certain pAmpC enzymes are prevalent in livestock and humans in some regions (Jacoby, 2009). Termed as the "rising star" of ß-lactamase, plasmid-mediated carbapenemases are the most common encoded carbapenems in bacteria although some bacteria have chromosomal-mediated carbapenemases. Several carbapenemases have been discovered and identified (Table 1). Osano *et al*., 1994 and Watanabe *et al*., 1991 in their research in the 1990s in Japan both reported the first case of isolated IMiPenem (IMP) carbapenemases in *Serratia marcescens* and *P. aeruginosa* respectively. Yigit *et al*., 2001 reported the first case of *Klebsiella pneumoniae* carbapenemases (KPCs) in the United States in 1996 while Lauretti *et al*., 1999 reported Verona integron-encoded metallo-β-lactamase (VIM) carbapenemases in Italy in 1999.

1.3.4 Extended-spectrum β-lactamases in *Rahnella* and *Ewingella*

Members of the genus *Rahnella* are commonly associated with plants while *Ewingella* is found with cultured mushrooms. They both are regarded as infrequent human opportunistic pathogens, which are easy to treat with specific antibiotic patterns. *Rahnella* and *Ewingella* are naturally resistant to several β-lactam antibiotics, which is mediated by an Amber class A and an Amber class C β–lactamase, respectively. The resistance gene of *Rahnella*, bla_{RAHN}, is located on a non-mobile plasmid belonging to the pEA29 family, associated

commonly in plant bacteria. bla_{RAHN} has been suggested to be present in prehistoric times before the divergence into genomospecies of *Rahnella*. bla_{RAHN} has developed and varied into bla_{RAHN-1} and bla_{RAHN-2} found in *Rahnella* genomospecies 2 and *Rahnella aquatilis*, respectively (Rohzon *et al*., 2012). Because this gene is located on a non-mobile plasmid, the transfer of resistance genes from one pathogen to another has yet to be documented but several examples of chromosomal resistance genes that were transferred into pathogens have been documented, it cannot be excluded that bla_{RAHN} also may spread to other bacteria in the future. On the other hand, using different primer combinations, the amplification and sequencing of the (partial) AmpC gene of the *Ewingella americana* strains WMR82 and WMR121 showed 72% identity to AmpC of Serratia *proteamaculans* and approximately 67% AmpC of other Serratia species. The origination of this AmpC gene has yet to be found, therefore, further studies are necessary to assess whether the *Ewingella* ampC gene is chromosome or plasmid born and its potential for transfer needs to be investigated (Rohzon *et al*., 2012).

1.3.5 Virulence factors of extended-spectrum β-lactamases

Virulence is the ability of an organism to infect a host and cause a disease. Virulence factors are the molecules that help bacteria colonize a host. These factors are either secretory, membrane associated or cytosolic in nature. The cytosolic factors assist the bacterium to undergo quick metabolic, physiological, and morphological shifts. The membrane associated virulence factors assist the bacterium in adhesion and evasion of the host cell. The secretory factors aid the bacterium to pass through the innate and adaptive immune responses launched inside the host (Sharma *et al*., 2017). Infections resulting from ESBL producers are associated with serious adverse conditions because of ineffective therapy and the failure in the choice of an antibiotic active against these isolates. However, the increased incidence of mortality associated with ESBL producers may also be associated with the increasing virulence of these isolates. One such is the pathogenicity of *K. pneumoniae* where the expression of its virulence factors such as microbial biofilm formation, Serum resistance among others have been linked to severe conditions (Gharrah *et al*., 2017). In a study by Pitout *et al*., 2005, several virulence factors were more prevalent among *E. coli* isolates that produced CTX-M enzymes compared to producers of TEM and SHV ESBLs. CTX-M producers were more commonly positive for *afa/dra* (Dr-binding adhesins), *iha* (putative adhesin-siderophore receptor), *sat* (secreted autotransporter toxin), and *kpsM* II. In contrast, non-CTX-M producers significantly more often exhibited *ireA* (iron-regulated element) and *cvaC* (colicin [microcin] V). Other virulence factors are colonization factors (CFs) that help enterotoxigenic *E. coli* attach to and colonize cells in the small intestine, allowing bacterial cells to produce heat-labile enterotoxins (LT)

and/or heat-stable enterotoxins (STh or STp) that target vital cellular processes (Margulieux *et al*., 2018). *Rahnella* possess root adhesins that mediates adherence to epithelial cells during bacterial invasion. Other virulence factors are lipopolysaccharide endotoxin and homoserine lactone-based autoinducer molecule thought to function in quorum sensing. *Rahnella* naturally expresses a chromosomally encoded extended-spectrum Amber class A beta-lactamase. It can be treated with ceftazidime, imipenem, and piperacillin-tazobactam (Tash, 2005).

1.3.6 The rise of antibiotic resistance and importance of finding new treatments.

The raising spread of drug-resistant bacteria has been of concern to the medical sector and society. As a result of the expensive cost of production of antibiotics, as well as a quick turnaround in acquisition of resistance to these antibiotics by bacteria, very limited new antibiotics are imminent. This is more observed as only four new classes of antibiotics have been approved since the seventies (Eriksson, 2015, Cooper and Shlaes, 2011). Nonetheless, fidaxomicin and bedaquiline are two classes of antibiotics approved over the last couple of years with a narrow spectrum for treatment of gram-positive *Clostridium difficile* and *Mycobacterium tuberculosis* respectively (Butler *et al*., 2013). The acquisition and spread of antibiotic resistance between bacterial species are a quick process (Davies, 1994). This ease of spread has become a major health concern as resistant bacteria can be isolated within months after introduction in most new types of antibiotics. Multi-resistant bacteria have become a global pandemic and peak occurrences have been attributed in countries where there are large antibiotic prescription routines or over-the-counter distribution of antibiotics (Eriksson, 2015). Since 2004, this problem has been tackled by governments by making numerous recommendations and restrictions to antibiotic use. Some of these recommendations were reported in the O'Neill report (2016) of tackling drug-resistant infections globally. One of such restrictions was the 2006 European Union ban (Regulation 1831/2003/EC) on additives for use in animal nutrition. All efforts have been focused on decreasing the spread of multi- drug resistant bacteria by reducing or ceasing the use of antibiotics for treating minor infections in society. Carbapenems, which are regarded as the last line of action for treatment against resistant bacteria have also seen emerging resistant isolates. This buttresses the importance of not only reducing the use of antibiotics but also looking at different treatment options for bacterial infections. This has led to a renewed interest in research into bacteriophage (phage) antimicrobial derived compounds as alternative or adjuvants forms of treatment (Eriksson, 2015).

In the last few years interest in phage therapy has grown as demonstrated by the number of papers published on the topic: 700 as of October 2017 (using "phage therapy" as a search term in the PubMed search engine). In Poland, the Hirszfeld Institute of Immunology and Experimental Therapy provides phage treatment for patients that have been through numerous ineffective antibiotic treatments whilst the Eliava Institute in Georgia, produces and commercializes phage cocktails, which are a mixture of phages targeting etiologic agents of specific diseases, such as intestinal disorders or skin infections (Kutter., 2010). Yet phage therapy won't be taken seriously until controlled clinical trials are published in major medical journals. A detailed description of the composition of the drug may aid the approval of phage cocktails by the European Medical Agency and the US Food and Drug Administration (FDA). As biological entities, phages are designed to mutate over time. Additionally, phages multiply at the site of infection, which has two-sides: it has the advantage of effective treatment even at low doses, but on the other side, it implies a complex pharmacokinetic that cannot be simply reconstructed. Controlled human clinical trials of phage therapy are required by the FDA, which also stresses the need of several phage therapy trials conducted against different infectious diseases (Brüssow, 2012).

1.4 Phage Biology and classification

Bacteriophages (phages) are infectious agents that replicate as obligate intracellular parasites in bacteria. They are viruses that rely on bacterial metabolism and replication machinery to produce and transfer their progeny. The genetic material of phages comprises of double-stranded DNA (dsDNA; the vast majority), single-stranded DNA (ssDNA), single-stranded RNA (ssRNA), or double-stranded RNA (dsRNA; very rare) (Holmes and Jobling, 1996). Also, the Baltimore classification system takes into account combination of their nucleic acid (DNA or RNA), strandedness (single-stranded or double-stranded), reading frame orientation, and method of replication, placing viruses into one of seven groups designated by Roman numerals and depending on their mode of replication and genome type (Baltimore, 1971). The electron microscope analyses allowed the discovery of different phage morphologies; phages can be tailed, polyhedral, filamentous, or pleomorphic, and some have lipid or lipoprotein envelopes (Ackermann, 2006). Since 1966, the International Committee on Taxonomy of Viruses (ICTV) have tried to taxonomically classify phages based on their genome and the morphology. Tailed phages constitute the majority (96%) of bacterial viruses. They belong to the order *Caudovirales* (from latin cauda, tail), which have double stranded DNA (dsDNA) as genetic material. The families in this order are *Siphoviridae* characterized with a noncontractile and flexible tail, *Myoviridae*, characterized by a straight contractile tail and *Podoviridae*, characterized by having a short tail (Figure 6) (Ackermann, 2005; Ackermann, 2009).

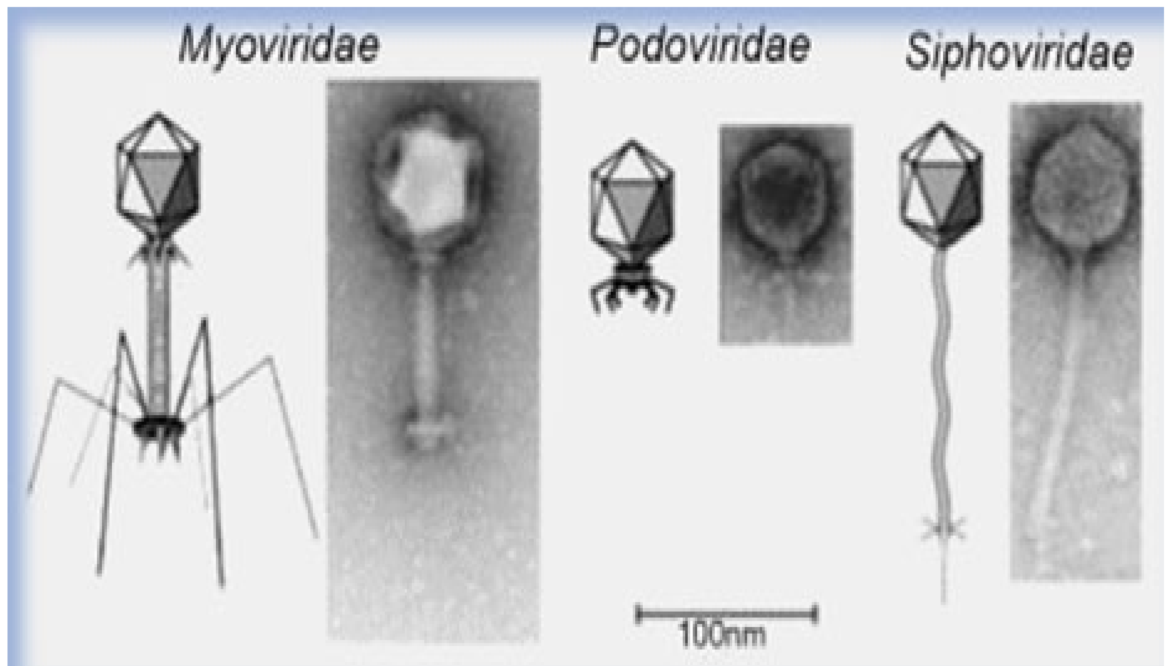

Figure 6. Families in the order of Caudovirales. (Harper *et al.*, 2011)

1.5 Phage Infection

Phages have various possible life cycles which, along with interaction with their physical environment, dictate their role in bacterial/archaeal biology (Clokie *et al.*, 2011). Viruses show two life cycles (Figure 7): lytic or lysogenic cycles. In the lytic cycle, the (lytic or virulent) phage lyse the bacterial cell upon infection, introducing their genetic material into the host and redirecting the host metabolisms towards the production of new phage, which are released during the lysis of the cell. In the lysogenic cycle, the genome of the (temperate or lysogenic) phage characteristically remains in the host in a prophage stage (inactive) and reproduces along with the host, creating a symbiotic relationship that in some cases results in mutualism, where the prophage, the integrated phage, provide fitness and evolutionary advantages to the lysogenic bacterium until the lytic cycle is induced (Ackermann, 1987; Weinbauer, 2004).

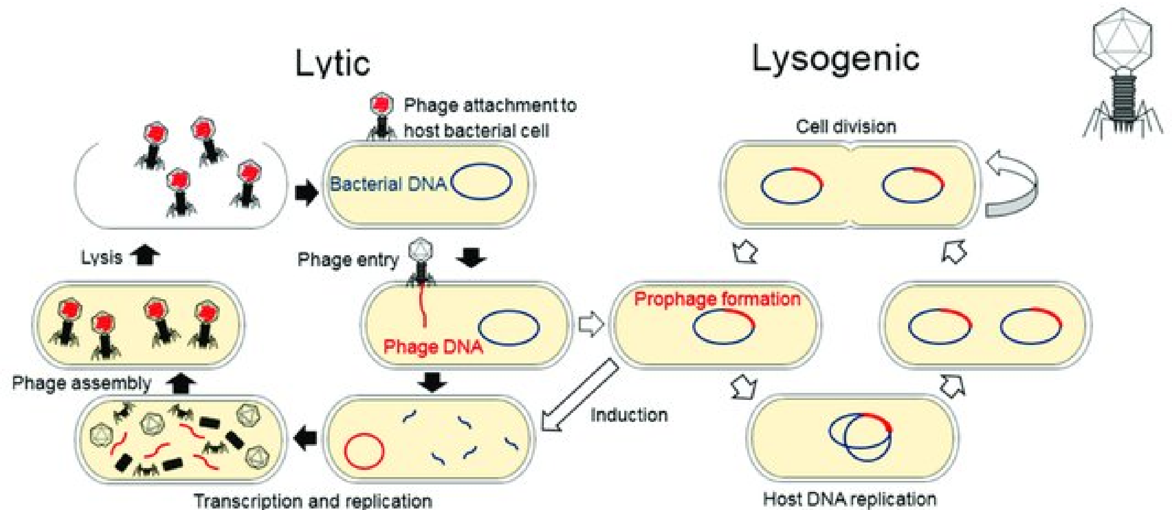

Figure 7. Bacteriophage Lifecycle: Lytic phages attach and infect a bacterial cell which results in the reproduction of phages and lysis of the cell host and the lysogenic cycle results in the integration of a phage genome into the bacterial genome. Some lysogenic phages do not integrate into the genome and remain in the cell as a circular or linear plasmid (not depicted here) (Batinovic *et al*., 2019).

The life cycle of a phage is separated into several stages: adsorption, separation of nucleic acids from protein coat, expression and replication of the nucleic acids, virion assembly, release, and transmission (Duckworth, 1987). Virion adsorption on host cell surface is usually explained as the process involving 2 stages: reversible and irreversible binding. The initial step of adsorption to a cell surface is reversible presenting an opportunity for phage to progress to an infection or remain dormant. During the second stage, the adsorption of phage is an irreversible binding between bacterial surface receptors and phage tail fibres. Subsequently, phage enzymes make the cell wall penetrable and the genetic material is injected into the cell. At this stage, the injected phage genetic material can either remain in the cytoplasm or be incorporated into the host genome. However, for all viruses, there is a stage where the injected phage genetic material is not integrated into the host genome but exists within the cytoplasm of the host cell. This is the stage where genomes, tails and capsids are formed before the genomes are packaged into the capsids, genome replication, and gene expression occurs (Weinbauer, 2004). There are 3 stages that describes the life cycle of phages from adsorption to cell lysis: the latent period where no phage is seen outside the host genome; the eclipse period where matured phages are assembled from genomes and capsids; and the rise period where the mature phages are released by cell lysis (Rakhuba *et al*., 2010; Weinbauer, 2004). Phages and bacteria have coevolved in a fierce arms race that has involved the development of strategic defence mechanism {such as the restriction-modification system; CRISPR (clustered regularly interspersed palindromic repeats) and the abortive infection system} by bacteria and the phage counterattack with restored infectivity.

1.6 Phage evolution

The evolution of bacteriophages has been attributed to different mechanisms including DNA replication errors, environmental mutagens and HGT mechanisms. The large diversity seen among bacteriophages comes from single point mutations occurring during genome replication. These minimal rate mutations are then integrated into phage offspring's (Eriksson, 2015, Duffy *et al*., 2008).

In a process called recombination, DNA is easily integrated into genomes by exchanging or recombining of genetic material, whilst in prokaryotic cells, genetic materials are exchanged through HGT mechanisms and in some cases the integrity of the genome is preserved through DNA repair mechanisms. In general, 4 types of naturally occurring recombination have been described: homologus, non-homologous, site-specific, and replicative recombination (Eriksson, 2015).

The ever-evolving coexistence of phages and bacteria is responsible for bacteria diversification. Phages contribute through lysogenic conversion or transduction, to the HGT between microbial genomes. The three classical mechanisms for HGT in prokaryotes are transformation, conjugation, and transduction. Historically, conjugation and transformation were the major contributors to bacterial HGT, however transduction plays important roles in the transfer of antibiotic resistance genes and in the acquisition of novel genes during intra-specific bacterial competition. The characteristics of transduction and lysogenic conversion complement those of other mechanisms of transfer and could play a key role in the spread of adaptive genes between communities (Schneider, 2017; Touchon *et al*., 2017).

1.7 Bacterial defence system against phages

In the different stages from when phages adhere to the cell, to when they are assembled and released into the environment, bacteria have developed mechanisms to defend against phage infection at every stage (Figure 8). This bacterial defence system against phage invasion can be categorised into three: adsorption inhibition, restriction, and abortive infections (Hyman and Abedon, 2010).

1.7.1 Adsorption inhibition

Adsorption inhibition is the primary defence mechanism of the bacteria. It does this by blocking the attachment and transfer of bacteriophage genome into bacteria host cell. The part of the bacterial cell wall that phages binds to is usually a conserved part of the cell wall. Phages can bind to different surface structures on the bacterial cell wall before adsorption, such an example is T5 phage binding to an outer membrane transport protein FhuA (Flayhan *et al*., 2012). Some systems of adsorption inhibition include bacterial surface

receptors which can become resistant to bacteriophages adsorption by changing the structure on their cell surface receptor thereby blocking phage receptors. This process ensures bacteria are protected from infection (Figure 8A). It can also lead to a process called superinfection exclusion (Sie) where host cells are granted immunity against subsequent infections by specific phages (Labrie *et al*., 2010); external polysaccharides of bacteria which can cover the cell in a capsule layer which is usually described as a virulence factor since it protects bacteria from phage infection and animal immune system. However, phages have developed the capability to digest these polysaccharide capsules; biofilm production where bacteria can provide alternative defence mechanisms by producing an outer protective polymer layer or biofilm which comprises of clusters of microorganisms, that can stick to surfaces such as epithelium in animals. (Hall-Stoodley *et al*., 2004). These polymer layer consist of different types of extracellular polymeric substances including proteins, lipids, DNA, and polysaccharides. These aggregates are physically joined, granting protection to these microorganisms against phages, antibiotics, and other environmental factors (Bjarnsholt, 2013).

1.7.2 Restriction modification systems

In what is seen as the secondary defence mechanism, bacteria can restrict the invasion of foreign DNA. Bacteria have developed several defence systems towards that goal including: the digestion of foreign DNA and the protection of the host DNA. The host DNA avoids restricting its own DNA by modifying it through methylation. Restriction - modification (RM) systems function to protect bacterial cells from invading foreign un-methylated DNA molecules. The RM systems are divided into several categories depending on enzyme composition and cofactor requirements, recognition sequence sites, and cleavage position. Found in 80 % of sequenced bacterial genomes, type II RM system is the most published in literature (Wilson and Murray, 1991). The RM systems comprises of a pair of opposing intracellular enzyme activities: a restriction endonuclease (REase) and a DNA methyltransferase (MTase), which interacts with specific nucleotide sequences in DNA. The REase explicitly marks and cuts double-strand DNA at certain nucleotide sequences, while the MTase methylates (adds one methyl group) cytosine or adenine residues in the same nucleotide sequence. The enzymes co-exist simultaneously at various molarity, with REase being the most abundant. Interestingly, phages have developed the capability to avoid digestion by RM systems, e.g., base modification to prevent recognition, DNA masking and endonuclease blocking (Samson *et al*., 2013).

1.7.3 CRISPR-Cas

The clustered regularly interspaced short palindromic repeats (CRISPR)-Cas (CRISPR-associated proteins) are found in many bacteria and the CRISPR-Cas system provides adaptive immune system to prokaryotes by integrating plasmid and viral DNA fragments in loci of CRISPR on host chromosome (Westra *et al*., 2012a, Wiedenheft *et al*., 2012). This is a defensive method that functions in three stages: adaptation, expression, and interference. During the adaptation phase, resistance is acquired by integrating foreign DNA into the CRISPR array. During the expression phase, *cas* genes are transcribed and translated whilst during the interference stage, *cas* proteins cleave complementary nucleic acids conferring immunity against a subsequent infection by a phage with an identical sequence to the acquired DNA (Westra *et al*., 2012b, Swarts *et al*., 2012). The CRISPR array, keeps track of prior infections experienced by the cell. When viruses infect the cell for the first time, the specific Cas proteins cleave the invading genetic material and introduce the resulting snippets into a CRISPR array. These snippets are used to recognize a second invasion from the same phage and to promptly inactivate the invading DNA/RNA (Richter, *et al*., 2012). The CRISPR-cas system is divided into three subtypes: type I, II and III. This classification was made based on signature genes present in each type and each type uses a unique set of Cas proteins along with CRISPR RNAs (crRNA) for CRISPR interference(Shabbir *et al*., 2019). In contrast to the type I and type III systems that utilize a large multi-Cas protein complex for crRNA binding and target sequence degradation, type II CRISPR systems uses a unique DNA endonuclease, Cas9, to recognize dsDNA substrates and cleave each strand with a distinct nuclease domain (Jiang and Doudna, 2017; Hille and Charpentier, 2016). During this silencing process, an additional small noncoding RNA, called the *trans*-activating crRNA (tracrRNA), base pairs with the repeat sequence in the crRNA to form a unique dual-RNA hybrid structure. This dual-RNA guide directs Cas9 to cleave any DNA containing a complementary 20-nucleotide (nt) target sequence and adjacent protospacer adjacent motif (PAM) (Jiang and Doudna, 2017). Bondy-Denomy *et al*., 2013 observed that *Pseudomonas aeruginosa* temperate phages using the crRNA/Cas complex could overcome restriction by the CRISPR-Cas system by cleaving foreign DNA molecules at sites complimentary to the crRNAs, thus granting resistance to phages and other invading DNA molecules.

1.7.4 Abortive infection

Abortive infection (Abi) is a bacterial defence mechanism employed by bacteria against phages to initiate autolysis or cause cell to arrest phage infection cycle. This process decreases, or eradicates, production of phage progeny and protects bacterial population by an act like known as “altruistic suicide” where infected bacterial cells die abruptly (Figure

8C). It can be facilitated by toxin-antitoxin (TA) systems. Different types of Abi systems have been published with their principle of operation reliant on a single gene. Most are domiciled on plasmids with some exceptions where Abi system is part of the bacterial genome (Samson *et al*., 2013, Eriksson, 2015).

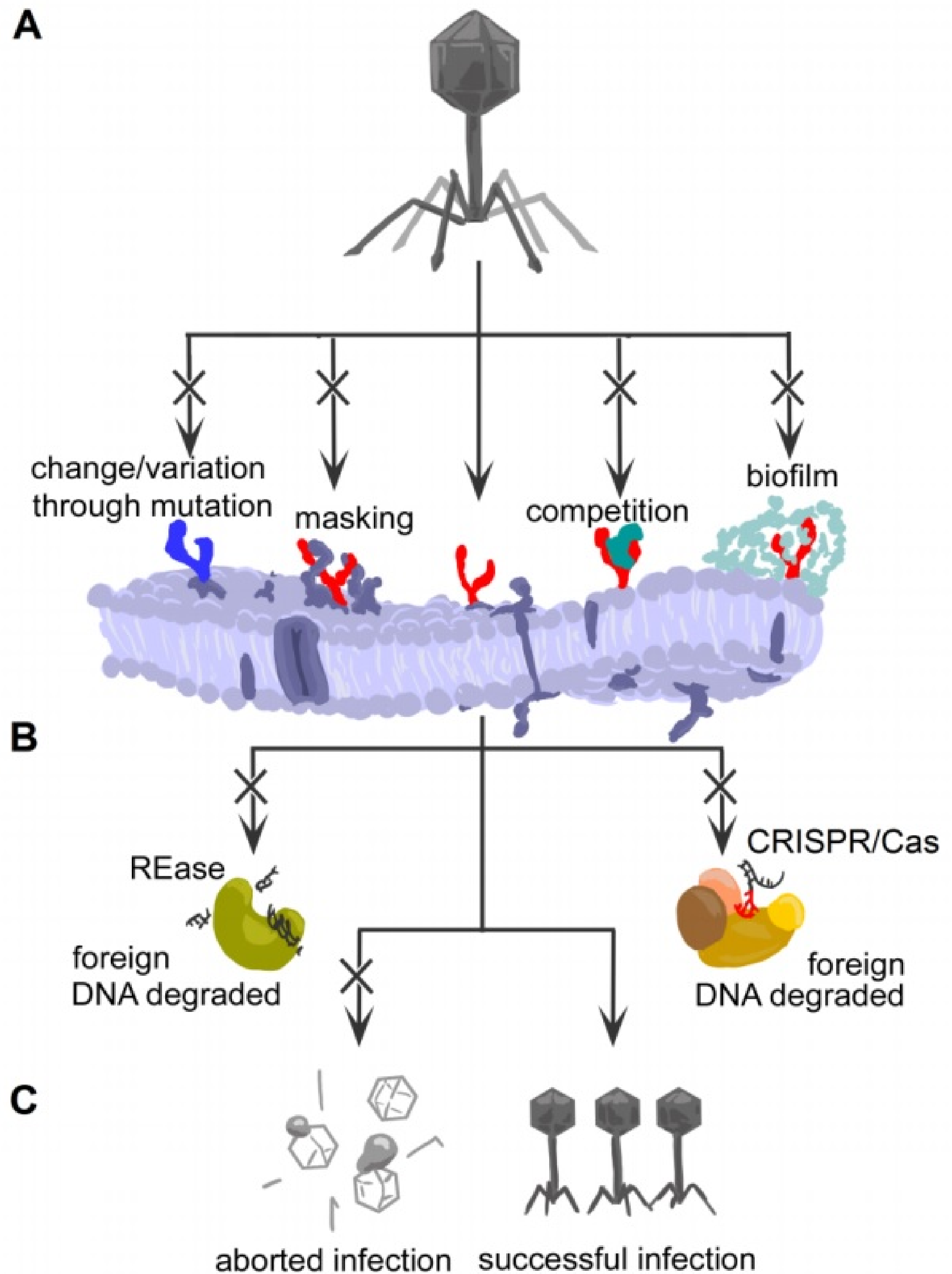

Figure 8. Bacterial phage defense mechanisms. A) Adsorption inhibition, B) Restriction, C) Abortive infection (Eriksson, 2015).

1.8 History of phages and phage therapy

Traditionally, the discovery of bacteriophages is traced to the papers of Twort and D'Hérelle. However, earlier studies suggested the presence of phage like antibacterial activity. One such was reported in 1896 by Ernest H. Hankin in the Jumna and Ganges rivers. The British chemist described the first inkling that phages existed, when he discovered the potential of these waters to stop the spreading of cholera in the villages close to the rivers (Abedon *et al*., 2011). Not until 1915 when British bacteriologist Frederick Twort published an article in the renowned journal "Lancet" describing the existence of a glassy, transparent material that grew in Micrococci cultures. It was however not apparent if it was a virus, an enzyme produced by the same bacteria, or a small bacterium. At that time, it was difficult to obtain proof of their existence as pathogenicity towards higher organisms was the only evidence available of the presence of an "ultra-microscopic virus" (Twort, 1915).

Two years later, in 1917, the French-Canadian Félix D'Herelle coined the name 'bacteriophages' for these infectious agents lysing bacteria, when he discovered invisible microbe that was present in the bacteria-free filtrates of stool samples from dysentery patients (Roux, 2011; Summers, 2001). D'Herelle was credited with developing the idea of 'phage therapy', a therapeutic and prophylactic treatment aimed at taking advantage of phage selectivity in the cellular destruction of pathogenic bacteria while remaining completely innocuous to host cells. D'Herelle's approach led to a lot of international success where phages were used to treat dysentery in Brazil, Senegal, Egypt while the impact of phage against typhoid and paratyphoid were studied in Britain, Italy, and Greece. In USA, anti-staphylococcal phage suspensions were produced and distributed to many hospitals and during World War II, phage therapy was used by the German army (Fruciano and Bourne, 2007). Bacteriophage therapy was widely used around the world in the 1930s and 1940s to treat various infections.

However, the lack of understanding in phage biology, a lack of knowledge in phage specificity, the use of single type of phage to treat bacterial infection, improper phage preparation leading to phage inactivation, led to controversies around their effectiveness. With the introduction of antibiotics, commercial production of therapeutic phages stopped in most of the Western world. However, in some Eastern European countries and in the former Soviet Union, therapeutic phages have continued to be used instead or as an adjuvant with antibiotics. "Two such institutions actively involved in therapeutic phage research and production were, the Eliava Institute of Bacteriophage, Microbiology, and Virology (EIBMV) of the Georgian Academy of Sciences, Tbilisi, Georgia, and the Hirszfeld Institute of Immunology and Experimental Therapy (HIIET) of the Polish Academy of Sciences, Wroclaw, Poland" (Sulakvelidze *et al*., 2001; Semler *et al*., 2012).

1.9 Mechanism of Phage Therapy

The idea of using phage therapy to treat clinical infection involves three basic steps. Firstly, the root cause of the infection must be determined. The causative agent must be isolated and characterized to evaluate the sensitivity of the strain. Secondly, a phage cocktail preparation, preferably with a high titer of a variety of host receptors effective against the corresponding strain, must be made. Finally, at the localised site of infection, this phage preparation must be administered correctly to the patient (Semler *et al*., 2012, Bradbury, 2004). According to published literature, phage preparation can be administered in several ways including oral, rectal, topical, intravenous, and via an aerosol (Sulakvelidze and Morris, 2001).

The pharmacokinetics of phage treatment are unique in relation to any medication presently utilized. An example is the inverse relationship seen with a chemical drug where concentration decreases after initial administration. The reverse is valid for phage treatment where the dosage of phage preparation administered is increased until the infection is cleared, because the initial phage dosage replicates. This is known as active phage therapy whilst a situation where the initial phage dose administered is effective enough in clearing the infection without replicating is known as passive phage therapy (Payne and Jansen, 2003). While it is untrue to suggest that all that is required for a successful active phage therapy is a low phage titer preparation, Payne and Jansen (2003) using a numerical model mimicking the pharmacokinetics of phage treatment observed that bacterial density of the infection is key in choosing the appropriate concentration needed for a successful treatment. They argued that a less dense bacterial population could not support an active phage therapy because phages would be unable to replicate rapidly enough to yield and sustain the high concentrations that are needed to maintain a high bacterial mortality rate. However, in situations like this, to entirely clear the infection high titer phage preparation must be administered, in a similar plan to antibiotic therapy (Levin and Bull, 2004).

In summary, wastewater is a large reservoir of microorganisms with antibiotic resistance genes and have become increasingly significant in the spread of multi-drug resistant microbes. Although the focus has been put in limiting the spread through creation of wastewater treatment plants where effluent is recycled for use in the environment, this can also serve as a route of infection to humans with the effluent thought to be a hotspot for antibiotic resistance genes. The spread of infections caused by ESBL producing bacteria have added to the already increasing financial burden of healthcare providers. Increased duration of hospital stays, prolonged treatment and additional nursing requirements all contribute to the increased costs of patient care. In addition to the increased cost to the NHS, the spread of ESBL producing bacteria within the hospital and community settings

has been one of the most significant epidemiological challenges in recent years, increasing predicaments for clinicians due to limited treatment options. Reduction in treatment options has forced carbapenems, particularly meropenem, to be the treatment of choice, however resistance because of overuse of this agent is also a concern. The application of bacteriophages to control microbial populations in environmental situations has been used successfully in medicine and food technology industries. The control of ESBL-producing bacteria is likely to be an important consideration in the future of multiple drug resistant pathogen control.

1.10 Statement of aims and objectives.

This project aims to contribute towards the expansion of different varieties of bacteriophage cocktails that can be used to kill pathogens in treated wastewater. The specific aim of this project is to isolate and characterize bacteriophages targeting different bacteria, which may be producing Extended Spectrum Beta-Lactamase (ESBL). These bacteria exhibit resistances to a wide variety of modern antibiotics making them difficult to eradicate. Specific objectives of the investigation are as follows:

- Collection of water samples from Petersfield water plant for isolation of ESBL producing bacteria.
- Identification and characterisation of environmentally ESBL producing bacteria for isolation of bacteriophages.
- Isolation and characterisation of bacteriophages targeting specific bacteria strains, which may show antibiotic resistance because of ESBL production.

2.0 Materials and Methods

2.1 Media preparation

Different media were used in this study. LB medium was used for initial growth of environmental samples, for morphology study of isolated bacterial colonies and for preparation of serial dilution of the samples. LB agar was used for growth of isolated bacteria and counting of live bacteria when studying their growth. Mueller-Hinton agar was used for antimicrobial susceptibility, ESBL and AmpC production tests. Tryptone soy broth (TSB) and Tryptone soy agar (TSA) were used for bacterial growth for extended biochemical test. Media composition and preparation were as follows:

2.1.1 LB medium

Tryptone (10 g), Yeast Extract (5 g) and NaCl (10 g) (Fisher Scientific) were dissolved in 1 L of dH_2O, adjusted the pH to 7.0 and autoclaved at 121°C for 15 min.

2.1.2 LB agar

Agar Bacteriological (15 g) (Fisher Scientific) was added to 1 L of LB medium and autoclaved at 121°C for 15 min. The LB agar was chilled to 60°C in a water bath and approximately 25 ml poured into each Petri dishes.

2.1.3 Mueller-Hinton Agar

Mueller-Hinton Agar (38 g) (Fisher Scientific) was added to 1 L dH_2O and autoclaved at 121°C for 15 min. The medium was chilled up to 60°C in a water bath and approximately 25 ml was poured into each Petri dishes.

2.1.4 Tryptone soy broth

TSB (30 g) (Fisher Scientific) was added to 1 L of dH_2O and autoclaved at 121°C for 15 min.

2.1.5 Tryptone soy agar

TSB (30 g), Agar Bacteriological (15 g) (Fisher Scientific) were added to 1 L dH_2O and autoclaved at 121°C for 15 min. The TSB agar was chilled to 60°C in a water bath and approximately 25 ml poured into each Petri dishes.

2.1.6 Soft agar

Agar Bacteriological (0.6 g) (Fisher Scientific) was added to 100 ml of LB medium and autoclaved at 121°C for 15 min. The soft agar was chilled up to 47°C in a water bath and 3 ml was used for the overlay technique (page 38).

2.1.7 Bacteriophage buffer

Ten ml of 1M Tris-HCl (pH 7.5), twenty ml of 5M NaCl, ten ml of 1M $MgSO_4 \cdot 7H_2O$ was added to 960 ml of sterile dH_2O.

2.2 Strains used in this study

Seventeen bacterial strains were used in this study (Table 2). Twelve were isolated from water samples obtained from the Petersfield water plant while 5 were obtained from the University of Portsmouth culture collection.

Table 2. Strains used in this study.

Strains used in the study	Sources
EwS1	Isolated from water samples
EwB2	
**E. coli* (strains 1-6)	
**Pseudomonas* (strains 1-4)	
Serratia marcescens ATCC 17 AW	Laboratory culture collection
Hafnia alvei UoP culture collection	
Klebsiella pneumonia ATCC 13883	
E. coli K802	
Proteus mirabilis ATCC 7002	

* Shows *E. coli* and *Pseudomonas* strains isolated from wastewater.

2.3 Collection of water samples and bacterial enrichment

Environmental samples were collected from water treatment tank (site 4) from the Petersfield Water Treatment plant Hampshire, UK (Figure 9). The samples (100 ml) were collected into a sterile plastic container, packaged, and transported to the laboratory for bacteriological analysis in accordance with the Carriage of Dangerous Goods and Use of Transportable Pressure Equipment Regulations 2009. The samples for bacterial enrichment were diluted 1:50 with LB medium (Bertani, 2004) and were cultured under aerobic conditions for 4 hr at 30°C.

Figure 9. Schematic view of Petersfield Water Treatment plant. Untreated Effluent enters at Site 0 and is screened. Water containing more dense particles removed and taken underground to Site 1 for sludge processing independent from the rest of the plant. The remaining water moves through a pipe where ferric sulphide is added. The large volume of water is split in half into site 2 and site 3, for ease of processing before it is moved to the start of a series of trickling filters. The contaminated water is spread, via mechanical rotating arms, over basins containing large stones. Aeration of the water favours the breakdown of organic materials as it is passed over biofilms present on the gravel. Water is then passed through a holding tank (Site 4) before ejection into the surrounding environment. The reed beds are used to handle overflow, resulting when the rainfall is heavy. This provides some treatment before the water is released.

2.4 Isolation of extended spectrum beta lactamase producing bacteria

Ten times serial dilution of the enriched samples were prepared and used for isolation of extended spectrum beta lactamase (ESBL) producing bacteria by spreading of 200 µl onto

chromogenic medium (Oxoid Brilliance ESBL Agar). The plates were sealed and incubated inverted at 30°C for 48 hr. For further purification, the colonies with different colour and morphology were picked up with a sterile loop, re-streaked onto fresh chromogenic medium. Sealed and inverted plates were incubated at 30°C for 48 hr.

2.5 Identification and characterization of ESBL producing bacteria using microbiological techniques

2.5.1 Antimicrobial susceptibility test

Antimicrobial susceptibility test was carried out according to British Society for Antimicrobial Chemotherapy (BSAC) guidelines (Wootton, 2013). Bacterial colony was suspended in 5 ml of nuclease-free water (Sigma) and the optical density was adjusted to 0.5 McFarland standard by comparing the test and standard suspensions against a white background with a contrasting black line (Wootton, 2013). The inoculum (200 µl) was evenly plated over Mueller-Hinton Agar (Fisher Scientific) using sterile spreader. After inoculation, antibiotic discs contaning Amoxicillin (10 µg/ml), Amoxicillin-clavulanic acid (30 µg/ml), Meropenem (10 µg/ml), Cefotaxime (30 µg/ml), Ertapenem (10 µg/ml), Ceftazidime (10 µg/ml), Cefepime (30 µg/ml), Tigecycline (15 µg/ml), Amikacin (30 µg/ml), Gentamicin (10 µg/ml), Ciproflaxcin (1 µg/ml), Piperacilin-tazobactam (75/10 µg/ml), and Cefuroxime (30 µg/ml) (all Fisher Scientific), were evenly spaced on the plates. After 24 hr incubation at 30°C, the results were interpreted by measuring diameters of the zone of inhibition in millimetres (Figure 10) and comparing with acceptable zone diameter breakpoints for *Enterobacteriaceae* (Wootton, 2013). Bacteria were classified as sensitive, intermediate demonstrating low level resistance or resistant to each antibiotic.

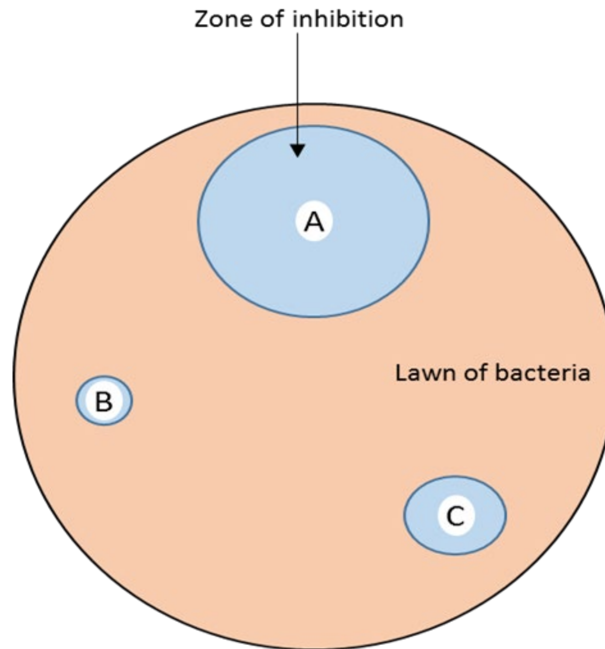

Figure 10. Schematic diagram of the results of antibiotic sensitivity test. Creamy coloured area is bacterial lawn and A, B and C are antibiotic impregnated disks. A - bacteria are susceptible to an antibiotic, B - resistant to an antibiotic, C – bacteria have intermediate resistance to an antibiotic. The diameters of zones of inhibition are measured to the nearest millimetre (zone edge should be taken as the point of inhibition as judged by the naked eye) with a ruler. These zones can have different sizes for different antibiotics.

2.5.2 ESBL and AmpC production

ESBL producing microorganisms can be resistant to AmpC or have both AmpC and ESBLs resistance. To differentiate between these alternatives, the ESBL and AmpC disc diffusion test (MAST Group Ltd) was performed (Derbyshire *et al.*, 2009). This method uses four test discs (A - cefpodoxime, B - cefpodoxime plus an ESBL inhibitor, C - cefpodoxime plus an AmpC inhibitor and D - cefpodoxime plus ESBL and AmpC inhibitors). Single bacterial colony was suspended in 5 ml of nuclease-free water (Sigma) and the optical density was adjusted to 0.5 McFarland standard according to BSAC guidelines (Wootton, 2013). Each inoculum (200 µl) was evenly spread over the Petri dishes with Mueller-Hinton Agar and four identification discs were placed onto the agar plate. The plates were incubated at 30°C for 24 hr. Calculation of zone of inhibition diameters of each of the four discs identifies the ESBL, AmpC or both ESBL and AmpC determinants in isolates (Figure 11). The results were determined and interpreted by using the MAST ESBL calculator spreadsheet version 10.3, available from, http://www.mastgrp.com/Users_Details.asp.

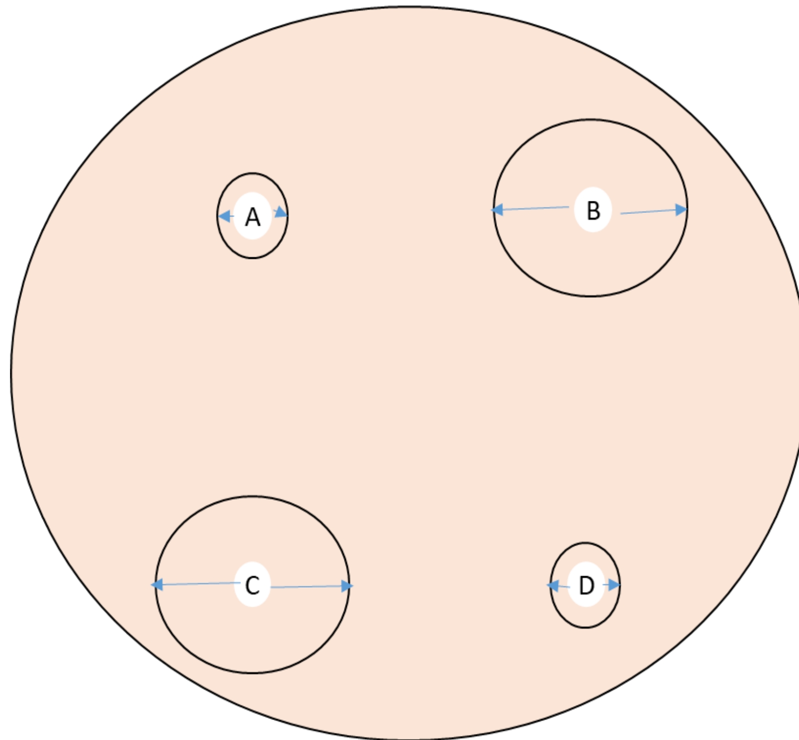

Figure 11. Schematic diagram for calculation of bacterial growth inhibition zone discs for determination of antibiotic resistance type. The above calculation corresponds to bacteria having an ESBL resistance only as the zone diameter of Disc B – zone diameter of Disc A and zone diameter of Disc D – zone diameter of Disc C was ≥ 5 mm and the difference of each zone diameter of Disc B – zone diameter of Disc D and zone diameter of Disc A – zone diameter of Disc C was < 4 mm.

2.5.3 Identification of isolated ESBL producing bacteria using API 20 E strip test.

The API 20 E system (Biomerieux) uses a standard principle of assessing the enzymatic and metabolic activities that are specific for bacteria belonging to *Enterobacteriaceae*. The inoculum was prepared by suspending single bacteria colony in 5 ml of 0.9 % NaCl solution, the optical density (OD) was adjusted to 0.5 McFarland standard according to BSAC guidelines (Wootton, 2013). The inoculum was evenly distributed within the 20 cupules of the API 20 E strip; ensuring that wells arginine dihydrolase (ADH), lysine decarboxylase (LDC), ornithine decarboxylase (ODC), sodium thiosulfate (H_2S) and urea (URE) were sealed with mineral oil due to the requirement for anaerobic conditions and sodium pyruvate and gelatine (VP and GEL) wells were filled completely. The strip was incubated at 30°C for 24 hr. After incubation, tryptophan deaminase (TDA reagent), Indole (James reagent) and VP (VP1 and VP2 reagents) were added into the cupules for TDA, IND and VP, respectively. The colour changes in cupules were registered and converted into a seven-digit code using the APIWEB data base (Biomerieux), available at

https://apiweb.biomerieux.com. This code can then be converted to a bacterial identification.

2.5.4 Biolog GEN III extended Biochemical test

Biolog GEN III (BIOLOG) is a single 96- well microplate consisting of 94 biochemical tests: 71 carbon sources and 23 chemical sensitivity assays. All microplates were inoculated according to the protocol A. Overnight bacteria cultures grown in tryptone soy broth were streaked onto tryptone soy agar plates and incubated for 48 hr. A single bacterial colony was picked up using a sterile swab and carefully mixed with “inoculating fluid IF-A”. The turbidity was adjusted to a range of 90 – 98 % transmittance at 590 nm by addition of more cells if cell density was too low or inoculating fluid, if density was too high. Each microplate well was inoculated with 100 µl of the cell suspension. After 22 hr of incubation, the colour intensities in wells were observed and recorded as positive in wells having a noticeable purple colour, borderline in wells having a faint purple colour or negative if no colour developed. This well colour development was imaged and sent to BIOLOG for Interpretation using a specialised software and these substrate-utilization profiles were compared with the Biolog software database for identification of the bacteria (BIOLOG).

2.5.5 Growth of isolated bacteria at different temperatures

A total of 8 flasks (250 ml) were used in this experiment. Each flask contained 50 ml of LB broth inoculated with overnight bacterial culture with at the ratio 1:10. For this experiment, the medium was prewarmed and the cultures were grown overnight before inoculation at the temperature to be used for the experiment. Each flask was incubated at its desired temperature of +5°C, +10°C, +15°C, +22.5°C, +25°C, +30°C, +32°C, +37°C with aeration at 150 rpm (SciQuip Incu-Shake MAXI). Each hour of the first 10 hr of incubation, 1 ml of culture was taken under sterile condition for optical density (OD) measurement at 600 nm using a spectrophotometer (Thermo Scientific Helios Epsilon). A ten times serial dilution down to 10^{-7} was made and from each dilution 100 µl was plated on LB agar for counting of living bacterial cells. All experiments were run in triplicate. Growth curves were made by plotting the OD values against temperatures.

2.6 Identification and characterization of ESBL producing bacteria using molecular biology techniques.

2.6.1 Purification of Genomic DNA

Total bacterial DNA was purified using the DNeasy Blood and Tissue kit (Qiagen®) according to the manufacturer’s instruction. The bacterial cells were incubated overnight in 5 ml of the LB at 30°C in a shaking incubator at 150 rpm (SciQuip Incu-Shake MAXI). Three

ml of cultures was harvested by centrifuging for 10 min at 5000 x g. The pellet was suspended in 180 µl Buffer ATL. 20 µl of Proteinase K (600 mAU/ml) was added, sample vortexed, incubated at 56°C for 1 hour 30 min. The suspension was then vortexed thoroughly for 15 sec. 200 µl of Buffer AL (lysis buffer) mixed with ethanol (96%) was added and thoroughly mixed. The mixture was transferred to the DNeasy Mini spin column and centrifuged for 1 min at ≥6000 x g. The column was consequently washed with 500 µl Buffer AW1, then with 500 µl Buffer AW2 and centrifuged for 3 min at 20000 x g to dry the membrane. DNA was eluted from the membrane with 100 µl Buffer AE into a fresh tube. DNA concentration and quality were estimated spectrophotometrically (NanoDrop, Technologies Inc.). The stock solution of DNA for PCR reactions was prepared to make a final concentration of 5 ng/µl.

2.6.2 Purification of Plasmid DNA

Plasmid DNA from bacterial cells was purified using the Zyppy miniprep kit (ZYMO Research) according to manufacturer's manual. Briefly, 3 ml of overnight bacterial culture was centrifuged for 90 sec at 16000 x g. The pellet was suspended in 600 µl of sterile dH_2O. 100 µl of 7X Lysis Buffer was added and mixed thoroughly. Complete lysis was confirmed by the change of colour of the suspension from opaque to clear blue. Cold Neutralization Buffer (350 µl) was added and the suspension mixed thoroughly. The sample turns yellow when the neutralization is completed. The suspension was centrifuged for 4 min at 16,000 x g, the supernatant transferred into the Zymo-Spin™ IIN column and centrifuged for 15 sec at 16,000 x g. The column was washed with 200 µl of Endo-Wash Buffer, then with 400 µl of Zyppy™ Wash Buffer. The column was transferred into a clean microcentrifuge tube and DNA was eluted with 30 µl of elution buffer. DNA concentration and quality were estimated spectrophotometrically (NanoDrop, Technologies Inc.) and analysed by electrophoresis in 1 % agarose gels.

2.6.3 Multiplex PCR Amplification of TEM, CTX and SHV genes

Plasmid and chromosomal DNA were used for multiplex PCR amplification with 3 sets of primers to detect TEM, CTX, SHV genes (Monstein *et al.*, 2007). Multiplex PCR reactions were set up using 12.5 µl of 2x GoTaq® G2 Hot Start Green Master Mix (Promega, UK), 1 µl (10 µM) of each forward and reverse primer (Table 3) and 6.5 µl of bacterial DNA (5 ng/µl) in a final volume of 25 µl. PCR amplification conditions were as follows: initial denaturation step at 95°C for 5 min; 30 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 s, extension at 72°C for 2 min, followed by a final extension step at 75°C for 10 min using the T100TM Thermal Cycler (Bio-Rad). The PCR products were analysed by

electrophoresis in 1.2 % agarose gels and documented using molecular imager® Gel Doc™ XR+ imaging system (Bio-Rad).

Table 3. Primer sequences for PCR amplification of 16S rRNA gene and multiplex PCR analysis of CTX, TEM, SHV, and OXA group I, II, III genes. (Monstein *et al*., 2007; Bert *et al*., 2002).

Primer Name	Sequence 5' to 3'	Amplicon Size (bp)
Bla_{CTX} Forward	ATGTGCAGYACCAGTAARGTKATGGC	593
Bla_{CTX} Reverse	TGGGTRAARTARGTSACCAGAAYCAGCGG	
Bla_{TEM} Forward	TCGCCGCATACACTATTCTCAGAATGA	445
Bla_{TEM} Reverse	ACGCTCACCGGCTCCAGATTTAT	
Bla_{SHV} Forward	ATGCGTTATATTCGCCTGTG	747
Bla_{SHV} Reverse	TGCTTTGTTATTCGGGCCAA	
OXA Group 1 Forward	TCA ACA AAT CGC CAG AGA AG	276
OXA Group 1 Reverse	TCC CAC ACC AGA AAA ACC AG	
OXA Group 2 Forward	AAG AAA CGC TAC TCG CCT GC	478
OXA Group 2 Reverse	CCA CTC AAC CCA TCC TAC CC	
OXA Group 3 Forward	TTT TCT GTT GTT TGG GTT TT	427
OXA Group 3 Reverse	TTT CTT GGC TTT TAT GCT TG	
8F	AGA GTT TGA TCC TGG CTC AG	1500
1492R	GGT TAC CTT GTT ACG ACT T	

2.6.4 Multiplex PCR Amplification of OXA group genes

DNA from purified bacterial cells were used for multiplex PCR amplification with 3 sets of primers to detect OXA groups I, II, III genes (Bert *el al*., 2002). Multiplex PCR reactions were set up using 12.5 µl of 2x GoTaq® G2 Hot Start Green Master Mix (Promega), 1 µl (10 µM) of each forward and reverse primer (Table 3) and 6.5 µl of bacterial DNA (5 ng/µl) in a final volume of 25 µl. The template DNA was amplified using T100TM Thermal Cycler using the same cycling conditions as described for TEM, CTX and SHV multiplex PCR. The

PCR products were analysed by electrophoresis in 1.2 % agarose gels and documented by molecular imager® Gel Doc™ XR+ imaging system (Bio-Rad).

2.6.5 PCR amplification of the 16S rRNA gene

Total genomic DNA was used for PCR amplification of 16S rRNA gene. PCR reactions were set up using 10 µl of bacterial DNA (5 ng/µl), 1 µl (10 µM) of each forward and reverse primer (Table 3) (Turner, 1999) and 12.5 µl 2x GoTaq Green master mix (Promega) and 0.5 µl of nuclease-free water (Sigma) in a final volume of 25 µl. PCR amplification conditions were as follows: initial denaturation step at 94°C for 2 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 45 s followed by the final extension step at 72°C for 15 min (Lane, 1991) using the T100TM Thermal Cycler (Bio-Rad, UK). The PCR products were analysed by electrophoresis in 1.2 % agarose gels and documented using molecular imager® Gel Doc™ XR+ imaging system (Bio-Rad).

2.6.6 Preparation of agarose gels for electrophoresis

Agarose gels 1-1.2 % (Sigma) were made by mixing the required amount of agarose with 100 ml of 1 x Tris-acetate EDTA (TAE) buffer and melted using a microwave. The ends of the gel tray were sealed with small amount of agarose and the rest of the molten agarose was cooled down to 50°C, 6 µl of SYBR® safe DNA gel stain (Invitrogen) was added and all poured into a gel tray, a comb was inserted, and gel was left to solidify. The tray was placed into a gel box and covered with 1 x TAE buffer and the comb was removed. Promega 1 kb DNA ladder (8-10 µl) and samples were loaded. Gels were typically run at 150 volts for 1 hour and 15 min. The PCR products on the gels were visualised under UV light and images captured using molecular imager® Gel Doc™ XR+ imaging system (Bio-Rad).

2.6.7 Sequence analysis of the 16S rRNA gene

PCR products of amplified 16S rRNA gene were cleaned up using QIAquick PCR purification kit (QIAGEN) according to manufacturer's instructions. Briefly, five volumes of Buffer PB were added to 1 volume of the PCR sample. The mixture was added to a QIAquick spin column to bind DNA to the column membrane and centrifuged for 30–60 sec at 17,900 x g. The column was washed with 0.75 ml Buffer PE and centrifuged for 30–60 sec at 17,900 x g. The column was centrifuged for an additional 1 min at 17,900 x g to dry the membrane and placed into a clean tube. Fifty µl of nuclease-free water (Sigma) was added to the centre of the QIAquick membrane, left for 1 min at room temperature and DNA was eluted by centrifugation for 1 min at 17,900 x g. DNA concentration and quality were estimated spectrophotometrically (NanoDrop, Technologies Inc.). DNA solution (30-40 µl)

(10-50 ng/µl) was sent for sequencing (GATC Biotech Ltd). DNA sequences obtained were confirmed using the nucleotide BLAST program.

2.6.8 Whole Genome Sequencing

Whole genome sequencing was performed by MicrobesNG. Overnight culture was streaked out on a LB agar plate to cover around 1/3 of the plate with a lawn of bacteria. After incubation at 30°C for 48 hr, using sterile loop all bacteria were taken off the plate and added into the barcoded bead tube supplied by MicrobesNG. The content was mixed by inverting 10 times, tubes packed, and sent off immediately for sequencing.

2.6.9 Sequence and Phylogenetic Analyses

Sequence alignments were generated for 16S rRNA genes representing 53 taxa, and for the *suc* A, *pyr* G, *rpo* B, *rpl* B and *fus* A genes for 46 taxa using the MUSCLE (Multiple sequence alignment) programme (Edgar, 2004). An additional alignment was produced for a combination of 4 genes (*suc* A, *pyr* G, *rpo* B, and *rpl* B) using the sequence editor implemented in the SEAVIEW programme (Gouy *et al*., 2009). Parsimony analysis was performed on the 16S rRNA sequence matrix using PAUP 4.1 (Swofford, 2002), and the maximum likelihood analysis performed on the housekeeping genes using PhyML (Zhou *et al*., 2017). Genome comparison was performed using OneCodex software and their genome library (https://onecodex.com/platform/), while gene annotation analysis was made using Artemis (Carver *et al*., 2011) and Genome Comparison software (Lederman, 2015). Genome sequence alignments were constructed using Mauve (Darling *et al.*, 2004; Darling *et al*., 2010) implementing the HOXD scoring. Genomic islands for isolates *EwS1* and *EwB2* were identified using the on-line programme IslandViewer 4 (Dhillon *et al*., 2013, Bertelli *et al*., 2017) using the complete genomic sequence for *Rahnella aquatilis* HX2 (NR_076967) as the reference.

2.7 Isolation, identification, and characterization of bacteriophages

2.7.1 Preparation of bacterial cells for bacteriophage propagation

Discovered *EwS1* and *EwB2* bacteria isolated from wastewater were grown on agar plates and used in all experiments. Bacterial cultures for phage titer determination were prepared in vials (30 ml, Fisher Scientific) by inoculating a swab of bacterial culture into 5 ml LB and incubation with aeration (150 rpm, SciQuip Incu-Shake MAXI) at 30°C for 16-24 hr. For the procedure of bacteriophage inoculation, fresh bacterial cells in log phase were used. To achieve a multiplicity of infection (MOI) of 0.1, which is the number of virions that are added per cell during infection, the quantity of bacteria cells needed was 2.8-3.0 x 10^8 cfu/ml which

corresponds to absorbance 0.54–0.6 at 600 nm. Therefore, the bacterial cell overnight culture was inoculated with ratio 1:10 in LB and incubated with aeration at 30°C for approximately 4–$4^1/_2$ hr. Furthermore, for every experiment 1 ml aliquot was taken out for measurement of optical density and subsequent quantification of living bacterial cells to confirm the previous calculations.

2.7.2 Bacteriophage enrichment and isolation of bacteriophages

The water sample was filtered through 0.8 µm filter pore (Thermo Scientific) and the filtrate was centrifuged at 4,400 rpm (Awel MF 20-R) at 20°C for 1 hr and subsequently passed through 0.2 µm filter (Thermo Scientific) to obtain Cell-free supernatant (CFS). The CFS was used for enrichment of bacteriophages using isolated bacteria. Briefly, 5 ml of LB was inoculated with 50 µl of overnight bacterial culture and 0.9 ml of CFS. The vial was incubated overnight at 30°C with aeration at 120 rpm (SciQuip Incu-Shake MAXI). After enrichment, the mixture was centrifuged at 4,400 rpm (Awel MF 20-R) at 20°C for 1 hr and filtered using 0.2 µm syringe filter pore (Elkay). A serial dilution (SD) from 10^{-1} to 10^{-5} was prepared. Bacteriophage buffer (4.5 ml) and 0.5 ml of CFS were added to the first universal vial, mixed thoroughly, and marked as SD^{-1}. From the 1st vial (0.5 ml) was transferred into the 2nd bottle, labelled SD^{-2}. The same procedure was repeated until SD^{-5} was achieved. From each dilution (0.1 ml) mixed with 0.1 ml of bacterial overnight cultures and 3 ml of soft agar was poured onto bottom agar plate using overlay technique (Twenty-five ml of LB bottom agar were poured on Petri dishes. Soft agar was stored in 100 ml volumes, melted when required, and tempered to 47° C before use. Bacterial culture and bacteriophages were mixed in each overlay before pouring onto dried base plates. The soft agar was swirled to produce a uniform top layer and incubated at 30°C for 24 hr before inspection for plaque formation) and incubated at 30°C overnight. Plaque counts in pfu/ml were calculated by taking the average number of plaques for a dilution and the inverse of the total dilution factor (Baer and Kehn-Hall, 2014).

$$pfu/ml = \frac{Average\ number\ of\ plaques}{Dilution\ factor\ X\ \ plated\ volume}$$

2.7.3 Purification of bacteriophages

Single plaques of bacteriophages with different plaque morphologies were picked up by stabbing sterile Pasteur pipettes through the selected plaque and into the hard agar beneath. An agar plug containing phage was transferred into 1ml of bacteriophage buffer, suspended, centrifuged at 14,800 rpm (Sigma 1-14 Micro Centrifuge) for 10 min and filtered

using 0.2 μm syringe filter. A ten times serial dilution down to 10^{-5} were prepared as described before and the bacteriophages were grown on agar plates using overlay technique (page 38). The plates were incubated at 30°C overnight. For each isolated phage, this step was repeated three times to obtain bacteriophages of the same morphology and used for further purification.

2.7.4 Effect of $CaCl_2$ and $MgSO_4$ on the efficiency of bacteriophage growth

These cations are needed for bacteriophage growth. A total of 6 flasks (250 ml) were used in this experiment. Each flask contained 50 ml of LB was inoculated with bacteria (2.8 x 10^8 cfu) and bacteriophages (2.8 x 10^7 pfu) MOI 0.1. To the flasks were added 100 μl, 250 μl or 500 μl of stock solutions of 1M $CaCl_2$ or 1M $MgSO_4$ to a final concentration of 2 mM, 5 mM and 10 mM of each salt. The flasks were incubated at +30°C with aeration at 150 rpm (SciQuip Incu-Shake MAXI) for 16-24 hr. After incubation, the samples were centrifuged at 4,400 rpm (Awel MF 20-R) and filtered using 0.2 μm filters (Thermo Scientific). Serial dilution of the resulting CFS was prepared up to 10^{-7} and the bacteriophages were grown on agar plates using the overlay technique (page 38).

2.7.5 Large Scale purification of bacteriophages

(a) from agar plates with confluent lysis

Isolated bacteriophages were diluted serially in bacteriophage buffer to give a concentration that would provide confluent lysis for the isolated bacteria. 50 plates with almost confluent lysis were taken for phage purification. The soft agar layer scrapped from the base plate was transferred to a centrifuge tube, suspended in 5 ml of LB per plate and the contents were centrifuged at 4400 rpm (Awel MF 20-R) for 1 hr at + 4°C. The supernatant was then filtered through 0.2 μm filters (Thermo Scientific) into universal vials. The resulting CFS was serially diluted up to 10^{-7} and the bacteriophage titer was determined using the overlay technique.

(b) from liquid medium

250 ml flask with 100 ml of LB was inoculated with a fresh bacteria culture (4.4 x 10^9 cfu) and bacteriophages (4.2 x 10^8 pfu) with MOI 0.1. $CaCl_2$ (10 mM) was added and the flask incubated at 30°C with aeration at 150 rpm (SciQuip Incu-Shake MAXI) for 16-24 hr. After incubation, the samples were centrifuged at 4400 rpm (Awel MF 20-R), supernatants filtered through 0.2 μm pore-size filter (Thermo Scientific). The resulting CFS was serially diluted up to 10^{-7} and the bacteriophages were grown on agar plates using the overlay (page 38) and spot test techniques (Twenty-five ml of LB bottom agar were poured on Petri dishes. Soft agar was stored in 100 ml volumes, melted when required, and tempered to 47° C

before use. Bacterial culture (0.1 ml) was poured onto dried base plates and the soft agar was swirled to produce a uniform top layer and finally allowed to dry. Twenty µl of bacteriophage suspension from 10 times serial dilution was spotted on to the dried plate and incubated at 30°C for 24 hr before inspection for plaque formation).

2.7.6 PEG precipitation of Bacteriophage lysate

Cell free supernatant (250 ml) was transferred into a new container. PEG6000 (Fisher Scientific) was added to a final concentration of 10% and NaCl was adjusted to 0.5 M. Samples were mixed gently by swirling the tubes to completely dissolve PEG6000 and NaCl and left overnight at 4°C. The bacteriophages were pelleted by centrifugation at 4,400 rpm (Awel MF 20-R) for 1 hr at 4°C. Supernatants were removed, pellets were re-suspended in bacteriophage buffer and subjected to dialysis.

2.7.7 Dialysis of Bacteriophage lysate

Prior to dialysis, the dialysis membrane was hydrated by immersing in dialysis buffer for 2 min according to manufacturer's instructions. The dialysis chamber (3 ml Slide-A-Lyzer dialysis cassette, Fisher Scientific) was loaded with phage concentrate to a minimum volume of half of the cassettes maximum volume. Phage concentrate was dialyzed against 2 L of dialysis buffer at 4 °C on a stirring plate for 48 hr replacing the buffer solution three-four times. Finally, phage concentrate was carefully collected using a sterile plastic pipette and phage titer was determined by serially diluting the concentrate up to 10^{-7} and the bacteriophages were grown on agar plates using the overlay technique (page 38).

2.7.8 Transmission Electron Microscopy

Prior to TEM, dialysed phage sample was transferred into microcentrifuge tube and centrifuged at 14000 rpm (Sigma 1-14 Micro Centrifuge) for 2 hr. The pellet was resuspended in 50 µl of TEM phage buffer. One drop (5 µl) of phage suspension was applied to the dark and shiny surface of a Formvar-carbon-coated copper grid (200 mesh) and allowed to sit for 2-10 min. The grid was rinsed by using 60 µl of sterile dH_2O. Excess liquid was wicked off using filter paper. The grid was immersed in 2% uranyl acetate solution for 30 s, then passed through 3 tubes containing ultra-purified water (Fisher Scientific) with 10 sec in each. Excess stain was immediately wicked off by using a wedge of filter paper. The grid was then placed in a grid box, allowed to dry overnight, then stored in a desiccator until analysis.

Electron microscopic images of uranyl acetate stained phage were obtained by Prof. Cragg and Mrs. Christine Hughes using the JEM 2100 Transmission Electron Microscope (JEOL Ltd, Japan) operated at 80 kV. Dimensions of virions were measured on micrographs at

magnification of 30,000 times and with a measuring magnifier, calibrated at 100 nm intervals.

2.7.9 Host range determination of isolated bacteriophages

To determine host range, all newly isolated bacteria, as well as *Serratia marcescens*, *Hafnia alvei*, *Klebsiella pneumonia*, *E. coli K802*, and *Proteus mirabilis* from the laboratory collection were used. Briefly, 5 ml of LB medium was inoculated with 50 µl of overnight bacterial culture and 0.9 ml of cell free supernatant (CFS). The vail was incubated overnight at 30°C with aeration at 120 rpm (SciQuip Incu-Shake MAXI). After enrichment, the mixture was centrifuged at 4,400 rpm (Awel MF 20-R) at 20°C for 1 hr and subsequently passed through 0.2 µm syringe filters (Elkay) to obtain CFS specific for that isolated bacteria. Serial dilution was made up to 10^{-6}. From each dilution (0.1 ml) with 0.1 ml of bacterial overnight cultures and 3 ml of soft agar was poured onto bottom agar plate using the overlay technique (page 38). This stage was repeated for all isolated bacteria.

2.7.10 Phage DNA isolation using the Phage DNA isolation kit.

Bacteriophage DNA was purified using the Phage DNA isolation kit (Norgen) according to manufacturer's manual. One ml of phage prepared from liquid culture was added into a 5 ml vial, 1:100 of DNase I (10 mg/ml) and 1:200 of RNase A (10 mg/ml) were added, mixed, and incubated at 37°C for 30 min. Then 1:40 of 20% SDS and 1:100 of Proteinase K (10 mg/ml) were added to the sample, mixed, and incubated at 37°C for 30 min. 500 µl of lysis buffer B was added and vortexed thoroughly, incubated at 65°C for 15 min and occasionally mixed during incubation by inverting the tube. Isopropanol (320 µl) was added and tubes vortexed briefly. The lysate (650 µl) was transferred to the phage DNA isolation column and centrifuged for 1 min at 6000 x g. This stage was repeated until the remaining lysate passed through the column. The column was subsequently washed three times with Wash solution A centrifuging for 1 min at 6000 x g. The column was centrifuged for 2 min 14000 x g to dry the resin membrane. DNA was eluted from the membrane with 75 µl of Elution buffer B. DNA concentration and quality were estimated spectrophotometrically (NanoDrop, Technologies Inc.).

2.7.11 Phage DNA isolation by Phenol-chloroform extraction

For bacteriophage DNA purification using phenol-chloroform. Phage supernatant (500 µl) was added into an Eppendorf tube, 1:100 of DNase I (10 mg/ml) and 1:200 of RNase A (10 mg/ml) was added, mixed, and incubated at 37°C for 30 min. 1:40 of 20% SDS and 1:100 of Proteinase K (10 mg/ml) were added to the sample, mixed and incubated at 37°C for 30 min. The sample was transferred into a pre-centrifuged (30 sec at 16,000 x g) phase lock gel tube (Fisher Scientific), equal volume of phenol: chloroform: isoamyl alcohol (25:24:1)

added, tubes vortexed or shaken by hand thoroughly. The mixture should turn milky. After 5 min centrifugation at 16,000 × g the upper aqueous phase was carefully removed using a pipette and transferred to a fresh tube. The above stage was repeated, and the aqueous phase was transferred into a clean Eppendorf tube. 1/10 volume of 3M sodium acetate and 2.5 vol of 96 % ethanol (Fisher Scientific) was added to the DNA solution and left at -20°C overnight. After 10 min centrifugation at 4°C at 14000 rpm (Fisher, Heraeus fresco 21 centrifuge) the supernatant was discarded. The pellet was washed three times with 1 ml of 70 % ethanol, slightly dried and re-suspended with water. DNA concentration and quality were estimated spectrophotometrically (NanoDrop, Technologies Inc.).

2.7.12 Phage DNA digestion with restriction enzymes

Digestions of phage DNA were carried out using the appropriate buffer systems supplied with the enzymes. Briefly, the restriction mixture consists of 2 µl 10X fast digest buffer (Fisher Scientific), 1- 6 ng bacteriophage DNA, sterile dH_2O and 1 µl fast digest *Bgl* II restriction enzymes (Fisher Scientific). Bacteriophage DNA were digested in final volume of 20 µl at 37°C for one hour in a mini dry bath (Fisher Scientific). In order to guarantee the correct incubation temperature, the dry bath had been pre-warmed for 30 min, before loading the samples onto 1.2 % agarose gel and electrophoretic separation run at 150 volts for 1 hour and 15 min. The gels were visualised under UV light and images captured using molecular imager® Gel Doc™ XR+ imaging system.

3.0 RESULTS

3.1 Isolation of ESBL producing bacteria.

Enterobacteriaceae belongs to the normal flora in humans but may cause infections. The increasing level of antimicrobial resistance among clinically relevant bacteria, especially the increasing prevalence of ESBL producing *Enterobacteriaceae* is of great concern around the world.

Wastewater treatment is crucial to environmental hygiene with these multi-resistant bacteria from various sources potentially released into the environment via effluent. Wastewater treatment plant effluents are considered hotspots for antibiotic resistant genes and for the spread of bacteria into the environment. Because these bacteria resistant to antibiotics are mostly enteric in origin, when excreted they can contaminate the environment and, through ingestion, enter new hosts. Therefore, focus of this study was put on isolating ESBL producing bacteria from treated wastewater, which after treatment is recycled to the public, potentially serving as a direct route for infections. The presence of antibiotic resistant bacteria also increases the potential risk of gene transfer to non-resistant hosts and so identifying such sources may help in finding new treatments, such as bacteriophages.

Isolates were recovered from enriched cultures, seeded with water from the final holding tank of treated effluent, by culturing them on chromogenic media (Brilliance ESBL Agar, Oxoid) specifically designed to select ESBL-producing bacteria by the inclusion of the antibiotic cefpodoxime. Colonies recovered from this medium can be coloured blue or pink, for *Escherichia* isolates, green for *Klebsiellia*, *Enterobacter*, *Serratia*, and *Citrobacter* isolates, brown for *Proteus*, *Morganella*, and *Providencia* isolates, and finally colourless colonies for *Salmonella*, *Acinetobacter* or other isolates. From one batch sampled during the autumn, the majority of colonies recovered were either dark blue or colourless with one colony that was coloured light blue. While all six dark blue colonies were identified as representatives of *Escherichia coli* and the four colourless colonies as members of the genus *Pseudomonas* according to brilliance ESBL agar manufacturer's manual, the light blue colony was not easily identified following the preliminary testing.

While brilliance ESBL agar is specifically designed to select ESBL-producing bacteria, all purified and identified bacteria were further analysed for the presence of specific ESBL genes by PCR. The presence of CTX gene was demonstrated by a 593bp DNA amplicon (higher band) (Figure 10 &11) and TEM by a 445bp amplicon (lower band) (Figure 11). It was found that the unidentified isolate was only harbouring the CTX gene (Figure 12), *E. coli* 1 & 2 isolates showed presence of CTX and TEM genes while *E. coli* 4 & 5 showed

presence of CTX gene only (Figure 13). Only *Pseudomonas* 1 isolate showed the presence of CTX gene (Figure 13). These observations confirm, in accordance with published literature, that CTX is the predominant ESBL type found (Bonnet, 2004). SHV and OXA groups I, II and III genes were analysed but none of isolated bacteria nor control strain were positive for the presence of these genes. These results confirmed that the light blue colony, *E. coli* 1, 2, 4, 5 and *Pseudomonas* 1 isolate were ESBL producing, which means having antibiotic resistance properties that can be a burden to the healthcare. Therefore, further identification was necessary.

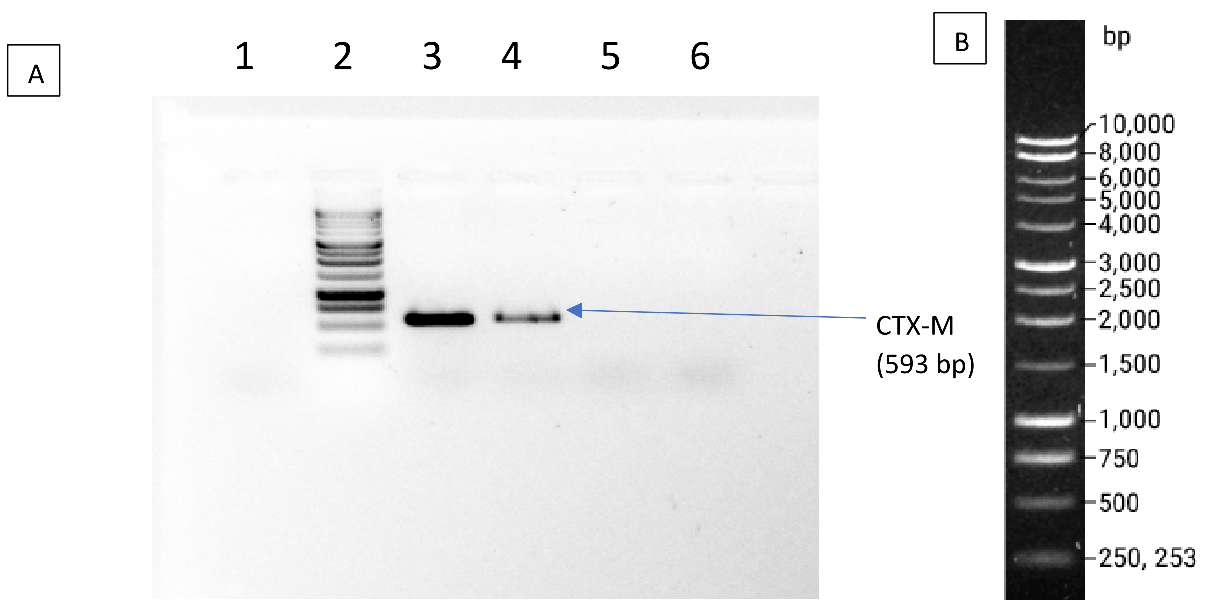

Figure 12. (A) Detection of ESBL resistance determinants SHV, TEM and CTX-M using primers pairs BlaSHV, BlaTEM & BlaCTX, (B) Promega provided image of DNA ladder band pattern. Lane 1: negative control with no added DNA in the PCR reaction, lane 2: 1 kb DNA ladder, lane3: PCR product amplified in *EwS1* DNA, lane 4: PCR product amplified in *Pseudomonas* 1 DNA, lane 5: PCR product amplified in *Pseudomonas* 2 DNA, lane 6: PCR product amplified in *Pseudomonas* 3 DNA. All *Pseudomonas* DNA were isolated from wastewater. The arrow in the figure points to the marker DNA, not the space where the expected determinant would be.

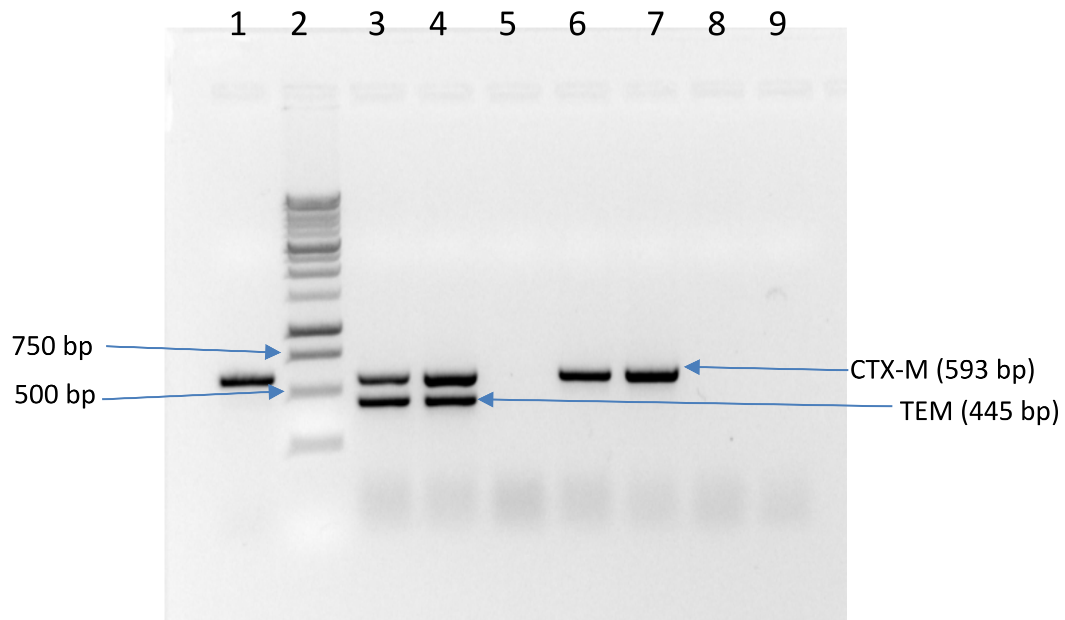

Figure 13. Detection of ESBL resistance determinants SHV, TEM and CTX-M using primers BlaSHV, BlaTEM & BlaCTX. Lane 1: positive control using DNA from *EwS1* ; lane 2: 1 kb DNA Ladder, positions of 500 bp and 750 bp bands are indicated by arrows; lanes 3,4,6,7,8,9: PCR products of amplified *E. coli* 1-6 DNA; Lane 5: PCR product of amplified *Pseudomonas* 4 DNA. The arrow in the figure points to the marker DNA, not the space where the expected determinant would be.

For further identification, API 20 E and API 20 NE tests were used for identification of bacteria belonging to genus *Enterobacteriaceae* and non-fastidious, non-enteric gram-negative rods, respectively. Bacterial identification was performed using the BIomerieux database and it showed that all six strains of *E. coli* gave 99% similarity with *E. coli*, *Pseudomonas* gave 97% similarity with all 4 *Pseudomonas* strains isolated while the light blue colony gave 67% similarity with *Ewingella americana* reference (Table 4).

Table 4. Summary table of isolated strains identification by selective media and characterization by biochemical test, sequence analysis and multiplex PCR analysis.

Strains	Chromogenic medium		API TEST		16S rRNA Sequence analysis	Genes identified (amplicon size)
	Colour	Identification	20 E test	20 NE test		
1	Light Blue	No clear identification as per manufacturer manual	*Ewingella americana*	N/A	*Ewingella americana Klebsiella sp hafnia sp*	CTX (593 bp)
2	Cream	*Pseudomonas*	*N/A*	*Pseudomonas luteola*	*Pseudomonas sp*	CTX (593 bp)
3	Cream	*Pseudomonas*	*N/A*	*Pseudomonas luteola*	*Pseudomonas sp*	
4	Cream	*Pseudomonas*	*N/A*	*Pseudomonas luteola*	*Pseudomonas sp*	
5	Cream	*Pseudomonas*	*N/A*	*Pseudomonas luteola*	*Pseudomonas sp*	
6	Dark Blue	*E. coli*	*E. coli*	N/A	*E. coli*	CTX (593 bp) TEM (445 bp)
7	Dark Blue	*E. coli*	*E. coli*	N/A	*E. coli*	CTX (593 bp) TEM (445 bp)
8	Dark Blue	*E. coli*	*E. coli*	N/A	*E. coli*	CTX (593 bp)
9	Dark Blue	*E. coli*	*E. coli*	N/A	*E. coli*	CTX (593 bp)
10	Dark Blue	*E. coli*	*E. coli*	N/A	*E. coli*	
11	Dark Blue	*E. coli*	*E. coli*	N/A	*E. coli*	

In a bid to identify the unknown strain, sequence analysis of the 16S rRNA gene was performed. The nucleotide blast analysis of the DNA sequences confirmed the identification of *E. coli* and *Pseudomonas* strains. However, the sequence of the unknown strain showed 99% similarity for *Ewingella, Klebsiella* and *Hafnia* (Table 4). This result suggested that this bacterium is a novel one, because different colour on the chromogenic medium and the API test showed no similarity to *Klebsiella* or *Hafnia*, while the low similarity with *Ewingella* in the API test could not be definitive.

The novel bacterium was sub-cultured onto LB agar plates, incubated at 30°C and found growing as large irregular or small regular colonies, and these were thus given the

designation *EwS1* for the small colony isolate and *EwB2* for the large colony isolate. In an attempt to identify the colonies further, each isolate was subjected to physiological, and biochemical tests and to whole genome sequencing.

3.2.1 Physiological characterisation of isolates *EwS1 and EwB2*

The physiological characteristics of the isolates *EwS1* and *EwB2* were further characterised using Biolog, Microbial Identification System, which enables identification of aerobic gram-negative and gram-positive bacteria in the same test panel. The strips were inoculated with samples of an overnight culture of the test and control (*K. pneumoniae* ATCC 13883) strains and growth was recorded after 24 and 48 hr (Figures 14 and 15). The isolates used a wide range of carbohydrates as carbon sources except for α-D-glucose, D-fucose, and D-turanose (Figure 14a). Strangely, the isolates catabolise pectin, D-cellobiose, and D-galacturonic acid but not starch. A wide range of alcohol carbohydrates was found to be metabolised by the *EwS1* and *EwB2* isolates. This included salicin, which is an alcoholic β-glucoside found in the bark of some plants (willow) (Figure 14b). The isolates did appear to catabolise plant metabolites in preference to animal ones, with a lack of growth on myo-inositol (a cell signal transducer in mammalian cells) and γ-aminobutyric acid (the chief inhibitory neurotransmitter in mammalian cells).

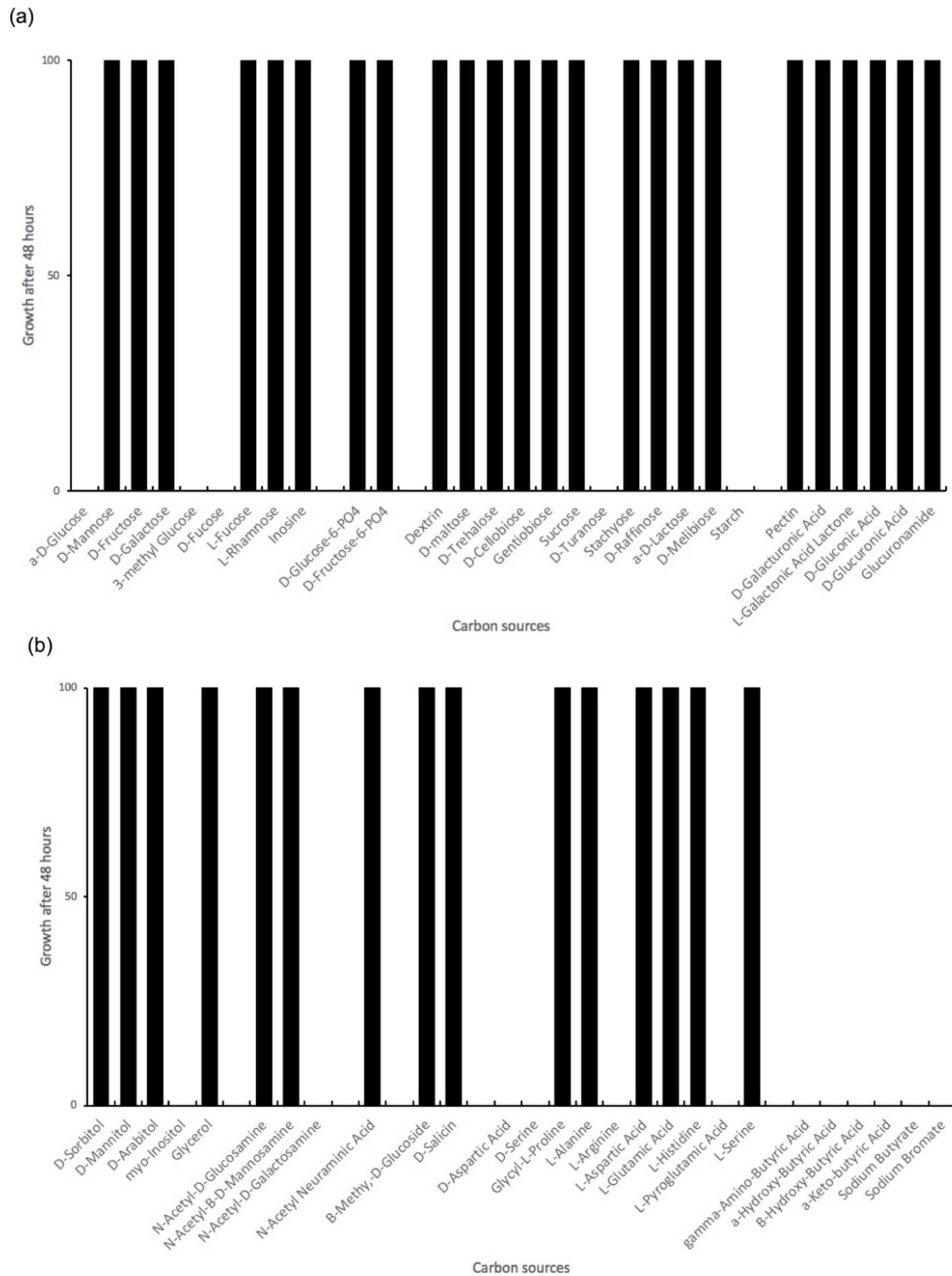

Figure 14. The physiological characteristics of the isolates *EwS1* and *EwB2* characterised using Biolog test strips. (A) and (B) show utilisation of carbon sources by the isolates after 48 hr incubation. the bars represent a positive reaction, while no bars indicate a negative reaction for the different tests in the panel. The figure is arbitrary denoting full growth compared to a standard and no growth compared to a standard.

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Both isolates grew well at pH6 and sodium chloride concentrations of 1 and 4%, but not at a pH of 5 or a sodium chloride concentration of 8% (Figure 15). Intestinal bacteria can cause aromatization of quinic acid (Cotran *et al*., 1960), while other intestinal bacteria can ferment mucic acid (Stemfield and Saunders, 1938). The isolates did not utilise mucic or quinic acid (Figure 15), suggesting that they might not function as intestinal bacteria. Butyric acid is a biologically active compound in humans and other animals, acting as an inhibitor of histone deacetylase or an energy metabolite to produce ATP, and a G protein-coupled receptor, while β-hydroxy-butyric acid is often excreted in urine. The isolates did not metabolise α-hydroxy-butyric acid, β-hydroxy-butyric acid, or α-keto-butyric acid (Figure 15).

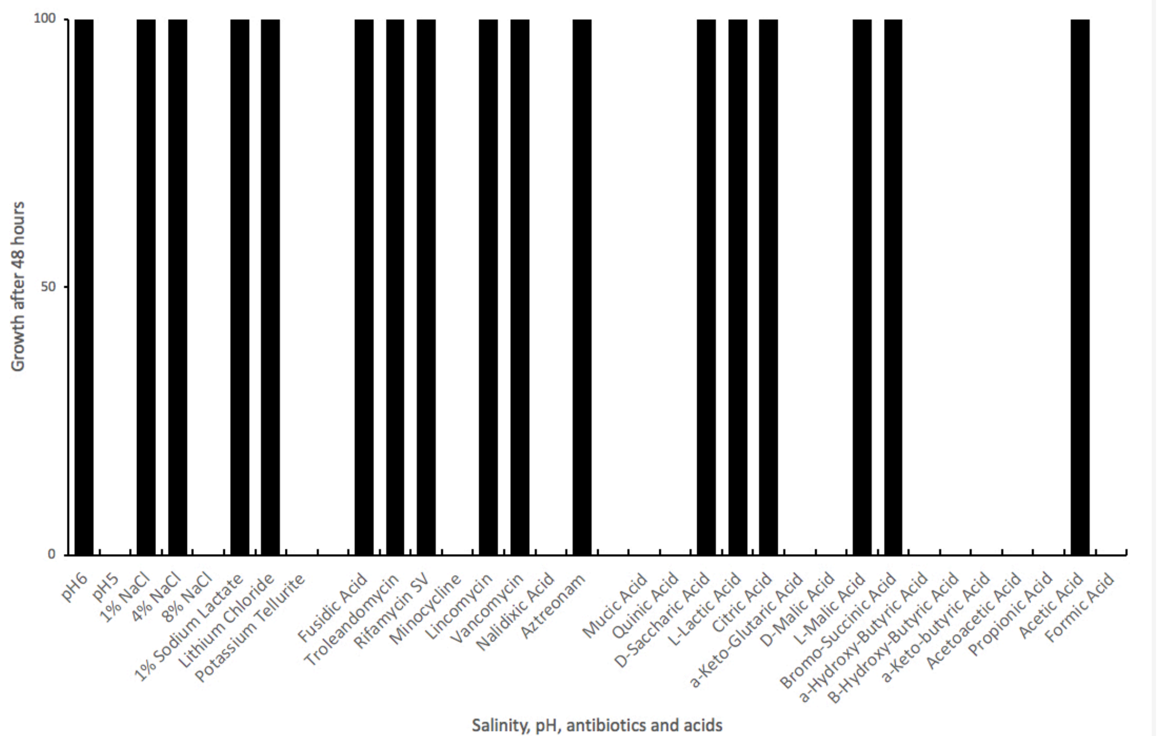

Figure 15. The physiological characteristics of the isolates *EwS1 and EwB2*.These assays were part of the 94 biochemical tests done using Biolog panel. This figure shows the data for salinity, pH, antibiotic resistance, and utilisation of acids by the isolates after 48 hr incubation. Positive reactions are denoted by the presence of bars.

3.2.2 Temperature requirements for growth of isolates *EwS1 and EwB2*

The growth temperature requirements of isolates *EwS1* and *EwB2* were determined at a range of temperatures from 5 to 37°C. The optimum growth for both isolates occurred at 25°C after 10 hr incubation (Figure 16). No growth was detected at 37°C. The exponential phase of growth at temperatures from 20°C to 32°C ended within 8 hr of incubation, whereas at 15°C it took significantly longer. However, these bacteria were capable of growth at 0°C to 7°C. Given that the optimal growth occurred between 20°C and 30°C, such bacteria could be classified as mesophiles. Although no growth was detected at temperatures of 37°C and above, the bacteria were not killed at these temperatures. Samples taken from cultures incubated at 37°C for 24 hr exhibited growth if the temperature was lowered to 30°C.

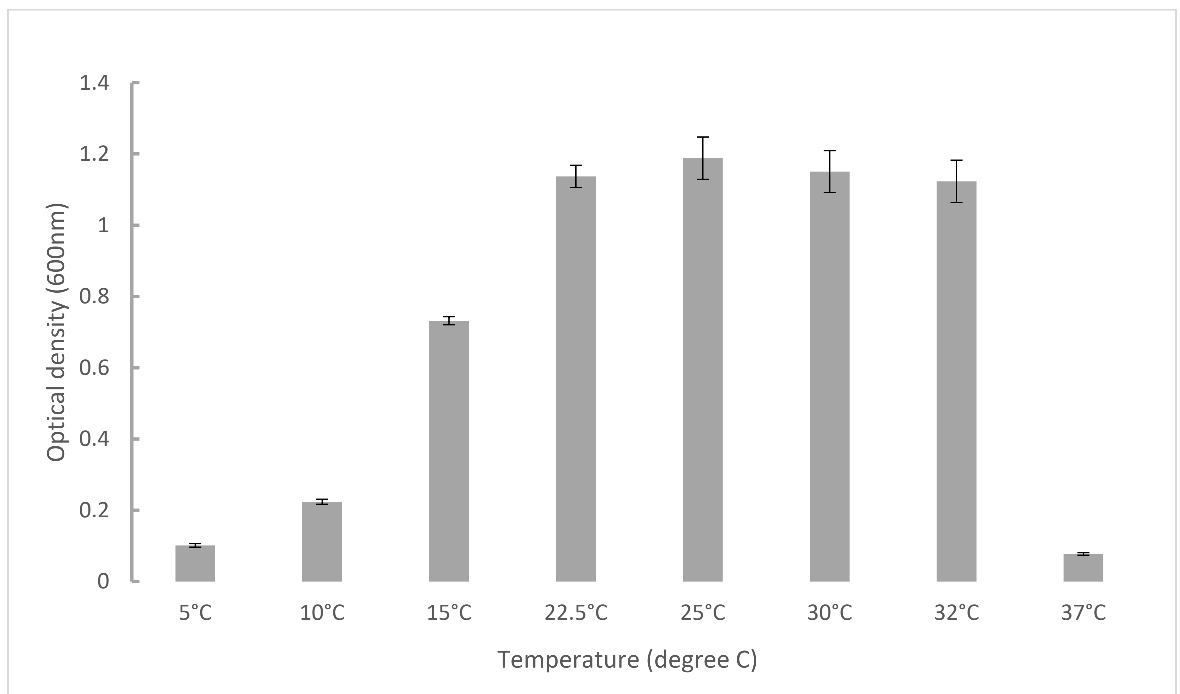

Figure 16. Histogram showing the effect of temperature on growth of *EwS1* and *EwB2* monitored by OD_{600}. the isolates were grown at different temperatures from 5-37°C for 10 hr and optical density was measured. The error bars represent the standard deviation.

3.2.3 Antimicrobial susceptibility of isolates *EwS1* and *EwB2*

The SHV, TEM, CTX-M and OXA genes on chromosomal DNA have already been tested, as described above, and shown in Figure 12 and 13. Next, plasmid DNA was isolated and tested for these determinants. Analyses revealed that both isolates contained a single plasmid of approximately 6 kbp. A multiplex PCR reaction was used to amplify SHV, TEM

and CTX-M determinants from chromosomal and plasmid DNA (Figure 17) the SHV specific amplicon appears as a band of 739 bp, the CTX-M as a 593 bp fragment and TEM as a 445 bp fragment. Both isolates showed the presence of CTX-M in chromosomal and plasmid DNA, while the TEM determinant was present in plasmid DNA (Figure 17).

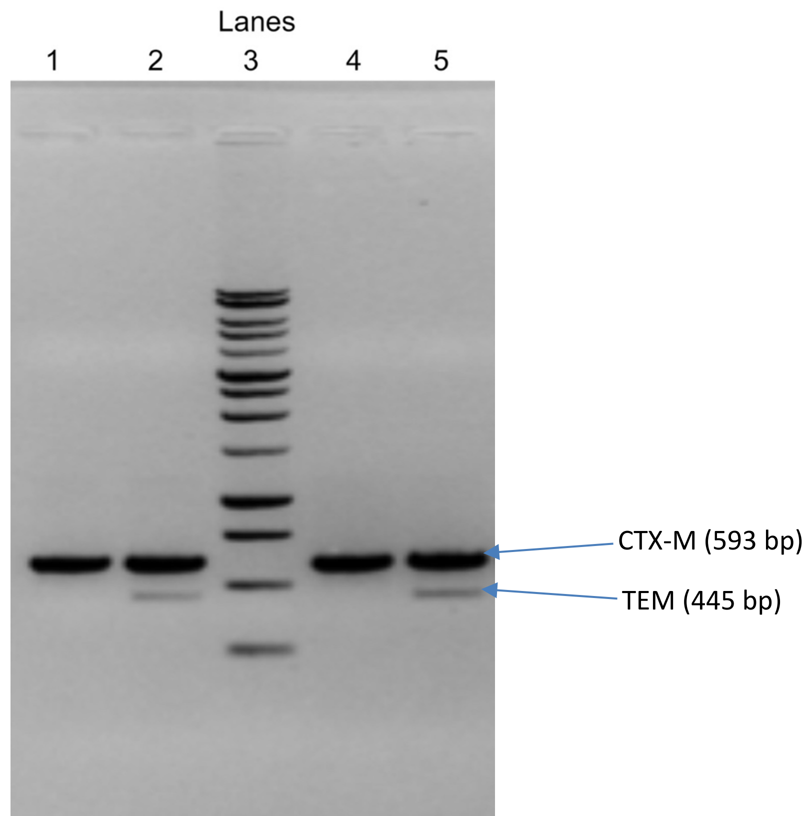

Figure 17. An example image of agarose gel electrophoresis of PCR products obtained using primers pairs BlaSHV, BlaTEM & BlaCTX. Lane 1: PCR product amplified in *EwS1* chromosomal DNA, lane 2: PCR product amplified in *EwS1* plasmid DNA, lane 3: 1 kb DNA ladder. lane 4: PCR product in amplified *EwB2* chromosomal DNA, lane 5: PCR product amplified in *EwB2* plasmid DNA.

The initial evidence for the presence of other antibiotic resistance determinants in isolates *EwS1* and *EwB2* was provided by the results from the Biolog strip test, where both isolates grew in the presence of fusidic acid, troleandomycin, rifamycin, lincomycin, vancomycin and aztreonam (Figure 15).

Next, antimicrobial susceptibility disc diffusion tests were performed for 13 antibiotics. Both isolates demonstrated a broader spectrum of antibiotic resistance in comparison to the control. The isolates showed resistance to 5 antibiotics: amoxicillin, cefuroxime, cefotaxime, gentamicin, and piperacilin-tazobactam but not to meropenem, ertapenem, ceftazidime, ciproflaxin (Table 5).

Table 5. Antibiotic sensitivity of isolates *EwS1* and *EwB2*. The diameter is the length of the zone of inhibition. the values were compared with the minimum and maximum values with in BSAC guidelines to determine the resistance or sensitivity to antibiotics. N=3

Antibiotics	*EwS1*		*EwB2*		Control *K. pnuemoniae*	
	Result	Diameter (mm)	Result	Diameter (mm)	Result	Diameter (mm)
Amoxicillin (10 µg/ml)	R	-	R	-	S	18
Amoxicillin- clavulanic acid (30 µg/ml)	S	22	S	22	S	34
Meropenem (10 µg/ml)	S	24	S	26	S	24
Cefotaxime (30 µg/ml)	R	-	R	-	S	6
Ertapenem (10 µg/ml)	S	28	S	26	S	22
Ceftazidime (10 µg/ml)	S	6	S	6	S	4
Cefepime (30 µg/ml)	S	28	S	24	S	32
Tigecycline (15 µg/ml)	S	20	S	20	S	24
Amikacin (30 µg/ml)	S	4	S	4	S	4
Gentamicin (10 µg/ml)	R	-	R	-	R	
Ciproflaxcin (1 µg/ml)	S	22	S	24	S	30
Piperacilin-tazobactam (75/10 µg/ml)	R	-	R	-	R	
Cefuroxime (30 µg/ml)	R	-	R	-	S	28

- S – Sensitive
- R – Resistant

2.4 Molecular identification of isolates *EwS1* and *EwB2*

In order to clarify the identity of isolates *EwS1* and *EwB2* the 16S rRNA gene was PCR amplified and sequenced. The two sequences were identical, consisting of 1540 nucleotides with a base composition of A: 25.5%, C: 22.3%, G: 31.4% and T: 20.6%. Blast similarity searches (Table 6) identified some close matches of these sequences with those from uncertain species *Hafnia* sp strain 270 (AM403659) at 99.73%, *Serratia* sp HC3-9 (JF312979) at 98.82, and *Aranicola* sp. NP34 (EU196321) at 100% sequence similarity. These matches are closely followed with sequences from recognised species: *Rahnella inusitata* OX0101 (MG576035) at 98.61% similarity, *Hafnia* alvei CBA7135 (CP021971) at 98.25% similarity; and *Ewingella americans* CIP81.94 (NR104925) at 98.18% similarity. Interestingly, the closest matches for the 16S rRNA sequences from the *EwS1* and *EwB2* came from *Enterobacterales* strains isolated from the Artic or Antarctic: *Aranicola* sp. NP34 was isolated from a cold saline (7.5% salt) sulfidic spring in the Canadian high Arctic, *Serratia* sp HC3-9 was isolated from artic cyanobacterial mats and *Hafnia* sp 270 was isolated from the Antarctic soil. All of these strains would appear to be cold adapted.

Table 6. Molecular identification of isolates *EwS1* and *EwB2* by blast similarity searches for 16S rRNA gene.

Query	Matches	% Identity	% coverage	E value	Acc. Number
EwS1 and *EwB2*	**Hafnia* sp strain 270	99.73	97	0.0	AM403659
	**Serratia* sp HC3-9	98.82	99	0.0	JF312979
	Rahnella sp. ERMR1:05	98.64	100	0.0	CP019062
	Hafnia alvei CBA7135	98.25	100	0.0	CP021971
	Ewingella americans CIP81.94	98.18	99	0.0	NR104925
	**Aranicola* sp. NP34	100.0	94	0.0	EU196321
	Serratia grimesli BXF1	97.92	100	0.0	LT883155
	Rahnella inusitata OX0101	98.61	97	0.0	MG576035

- (*) strains isolated from the Artic or Antarctic.

The 16S rRNA gene sequences from 46 taxa representing members of the *Enterobacterales* were aligned with those from the *EwS1* and *EwB2* isolates and their closest similarity matches, along with two outgroup of *Vibrio sp* to generate a matrix of 53 taxa. Parsimony analysis of this matrix was performed using random sequence addition with 10 replications and tree-bisection-reconnection (TBR) algorithm for branch swapping (Swofford, 2003). This generated 702 most parsimonious trees of 927 steps with a CI = 0.424, RI = 0.739, RC = 0.321 and HI = 0.566 and based on 221 parsimony-informative sites. In this analysis 5 other tree islands existed, ranging from 934 to 942 steps. Bootstrap analysis produced a consensus tree (data not shown) with little internal support within the *Enterobacterales*, except for a clade of the *EwS1* and *EwB2* isolates with *Hafnia* sp strain 270, *Serratia* sp HC3-9 and *Aranicola* sp. NP34 (100% support), and a clade of these sequences with those from *Rahnella* species (89% support). Maximum likelihood estimates of the parsimony trees revealed that different likelihood In (L) values existed for some trees within the population, ranging from - 6932.139 to -6940.375. A maximum likelihood analysis was performed on the 53 taxa data matrix using the model GTR (Swofford,2003) and estimated values for nucleotide frequencies as well as for the frequency of invariable/variable sites, together with a non-parametric bootstrap analysis. The resultant tree had a -In (L) -6924.159604 (Figure 18) and showed a low level of support for a split between the members of the *Yersiniaceae* and the rest of the *Enterobacteriaceae* (75% support), with the *EwS1*/*EwB2* sequences forming a well-supported clade with those from *Hafnia* sp strain 270, *Serratia* sp HC3-9 and *Aranicola* sp. NP34 (99% support) within the *Yersiniaceae*.

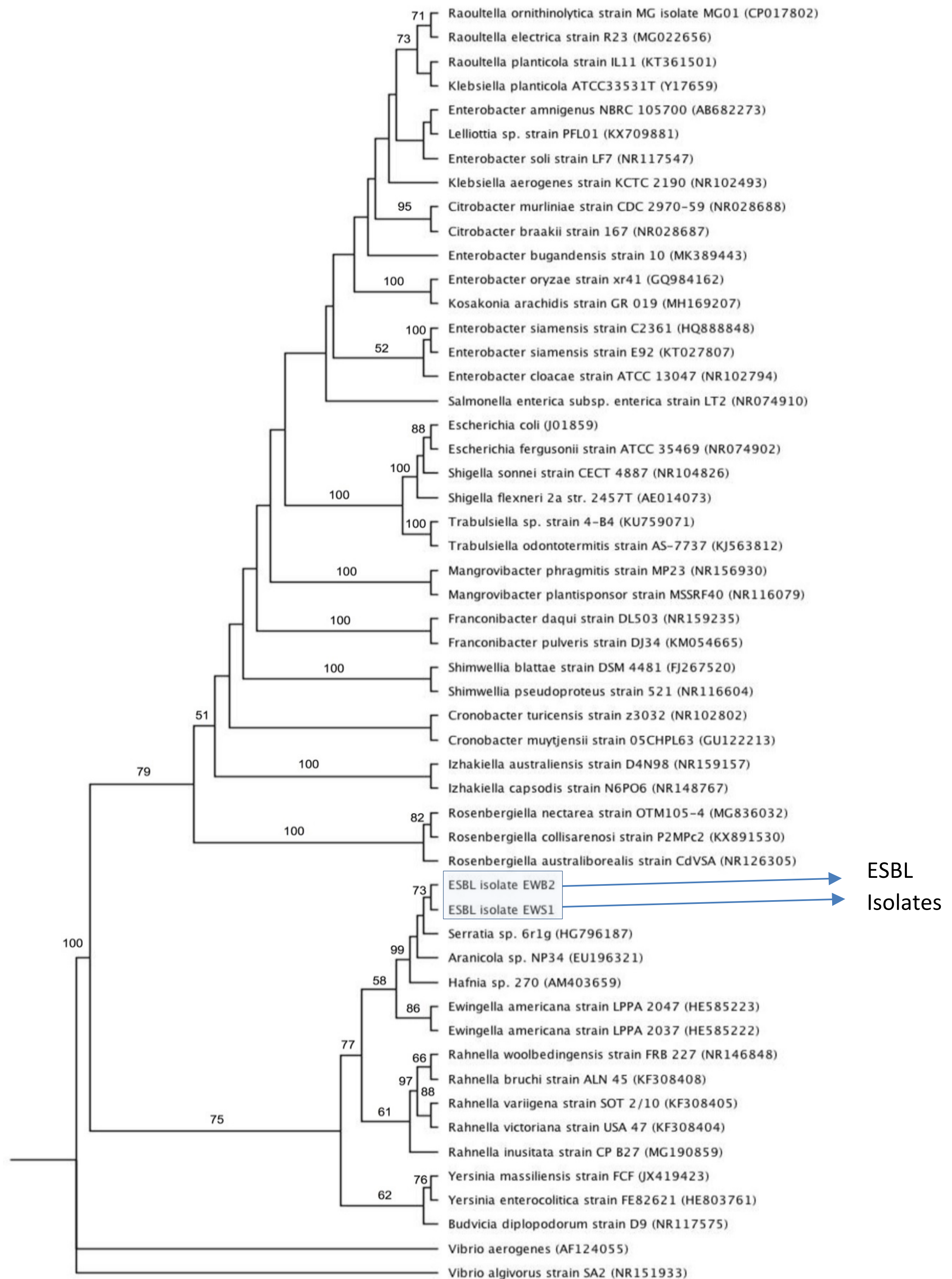

Figure 18. Consensus maximum likelihood tree showing the relationships between derived 16S rRNA gene sequences and those of two outgroup species of *Vibrio*, where In(L) = -6924.159604, Pinv = 0.696826, α = 0.478734. Bootstrap [DG1] values above 50% are shown above the branches. the ESBL isolates are the ones boxed**.**

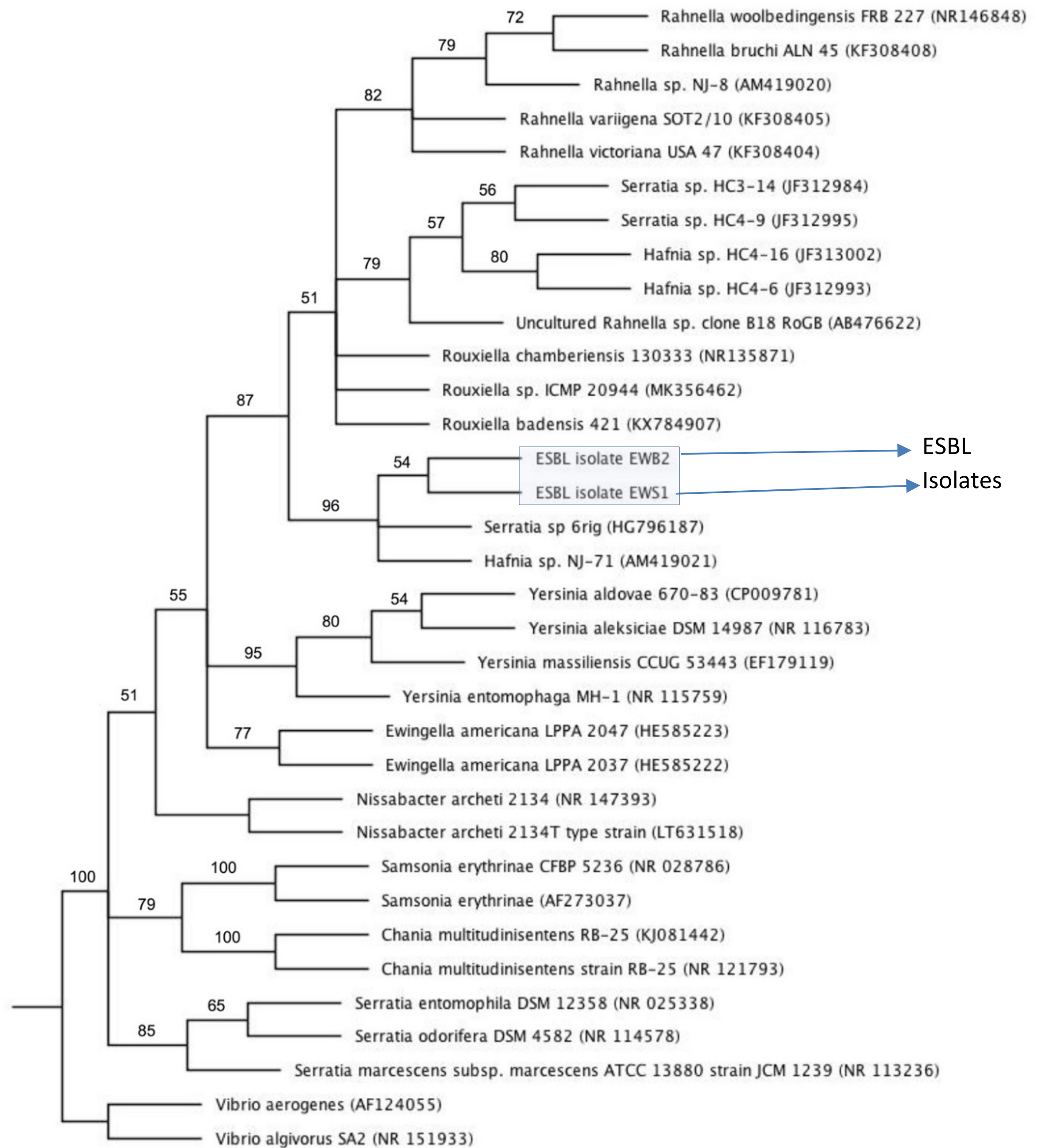

Figure 19. Maximum parsimony analysis of 16S rRNA gene sequences and those of two outgroup species of *Vibrio*. Strict consensus tree (CI = 0.549, RI= 0.744, RC = 0.408, and HI = 0.451) of 574 steps (PIC = 187). Representing 88 trees from 3 tree islands all of 574 steps, showing the relationship. *Yersiniacaea*.

A data matrix containing 32 OTUs representatives of the family *Yersiniaceae*, with 2 species of *Vibrio* as outgroup taxa, was constructed for 16S rRNA gene sequences. Maximum parsimony analysis of this data set produced 88 trees of 574 steps using 187 parsimony informative sites. Figure 19 shows that the strict consensus of these trees with bootstrap values for nodes. This analysis shows the ESBL isolates *EwS1* and *EwB2* formed a strongly supported clade (96%) with sequences from isolates recovered from cold environments (*Hafnia* sp. NJ-71 and *Serratia* sp. 6rig), which is separate from all other genera.

The relationship between taxonomic identification and sequence similarity for the 16S rRNA gene has been discussed by McDonald *et al* (2012) and Siegwald *et al* (2017): They recommended that sequence reads should be grouped into 3 bins: those showing 90-97% similarity that approximates to the family level; >97-99% similarity for genus level identity; and >99% similarity for species level identity. Table 7 shows the 16S rRNA sequence similarity matches for the isolates *EwS1* and *EwB2* with members of the genus *Rahnella* and *Ewingella*. Using the Ion Reporter rubrics, the isolates *EwS1* and *EwB2* belong to the genus *Rahnella*, as should *Ewingella americana*.

Identification according to 16S rRNA sequence similariity can be misleading, especially when dealing with closely related species. Brady *et al* (2013) adopted a multi-locus sequence approach to evaluate the significance of the genus *Enterobacter* concluding that the relationship inferred using 16S rRNA gene sequences was incompatible with that produced using four protein-coding essential genes. This conclusion was supported by Sato and Miyazaki (2017) who re-evaluated the cohesiveness of the genus using 194 genes from 609 ones known to be essential to *Escherichia coli*.

Table 7. Percentage sequence similarity for 16S rRNA gene sequences for *Rahnella* and *Ewingella* species

	EwS1	*EwB2*	*Rahnella chamberiensis*	*Rahnella badensis*	*Rahnella variigena*	*Rahnella victoriana*	*Rahnella inusitata*	*Rahnella woolbedingensis*	*Rahnella bruchi*	*Rahnella aquatilis*	*Ewingella americana*
EwS1	100	100	98.5	98.8	97.7	97.6	98.7	97.9	97.9	96.9	98.1
EwB2		100	98.5	98.8	97.7	97.6	98.7	97.9	97.9	96.9	98.1
Rahnella chamberiensis			100	99.1	98	97.8	99.7	98.3	98.3	97.8	98.1
Rahnella badensis				100	98.2	98.1	99.2	98.4	98.4	98	98.2
Rahnella variigena					100	99.6	98.1	99.5	99.4	100	98.2
Rahnella victoriana						100	98	99.1	99.1	99.6	98
Rahnella inusitata							100	98.3	98.3	97.9	98
Rahnella woolbedingensis								100	99.9	99.4	97.1
Rahnella bruchi									100	99.3	97.1
Rahnella aquatilis										100	98.2
Ewingella americana											100

3.2.5 Multi-loci phylogeny of house-keeping genes

Phylogenies based on ribosomal RNA small sub-unit gene sequences do not distinguish between closely related species sufficiently, while the API20E or VITEK 2 identification kits do not identity gram-negative, fermentative, oxidase-negative isolates well, often suggesting *Ewingella americana* or *Pantoea* sp. as possible identities. Multi-locus sequencing analysis or typing (Maiden *et al*., 1998) is a technique designed to molecularly classify closely related species, where sections of house-keeping genes are combined and analysed phylogenetically. This approach has been used successfully to classify closely related species of fungi and bacteria, including the Enterobacteriaceae where the genes *fus*A (translation elongation factor G), *pyr*G (CTP synthase), *rpl*B (50S ribosome protein L2), *rpo*B (β subunit RNA polymerase) and *suc*A (2-oxoglutarate dehydrogenase E1) were used to type *Pantoea*, *Rouxiella* and *Enterobacter* species (Delétoile *et al*., 2009; Le Flèche-Matéos *et al*., 2015, 2017; Paauw *et al*., 2008). Therefore, this method was applied in this study.

Sequences of the aforementioned five genes were obtained for 47 taxa, ensuring that they had the same origin, and aligned to those from isolates *EwS1* and *EwB2* to generate a 49 taxa matrix, with species of *Proteus* included as outgroup taxa. Matrices for each gene were constructed separately to test that the phylogenies produced were congruent. All genes except for *fus*A produced congruent trees (data not shown) so *fus*A sequences were removed from the matrix, which is the standard approach in phylogenetic analyses. Maximum likelihood analysis of the resulting matrix using the TN93 model and non-parametric bootstrap analysis produced a strongly supported tree (Figure 20) of In (L) = -86239.2, Ts/ts ratio = 5.384303, alpha = 0.871231 and P-inv = 0.517210. The matrix comprised 9394 sites, from which 59.52% were monomorphic, and from which the analysis identified 3303 separate patterns. The *EwS1* and *EwB2* sequences form a strongly supported clade (100% bootstrap) within a larger clade comprising sequences from *Rahnella* and *Rouxiella* species that is also strongly supported (100% bootstrap) and which forms a sister clade with sequences from species of *Yersina* and *Serratia* - at 94% bootstrap support. This group is separate from other members of the *Enterobacteriaceae*, and it is consistent with the results presented for the genome comparison study (Chapter 3, Figure 21, 22, and 23).

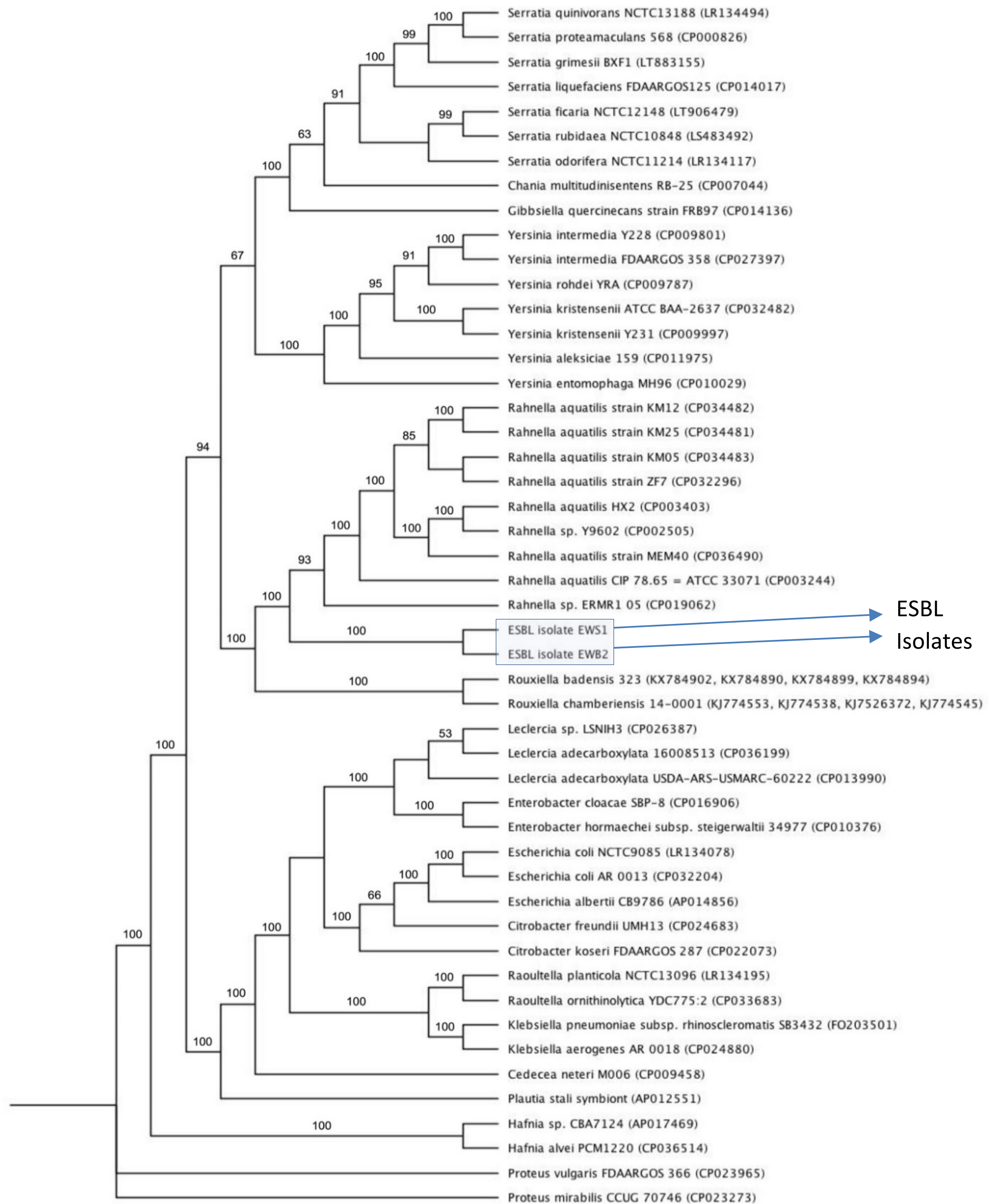

Figure 20. Four gene phylogeny (genes pyrG, rplB, rpoB and sucA) showing the relationship between the ESBL isolates *EwS1* and *EwB2* (boxed) within the *Yersiniaceae*. Maximum likelihood analysis of 9394 nuelotide sites using model TN93 with 4 rate classes generated 3303 patterns, 59.52% of which were monomorphic. Using estimated values of Ti/Tv ratio (5.384303), alpha (0.871231) and P-inv (0.517210, maximal likelihood analysis using nearest neighbor interachange (NNI) produced a tree of ln(L) = -86239.2. The figure shows a majority consensus cladogram of a bootstrap analysis with 100 repetitions, where the bootstrap values are shown above the branches.

The isolates *EwS1* and *EwB2* form a strongly supported clade with members of the genus *Rahnella*. The question remains whether these isolates are members of the *Ewingella*, *Rahnella* or whether they represent a new genus. To answer this question, the isolates were characterised for the key characteristics of the genera *Rahnella*, *Rouxiella*, *Ewingella*, *Serratia*, *Hafnia* and *Klebsiella* using biochemical test described by Bergey and Holt, 2000. Table 8 summarises these key characteristics.

Table 8. The key characteristics of the isolates *EwS1* and *EwB2*. compared to *Hafnia, Ewingella, Serratia, Rouxiella, Klebsiella* and *Rahnella*.

Characteristics	*EwS1/EwB2*	*Ewingella*	*Serratia*	*Rouxiella*	*Hafnia*	*Klebsiella*	*Rahnella*
Growth at 4°C	+	-	-		-	-	+
Growth at 37°C	-	+	+	+	+	+	+
Growth at 40°C	-	-					
Growth at 8% NaCl	-	+			-		
Mobility	+	+	+	-		-	±
Simon’s Citrate	-	+	+	+		+	+
Voges-Proskauer reaction	-	-	+	+	+	+	+
Methyl-Red	+	+	-		V	-	+
Urea hydrolysis	-	-	+	-		+	-
Sucrose	+	-	+	-	-		+
D-glucose, gas production	+	-		-			+
D-Sorbitol	+	-	+	-	-		
Glycerol	+	-	+	-			- *
L-Arabinose	+	-	-	+			+
Myo-Inositol	-	-	+	+		+	-
Melibiose	+	-	-	+	-		
Raffinose	+	-	-	-	-		+
Cellobiose	+	-	-	-	-		+
Maltose	+	-	+	-			+
D-Mannitol	+	+	+	+	+	+	+
L-Rhamnose	+	-	-	+			+
H_2S production	-	-	-				-
Lysine decarboxylase	-	-	+	V	+	+	-
Ornithine decarboxylase	-	-	+		+		-
Gelatin hydrolysis	-	-	+	-			-
Chitin	+	+					+
Lactose	+	-	-	-	-		+
Salicin	+		+		-		+
Trehalose	+		+	-			
D-Arabitol	+		V	-			-
different		14/32	12/32	12/32	11/32	7/32	4/32
similar		18/32	20/32	20/32	21/32	25/32	28/32

V- Variable

EwS1 and *EwB2* have a different profile to those of all the other genera. Specifically, the isolates are capable of growth at low temperature and cannot utilise citrate or possess lysine and ornithine decarboxylases. They have the ability to utilise cellobiose, pectin and chitin, which might be linked to the degradation of plant and insect material. Out of 32 biochemical/physiological tests, the two isolates show the greatest similarity to the genus *Rahnella* (Table 8).

Species belonging to the genus *Rahnella* are facultative anaerobes, nitrogen fixers that can utilise citrate, they can ferment a range of sugars including salicin, and do not possess lysine and ornithine decarboxylases. They do exhibit mobility but only at temperatures of 25ºC. They inhabit fresh water, soil, have been isolated from the guts of snails and beetles (Brenner *et al*., 1998; Park *et al*., 2007), and are recognised as being associated with plant roots. *Rahnella aquatilis* is known to cause disease in humans, specifically bacteraemia (from renal infection), sepsis, respiratory and urinary tract infections. It has also been shown to be involved in meat spoilage (Godziszewska *et al*., 2017). Its genome size ranges from 4.8 to 5.4 Mbp with a G-C content of 52.1% (Martinez, 2010). Furthermore, it can grow at temperatures from 4 to 10ºC (Tash, 2005) and a temperature sensitive isolate was recovered that could not grow on MacConkey or Blood Agar at 37ºC (Domann *et al*., 2003). The differences between the isolates *EwS1* and *EwB2* and members of *Rahnella* are few but significant: the inability to utilise citrate but to use glycerol and the lack of the Voges-Proskauer reaction.

3.2.6 Genome comparison

In order to obtain even greater understanding of these ESBL-producing bacteria and their taxonomic relationships, a step-by-step characterisation was done. The 16S rRNA gene was sequenced and analysed first, then a 4 gene analysis of putative genes, and now full genome sequencing. The genome size of isolates *EwS1* and *EwB2* were 52 and 51 Mbp, respectively with a GC content of 52.27% (53.37% and 53.38% respectively for gene coding regions) and base frequencies of 23.75% and 23.77% A, 25.93% and 26.01% C, 26.33% and 26.25% G and 23.97% and 23.95% T for *EwS1* and *EwB2* respectively (23.35% A, 25.86% C, 27.5% G and 23.27% T for gene coding regions of *EwS1*, and 23.33% A, 25.87% C, 27.5% G and 23.28% T for gene coding regions for isolate *EwB2*). Each isolate carried a plasmid of 10 kbp.

A comparison of genome sequences in FASTA format, with the One Codex genome library (https://app.onecodex.com/) was made to identify their taxonomic origins. The library comprises 53,193 bacterial, 27,020 viral, 1,724 fungal, 1,756 archaeal, and 168 protozoan genomes (83,863 including host). This analysis recognised that the genome sequence of isolates *EwS1* and *EwB2* represented a low-complexity sample of *Ewingella americana* (likely strain: *Ewingella americana* ATCC 33852). In the sample *EwS1*, 1.15% of reads (n=1) were specific to *Ewingella americana*. Overall, 72.41% of 87 reads were classified. An additional 1.15% of reads were classified but are non-specific or host reads. Similarly, In the sample *EwB2*, 23.46% of reads (n=19) were specific to *Ewingella americana*. Overall, 77.78% of 81 reads were classified. Histograms showing the proportions of read counts for

the genome matches at the family and genus levels are shown in Figure 21. Both isolates have genomes that match ones found within the *Enterobacterales* at the 92% level for isolate *EwS1* and 87% for isolate *EwB2* (Figure 21a).

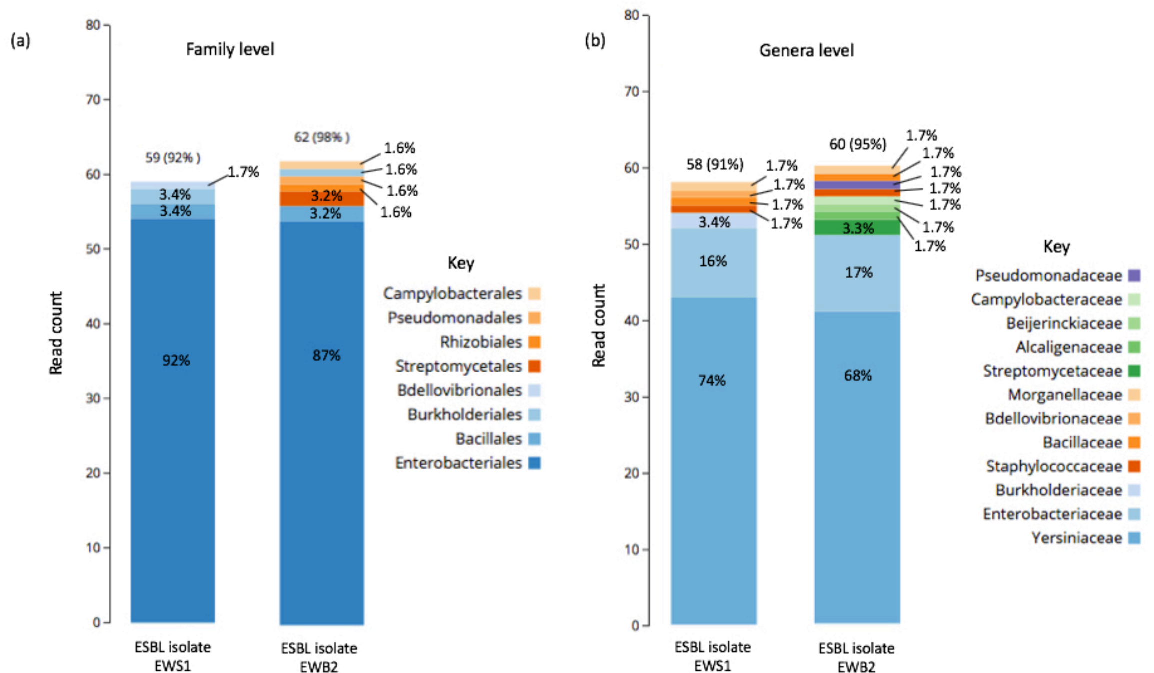

Figure 21. Histogram showing the proportion of read counts for the genome matches at the family and genus levels. (A) The figure shows at family level both isolates largely (87-92%) belonging to the *Enterobacterales* family. (B) the figure shows that at genera level, the most identified genera belonging to these isolates are *Yersiniaceae* (68-74%) and *Enterobacteriaceae* (16-17%).

At the genus level, both isolates have genome matches within the *Yersiniaceae* at 74% for isolate *EwS1* and 68% for isolate *EwB2*, with a smaller proportion for *Enterobacteriaceae* at 16% for isolate *EwS1* and 17% for *EwB2*. The mixed nature of these genomes suggests that they are novel, not being wholly represented by a single genus, as is the case for taxonomically defined species. Figures 22 and 23 shows the genome comparisons between the *EwS1* and *EwB2* with those form the One Codex library at the levels of genus and species, respectively. Unlike defined species, such as *Ewingella americana* ATCC 33852 (NZ JMPJ01000001, bioproject: PRJNA224116) and *Rahnella aquatilis* ATCC 33071 (NC 016818, bioProject: PRJNA224116) that have 100% of their genome sequence reads matching their designated library counterpart, the genome reads of isolates *EwS1* and *EwB2* display a range of matches. The principal sequence reads from the isolates matched those from *Ewingella americana* (46% for *EwS1* and 37% for *EwB2*) and *Rahnella victoriana* (15% for *EwS1* and 12% for *EwB2*). The analysis identifies that the isolates are made up of regions matching those from genera outside the *Enterobacterales*, representing 18% for *EwS1* and 25% for *EwB2* (Figure 22). Although the majority of reads matched those from

genera\species within the gamma-proteobacteria, small proportion were linked with genera/species from an alpha-proteobacteria and gram-positive bacteria, such as *Streptomyces* species (Figure 23).

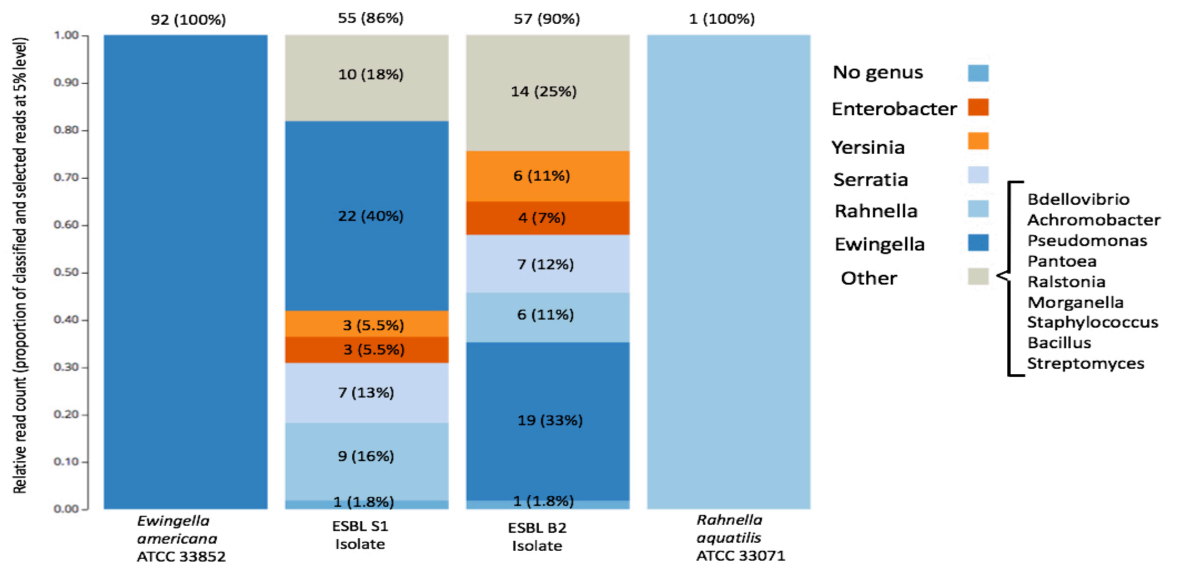

Figure 22. Histogram of the genome reads of ESBL S1 isolate (*EwS1*) and ESBL B2 isolate (*EwB2*) for genera level. The bars denoting *Ewingella americana* ATCC 33852 and *Rahnella aquatilis* ATCC 33071 shows 100% match of their genome reads to their assigned library reads. For isolates *EwS1* and *EwB2*, their genome reads match 33-40% for *Ewingella*, 18-25% for any of the listed other bacteria, 11-16% for *Rahnella*, 12-13% for *Serattia*, 5.5-11% for *Yersinia*, 5.5-7% for *Enterobacter* and 1.8% for no genus.

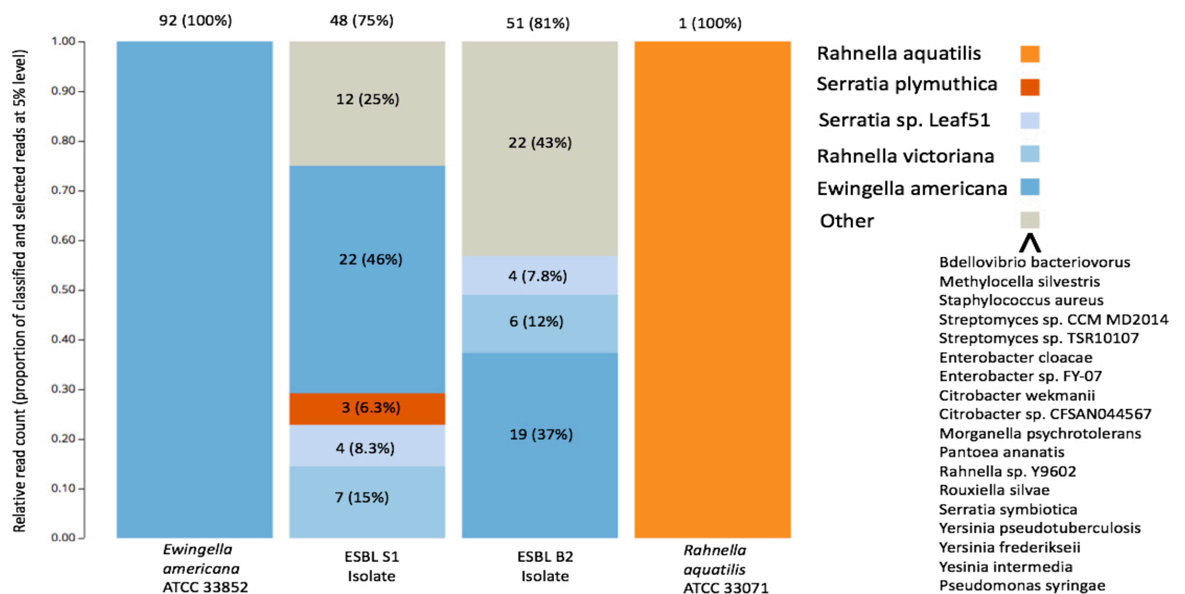

Figure 23. Histogram of the genome reads of ESBL S1 isolate (*EwS1*) and ESBL B2 isolate (*EwB2*) for species level. *Ewingella americana* ATCC 33852 and *Rahnella aquatilis* ATCC 33071 Bars shows 100% genome reads to match their corresponding species. while isolates *Ews1* and *EwB2*, their genome reads match 37-46% for *Ewingella americana*, 25-43% for other bacteria listed, 12-15% for *Rahnella victoriana*, 7.8-8.3% for *Serratia sp. leaf51*, isolate *EwS1* showed a 6.3% genome read to *Rahnella aquatilis*.

This cluster-based structure of a microbial genome is consistent with the Core Genome Hypothesis (CGH) definition of a bacterial species, where horizontal gene transfer has been important (Lan and Reeves,1996). The CGH predicts that a sub-set of genes, the core, is present in all members of a species that give the species its characteristics. These core genes display a neutral rate of evolution, whereas auxiliary genes will experience a variety of selective pressures (Riley and Lizotte-Waniewski, 2009).

Comparative genome analyses of six *Escherichia coli* strains, including the genome of *Shigella flexneri*, demonstrated that these strains had genomes with a highly conserved backbone of 3000 genes, punctuated by many 'sequence islands' that were strain-specific (Mau *et al*., 2006). Using the progressive alignment implemented in Mauve based on HOXD scoring (Darling *et al*., 2004, Bertelli *et al*., 2017), an alignment was constructed for the genomes of ESBL isolate EwB2 and *Rahnella victoriana* BRK18a (Acc. no. MAEN01000001) using the sequences from *Ewingella americana* NCTC12157 (Acc. no. UGGO01000001) as the reference (Figure 24). Despite the great similarity between the 16S rRNA gene sequences from these organisms, the genomes do not share a common conserved structural backbone. Instead, there exists a series of smaller homologous regions that appear to have undergone significant rearrangements. *Ewingella americana* had 79 local colinear blocks (LCB) between the two other genomes, while *Rahnella victoriana* had 83 and *EwB2* had 87. The weight of these blocks (sum of the matches within the block) is a measure of confidence that the LCB is a true genome rearrangement and the cut off for the minimum weight can be increased to identify rearrangements that are likely to exist or decreased to detect smaller genome rearrangements. The Mauve default minimum value to construct a co-linear block (3 times the minimum similarity match size) was used to construct the blocks in the three-genome comparison. This number of colinear blocks determined from this analysis was consistent with numbers detected between the different genomes of *Yersinia* (Chen *et al*., 2010).

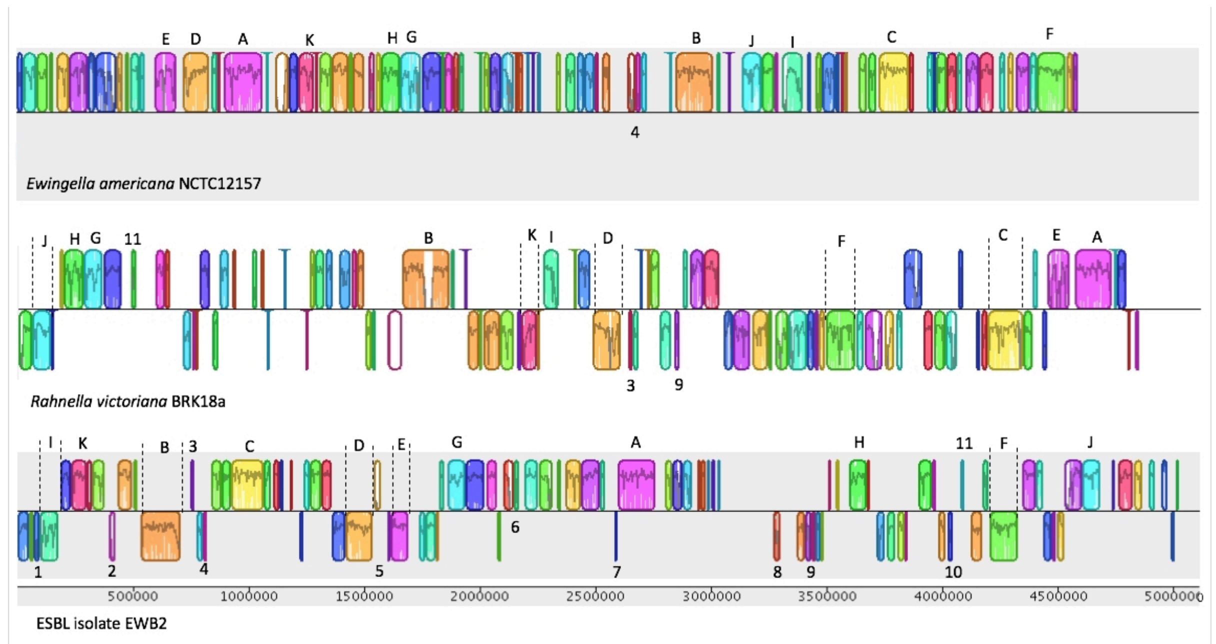

Figure 24. Three genome comparison of *Ewingella americana, Rahnella victoriana* and ESBL isolate *EwB2*. The reference sequences were from *Ewingella americana* NCTC12157 (Acc. no. UGGO01000001) showing that *Rahnella victoriana* and isolate *EwB2* had similar regions in their conserved backbone that has undergone rearrangement. the LCBs identified by a letter are some blocks present in all three genomes, whereas those identified by numbers are those absent or present in two genomes. the LCBs shown below the line are inverted blocks.

The LCBs of the alignment (Figure 24) are either present in all three genomes, present in two of the genomes or absent from two genomes. The common LCBs have undergone rearrangements, including location changes, inversions, and deletions, but contain common coding sequences (CDs) (Figure 25). Other blocks may not be present in all genomes and contain large deletions. Figure 26 shows an enlargement of region 5, which contains some similarity between the genomes but is dominated by a large deletion of approximately 55 kbp in *Ewingella americana* and *Rahnella victoriana*. The CDs present in this region for *EwB2* include the virulence factor *slr*B, a beta-lactamase coding region *bla*, and tellurium resistance coding protein region, *ter*A as well as features associated with a conjugative plasmid (*mob*C) and a transposon (Tn2501). These features suggest that the genes in this region may have been introduced by horizontal transfer. This pattern was repeated in other regions specific to genome of *EwB2*. This genome contained approximately 1000 kbp that was specific to this isolate and included CDs specific to iron acquisition, virulence factors, antibiotic resistance, metal resistance and secretion (Table 9) along with additional transposons Tn21, IS1111A, IS3A and IS4.

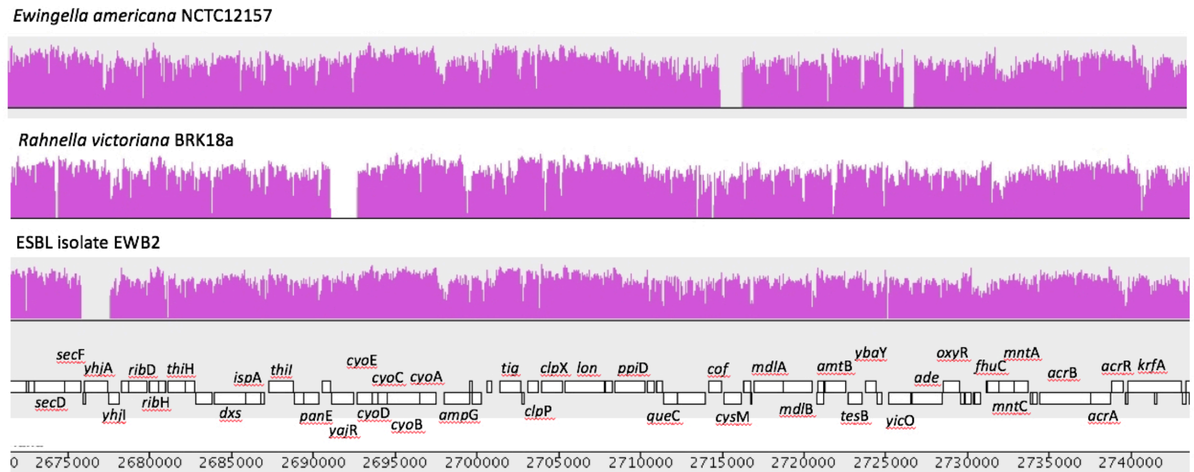

Figure 25. Region A containing CDs common to all three genomes. CDs include: secD (protein translocase subunit), tig (trigger factor), mntA (manganese binding lipoprotein), secF (protein translocase subunit), clpP (ATP-dependent CLP protease, proteolytic subunit), acrB (acriflavine resistance protein B), yhjA (cytochrome peroxidase), clpX (ATP-dependent CLP protease ATP binding subunit), acrA (acriflavine resistance protein A), yhjI (lipoprotein), lon (LON protease), acrR (HTH-type transcriptional regulator), ribD (riboflavin biosynthesis protein), ppiD (peptidyl-prolyl cis-trans isomerase), kefA (Potasium efflux system kefA), ribH (6, 7 – dimethyl-8-ribityllumazine synthase), queC (7-cyano-7deazaguanine synthase), thiH (thiamine monophosphate kinase), cof (HMP-PP phosphatase), dxs (deoxy – D-xylulose-5-phosphate synthase), cysM (cysteine synthase), ispA (farnesyl diphosphate synthase), mdlA (multidrug resistance-like ATP binding subunit), thiI (tRNA sulfurtransferase), mdlB (multidrug resistance-like ATP binding protein), panE (2-dehydropantoate 2-reductase), amtB (Ammonia channel), yajR (inner membrane transport protein), tesB (acyl-Co thioesterase), cyoE (protoheme IX farnesyltransferase), ybaY (lipoprotein), cyoD (cytochrome o ubiquinol oxidase protein), yicO (putative permease), cyoC (cytochrome o ubiquinol oxidase subunit 3), ade (adenine deaminase), cyoB (ubiquinol oxidase subunit 1), oxyR (hydrogen peroxide – inducible genes activator), cyoA (ubiquinol oxidase subunit 2), fhuC (metal transport system ATP binding protein CPn), ampG (Protein AMP G), mntC (manganese transport system membrane protein). Although Region 5 is a conserved block (located at different positions in the three genomes), deleted sections exist for each species where a gene is missing. For example, in the isolate *EwB2* genome there is a deletion for the yhJ A gene, which is present in the other genomes. Deleted regions are identified as gaps in the alignment.

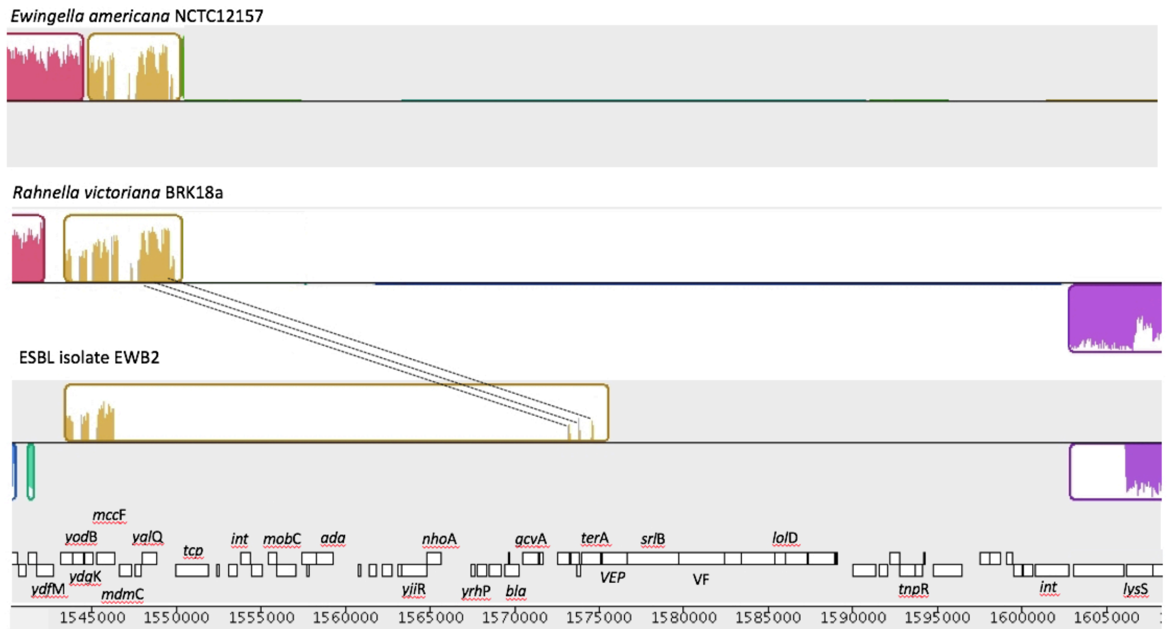

Figure 26. Region V containing CDs specific to ESBL isolate EWB2. The CDs include: ydfM (uncharacterized transporter), terA (tellurium resistance protein), yodB (cytochrome b561 homolog), VEP (virulence effector protein), ydgK (MFS-type transporter), srlB (virulence protein), mccF (microcin C7 self-immunity protein), VF (virulence factor), mdmC (O-methyl transferase), lolD (lipoprotein-releasing system ATP binding protein), yqlQ (oxidoreductase), tnpR (resolvase Tn2501), tcp (methyl accepting chemotaxis citrate transducer), int (integrase family), lysS (lysyl- tRNA synthase), mobC (MOB C protein), ada (bifunctional transcription activator), yijR (HTH-type transcription regulator), nhoA (N-hydroxylamine O-acetyltransferase), yrhP (membrane protein), bla (beta-lactamase containing domain protein), gcvA (glycine cleavage system transcription activator). Region V is primarily a deletion in *Ewingella* and *Rahnella*. The yellow block shows a region of some similarity between *Ewingella*, *Rahnella* and *EwB2*. The bottom gene map is for *EwB2*. The top alignments show how *Ewingella* and *Rahnella* match. This looks has an appearance of an integrated plasmid.

Table 9. Iron acquisition, virulence factors, antibiotic resistance, metal resistance and secretion.

Activity	Gene	Function	Activity	Gene	Function
Iron acquisition	fbpA	Fe (3+) binding periplasmic	Heavy metal	terZ	tellurium resistance
	fbpB	Fe (3+) transport permease		terA	
	fbpC	Fe (3+) ion import		terB	
	entF	enterobactin synthase		terC	
	entS	enterobactin exporter		arsH	arsenic resistance
	entE	Enterobactin synthase		tehB	
	entB	isochorismatase	Antibiotic resistance	macA	
	entC	isochrismate synthase			
Virulence	srfB	Virulence protein		mdtA	Multidrug transporter. confers resistance against novobiocin and deoxycholate. MdtABC requires TolC for its function
		virulence effector protein		acrD	aminoglycoside efflux pump
		virulence factor		mmr	methylneomycin resistance
	yeeJ	invasion		bla	Beta-lactamase CTX-M-2
	sprA	virulence protein		tcmA	tetracenomycin resistance protein
	sprB	mono ADP-ribosyltransferase		bla	
	shlA	hemolysin		bla TEM	Beta-lactamase TAM
	hpmB	hemolysin transporter		smvA	viologen resistance
	fepA	hemoglobin binding	Chemotaxis	trg	methyl accpeting chemotaxis protein III
	fhaB	filamentous hemagglutin		tcp	methyl accepting chemotaxis citrate transducer
	fhaC	filamentous hemagglutin transporter protein	Secretion	clp VI	VI protein
	hrtE	OMP usher protein		icmF	type VI secretion protein
	fimB	chaperone protein		datL	typw IV/VI secretion system
	ipfA	long polar fimbria protein A		impA	type VI secretion associated protein
	ipfB	chaperone		vgr	type vi secretion system
	ipfC	outer membrane protein			
	fimG			pqiA	paraquate-inducible protein A
	ydeR	fimbrial subunit		pqiB	paraquate-inducible protein B

The presence of genomic islands (GEIs) can be taken as evidence for horizontal gene transfer in the evolution of bacteria (Juhas *et al*., 2009). These elements are defined as discrete DNA segments between closely related strains, to which some past or present mobility can be attributed. As a result of selective pressure, these elements can code for a variety of functions important in the adaptation of the organism to the environment, and include complex traits such as pathogenicity, symbiosis, sugar and aromatic compound metabolism, mercury resistance and siderophore synthesis. GEIs can be detected using a number of models: the IslandPath-DIMOB model determines the presence of GEIs based on nucleotide bias and the presence of mobility genes (Hsiao *et al*., 2003), whereas the SIGI-HMM model uses codon usage bias combined with a hidden Markov model (Waack *et al*., 2006) and the IslandPick model which uses a comparative genomics approach to detection (Langille *et al*., 2008). IslandViewer 4 (Bertelli *et al*., 2017) implements all of these models, including Islander (Hudson *et al*., 2015), which uses a database of tRNA and tmRNA genes as integration sites for GEIs. Genome comparisons of *EwB2* and *EwS1* with reference sequences from *Rahnella aquatilis* HX2 identified 14 common islands based on SIGI-HMM and IslandPath-DIMOB models (Figure 27).

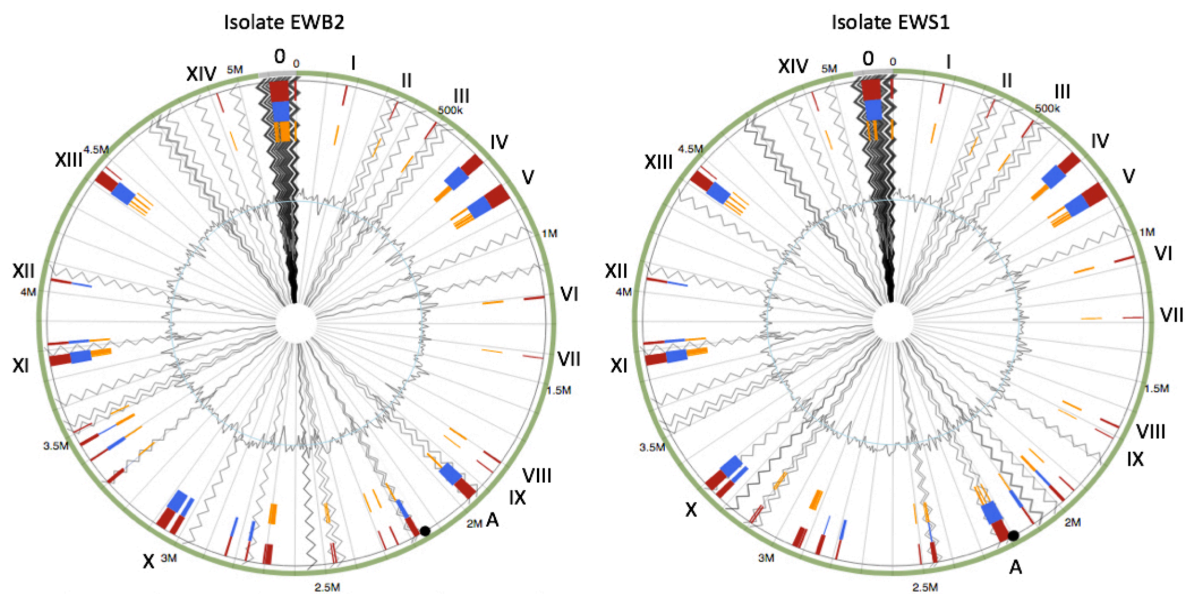

Figure 27. Circular genome maps of *EwB2* and *EwS1* showing the location of 14 Genomic Islands based on the integrated IslandPath-DIMOB (red), and SIGI-HMM (blue) models constructed using reference genome sequence from *Rahnella aquatilis* HX2. The grey areas denote gaps or missing sequences between the target and reference sequences.

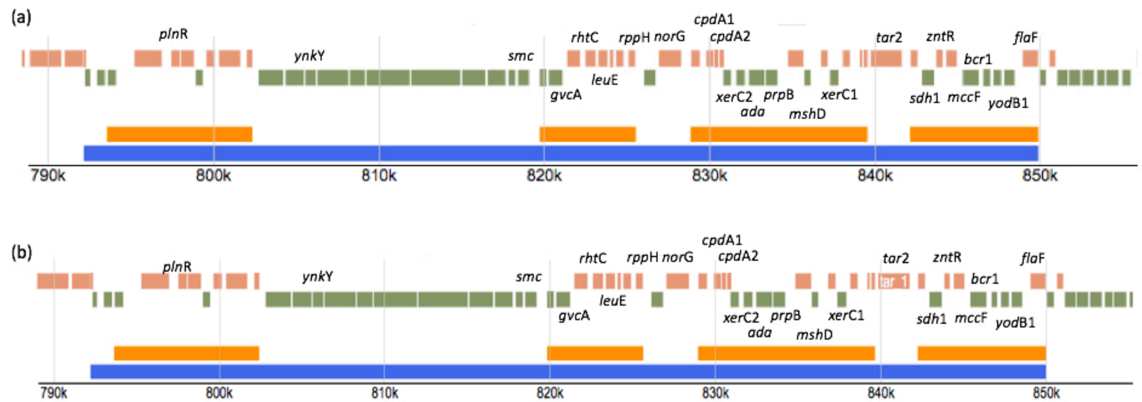

Figure 28. Genetic structure of GEI Region V for (a) isolate *EwB2* and (B) isolate *EwS1*. The Blue block represents the SIGI-HMM determined block, while the orange blocks were determined by the IslandPick model. The genes are present as pink-flesh or green blocks, representing different coding directions. Those genes readily identified by Blast searches are labelled.

An additional 11 potential regions were identified that were located at different sites. The architectures of the core putative islands are identical (Figure 28) between isolates *EwB2* and *EwS1*, whereas the potential strain specific regions contain blocks that are identical but integrated at different sites (Figure 29). Interestingly, many of these blocks were adjacent to or included deleted regions, like that depicted in Region A (Figure 29). Regions that may represent GEIs are present in genomic regions of *EwB2* and *EwS1* that are absent from the genome of *Rahnella aquatilis* HX2. Many of these islands have virulence genes or antibiotic resistance genes associated with them, although no pathogenic GEIs were identified in these analyses.

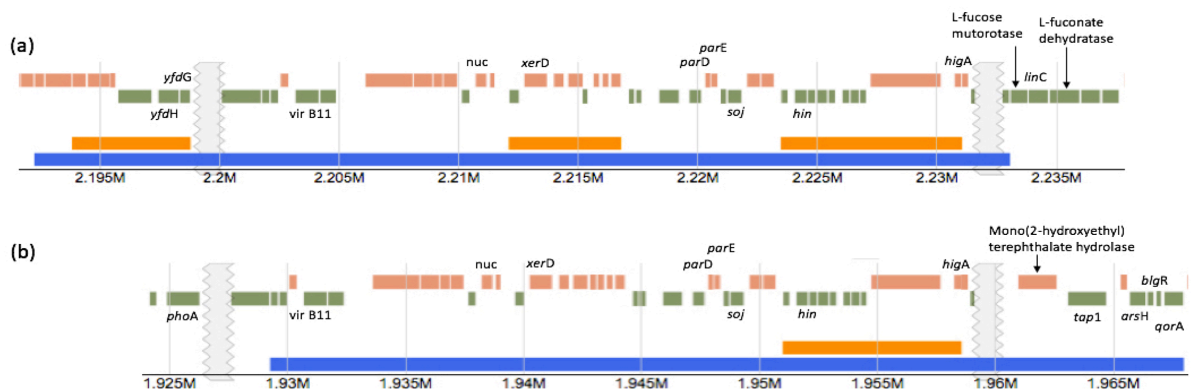

Figure 29. Genetic structure of GEI Region A for (a) isolate *EwB2* and (b) isolate *EwS1*. The Blue block represents the SIGI-HMM determined block, while the orange blocks were determined by the IslandPick model. The genes are present as pink-flesh or green blocks, representing different coding directions. Those genes readily identified by Blast searches are labelled. Deletions of regions from the comparison genome of *Rahnella aquatilis* HX2 are shown as gray areas.

A genome alignment between the sequences from isolates *EwB2* and *EwS1* was constructed using Mauve (Darling *et al*., 2004; Darling *et al*., 2010), allowing node

sequences from isolate *EwS1* to be aligned to those from isolate *EwB2* (Figure 30). The Mauve genome alignment procedure generates a global alignment of local colinear blocks that share sequence similarity. The algorithm finds local alignments, identifying multiple maximum unique matches (MUM) that it uses to create a guide tree using the neighbor-joining method. A sub-set of these MUMs are used as an anchor, called a local colinear block (LCB), a series of which are used to refine the alignment. Genome rearrangements, where regions may be re-ordered or inverted relative to related genomes, can be identified using the position of LCBs. From 87 contigs 23 LCBs were identified between the two genomes, with an average length of 268334.053 base pairs (and a median of 87294), with the longest LCB being 2854755 base pairs and the shortest 5019 bps.

The resolution level of the alignment can be altered by weighing LCBs. The weight of the LCB is defined as the sum of matches in that LCB, setting the level of mismatches can therefore change the resolution. The weight of an LCB provides a measure of confidence that it is a true genome rearrangement and selecting a high minimum weight during alignment, identifies genome rearrangements that are very likely to exist, whereas by selecting a lower minimum weight, some specificity can be traded for sensitivity to smaller genome rearrangements. So, by raising the minimum weight value the number of lower matching LCBs can be reduced (Darling *et al.*, 2004). Therefore, changing the minimum weights of each block to 83981 or 195952 reduced the number of LCBs from 10 (Figure 30b) to 4 LCBs (Figure 30a), respectively. Most of the structural rearrangements within the genomes has taken place between nucleotides 2854755 and 4068339, with the first 2854755 nucleotides being identical and minor rearrangements occurring between nucleotides 4068339 to 5109618. The number of complete coding sequences between the two genomes was 4623, with 106 SNPs. The LCBs within the 2854755 to 4068339 nucleotide regions were found compositionally similar but with major structural rearrangements of blocks. For instance, the gene composition and order within LCB 2, 3 and 4 are the same but in the reverse order in *EwS1* (Figure 31).

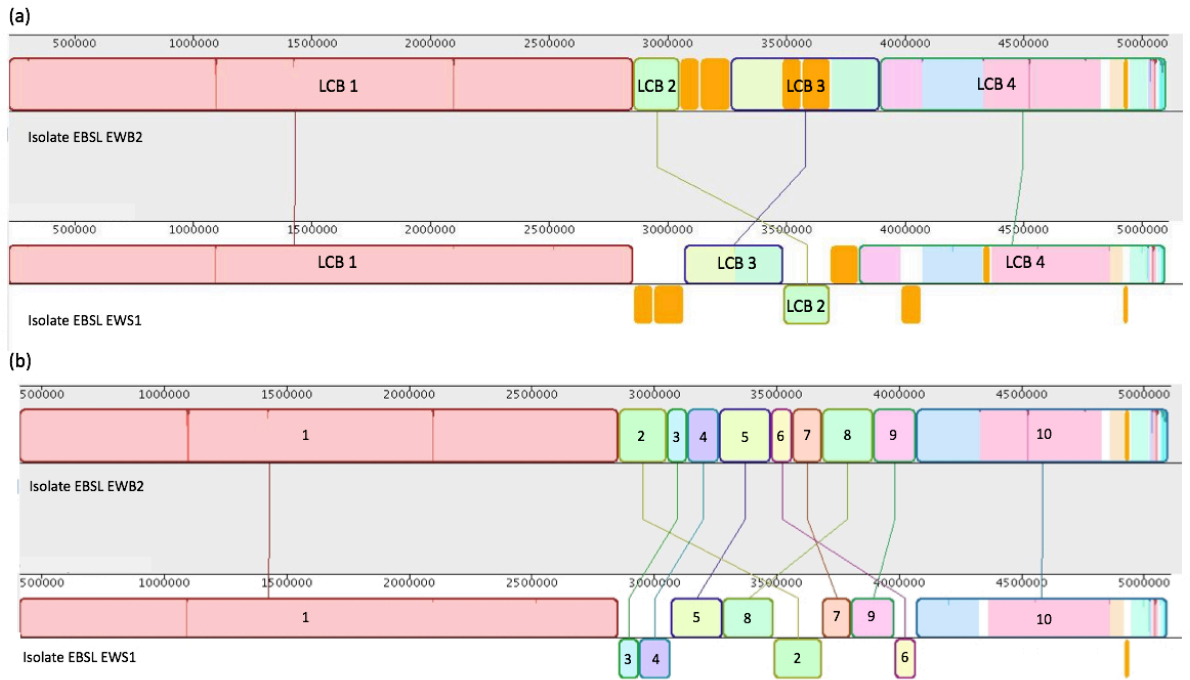

Figure 30. Alignment of the genome of isolates *EwB2* and *EwS1* using Mauve. (a) a minimum weight of 83981 generated 10 LCBs. (B) A minimum weight of 195952 reduced this number to 4 LCBs. The use of a lower arbitrary weight (greater mismatches between nucleotides) identified two conserved regions (LCB 1 and LCB 4) between the two genomes that were positionally similar. Other conserved regions (LCB 2 and LCB 3) showed rearrangements. The main rearrangements took place between nucleotide position 2854755 to 4068339. To identify conserved sections that had undergone rearrangement within this region, the weight was increased (b). Increasing the stringency for mismatches identified a further 8 LCBs (LCB 2 to 9), the rearrangements of which are shown in the above diagram.

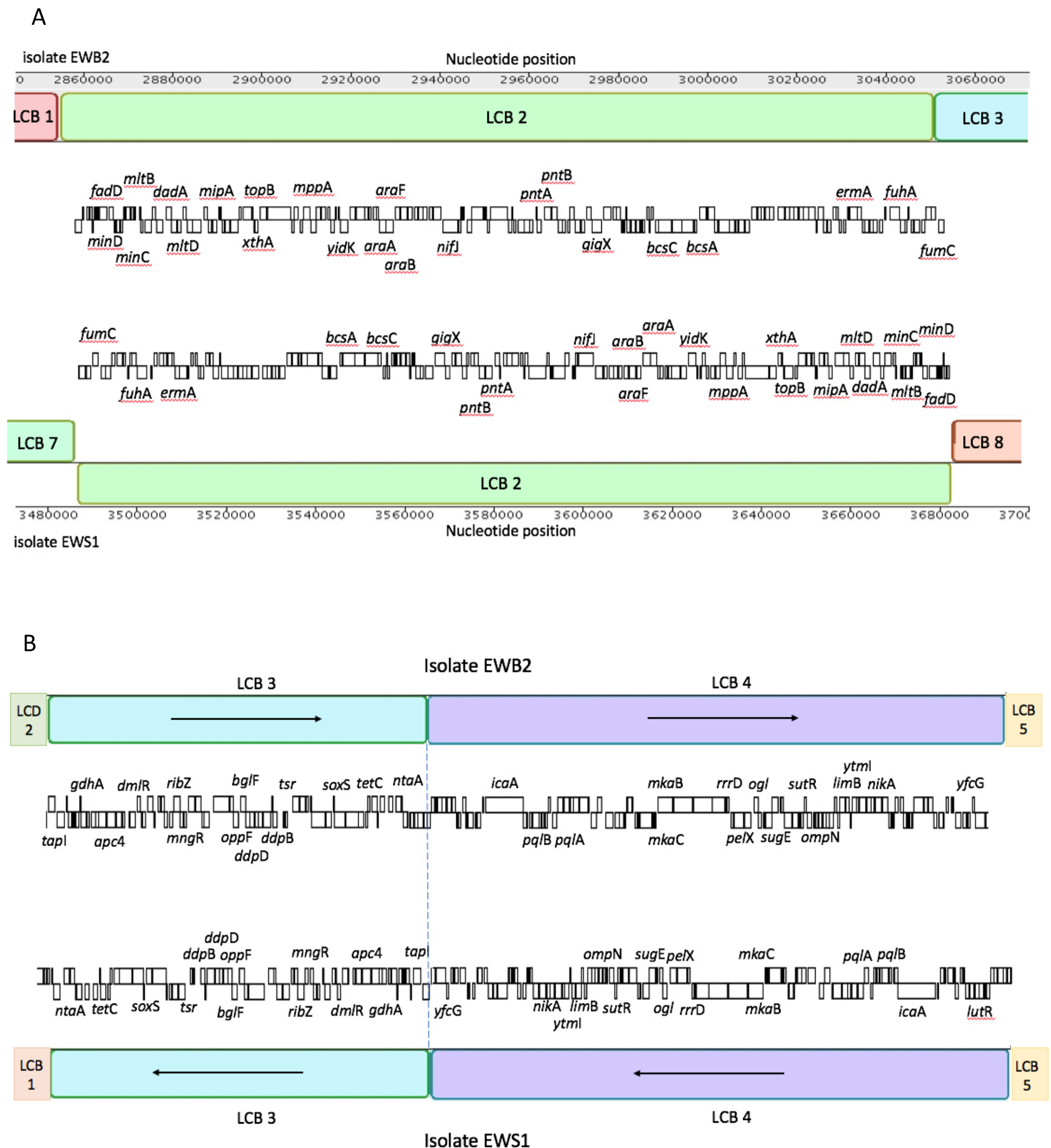

Figure 31. The genetic structures of LCB regions 2 (A), and 3/4 (B) for isolates EwS1 and EwB2. The regional LCBs were mapped using Genome Compiler version 2.2.BB and the appropriate gbk files.

3.2.7 Molecular features of the *EwS1* and *EwB2* genomes

The abilities of isolates *EwS1* and *EwB2* to degrade cellobiose, pectin, D-galacturonic acid, D-mannitol and D-sorbitol as well as having the genes (*pqi*A and *pqi*B) inducible by paraquat suggest that these organisms are adapted to interact or associate with plants in the environment. In support of this idea, the isolates also contain a putative *limB* gene coding for an oxygenase capable of degrading limonene, present in citrus fruit and a component of the herbicide Avenger, genes for Quercetin, which is a plant flavanol; and genes for the

degradation of acetophenone (putative gene yhhW), present in many plant-based foods. In addition to these, others putative genes exist in these isolates: *lic*C for lichenin transport (a complex glucan occurring in certain species of lichens), and *cyc*B gene for binding of cyclodextrin, a product of starch degradation). Nevertheless, it is unclear whether the isolates *EwS1* and *EwB2* are plant pathogens.

To be considered a plant pathogen, the isolates should have virulence or *avirulence* factors for: (1) phytotoxin production, (2) stress survival, (3) Quorum sensing, (4) type III secretion system, (5) biofilm formation and (6) cell wall degrading enzymes/system (Melotto and Kunkel, 2013). The analyses of genomes of *EwS1* and *EwB2* undertaken here, revealed that these isolates certainly contain putative genes coding for functions that would satisfy four of these categories: (i) Genes coding for a variety of stress responses, including acid (genes *asr*, *iba*G and *hde*A), heat (genes *ibp*A and B, *hsl*R), and cold (genes *csp*A, C, E and G) shocks as well as nutrient starvation (*cst*A, *rsp*B, *ssp*A and B), and cryoprotectant metabolite (genes *yeh*W, X, Y, Z) are present (Table 10). (ii) Genes that share similarities to already characterized genes associated with chemotaxis, (iii) Genes involved in quorum sensing and (iv) Genes involved in biofilm formation (Table 10) are also present, including members of the DMT (genes *rht*A, *yij*E, *ydd*G, *yed*A) and RhtB/LysE (genes *rht*B, *rht*C, *leu*E, *arg*O) families and their controlling protein Lpr. These families are involved in the export of amino acids, purines and other metabolites such as homoserine/homoserine lactone thought to play a role in quorum sensing (Zakataeva *et al*., 2006). The presence of these genes suggests that these isolates are adapted to cold environments (Chattopadhyay, 2006). No putative genes coding for phytotoxins, such as coronatine or syringolin, were identified, however other genes coding for animal/human toxins and virulence factors were detected (Table 11).

Table 10. Molecular features of the *EwS1* and *EwB2* genomes, with emphasis on genes responsible for antibiotic resistance, chemotaxis and stress responses.

Gene	Function	Gene	Function	Gene	Function
Chemotaxis		*ywr* O	General stress protein	*mda* B	Adriamycin
trg	Methyl-accepting chemotaxis protein III	*yeh*W, X, Y, Z	glycine betaine uptake system	*stt* H	Streptothricin
che A, B, R, Y, V, W	Chemotaxis proteins	*yhd* N	General stress protein	*yhe* I	multidrug resistance ABC transporter
qse G	Quorum-sensing regulator protein G	*oxy* R	Hydrogen peroxide-inducible genes activator	**Bacterial interactions**	
aer	Aerotaxis receptor	*ssp* A, B	Stringent starvation proteins A and B	*caf* 1A	F1 capsule-anchoring protein
tap 2, 3	Methyl-accepting chemotaxis proteins II and IV	*egt* B	Hercynine oxygenase	*sto* A	Sporulation thiol-disulfide oxidoreductase A
tar	Methyl-accepting chemotaxis protein II	*tau* A, B, C, D	Taurine import system	*soj*_2	Sporulation initiation inhibitor protein Soj
mot A, B	Motility proteins A and B	*ssu* A, B, C, D, E	aliphatic sulfonates utilization	*mli* C	Membrane-bound lysozyme inhibitor of C-type lysozyme
tsr	Methyl-accepting chemotaxis protein I			Barstar	Barstar
ytn P	putative quorum-quenching lactonase YtnP	**Antibiotic Resistance**		*big* R	Biofilm growth-associated repressor
Stress response		*acr* A, B, F, Z	Multidrug/aminoglycoside export proteins	*pga* D	Biofilm PGA synthesis protein PgaD
asr	Acid shock protein	*bcr* 2	Bicyclomycin resistance protein	*tab* A	Toxin-antitoxin biofilm protein TabA
bhs A	Multiple stress resistance protein	*bla*	Beta-lactamase, CTX-M	*rat* A	Ribosome association toxin
csp A, C, E, G	Cold shock proteins	*drr* A	Daunorubicin/doxorubicin resistance	*lic*B	antigenic determinant
cst A	Carbon starvation protein A	*bla*_ToHo1	Beta-lactamase, CTX-M2		**Solvent resistance**
lba G	Acid stress protein	*bla*_TEM	Beta-lactamase, TEM	*qac*A	Antiseptic resistance
ibp A, B	Small heat shock protein	*mdt* A, B, C, H, K, L, N	multi-drug resistance (Chloramphenicol)	*srp*A	Solvent efflux pump
hde A	Acid stress chaperone	*bmr* 3	Multi-drug resistance	*pad*C	Phenolic acid decarboxylase
hsl R	heat shock protein	*emr* A, B, D, E, K	Multi-drug resistance	*aae* A, B	p-hydroxybenzoic acid efflux
rsp B	Starvation-sensing protein	*fsr*	Fosmidomycin resistance	**Thermostable enzymes**	
srk A	Stress response kinase	*mdl* B, G	multi-drug resistance	02691	Thermostable carboxypeptidase
yce D	Stress response protein	*mex* A	Multidrug resistance	gntK	Thermoresistant gluconokinase
*typ*A	GTP-binding protein TypA/BipA	*mac* A	Macrolide export protein	bglB	Thermostable beta-glucosidase
osm Y	Osmotically inducible protein	*mfs*	spectomycin/tetracycline	nuc	Thermonuclease

yehW, X, Y, Z – glycine betaine uptake system – cryoprotection; typA - GTP-binding protein TypA/BipA – involved in cold adaptation

Table 11. Molecular features of the *EwS1* and *EwB2* genomes. This includes genes that can function is virulence and toxicity, genes that can bacteria to stick to surfaces, genes that help bacteria in metabolism, iron acquisition, metal resistance, transportation.

Gene	Function	Gene	Function	Gene	Function
Fimbriae and Pili		**Virulence & Toxins**		*bfr*	Bacterioferritin
yra I, H, J, K	fimbrial proteins	*cbt*A	Cytoskeleton-binding toxin CbtA	ytf E	Iron-sulfur cluster repair protein
smf	Major fimbrial sub-unit	*apxI*B	Toxin RTX-I translocation ATP-binding	*fie F*	Ferrous-iron efflux
yad V	fimbrial chaperone	*par*E, D	Toxin ParE1, Antitoxin ParD1	*iuc C*	Aerobactin synthase
csg A, B, C, D, E, G	Curli production	*hig*A, B	Antitoxin HigA1, Toxin HigB-2	aer	Aerobactin receptor
fik	Flagella regulator	*cdi*A	Toxin CdiA	*apb* C	Iron-sulfur cluster carrier protein
lpf A, B	fimbrial sub-unit, chaperone	*cbe*A	Cytoskeleton bundling-enhancing protein	*efe* U, O	Ferrous uptake system
fim D	OM usher protein	*vgr*G	Actin cross-linking toxin VgrG1	f*et* A, B	Iron export system
elf A	fimbrial sub-unit	*cmi*	Colicin-M immunity protein	i*sc* A	Iron-binding protein
flh A, B, C, D, E	Flagella assembly	*ccd*B	Toxin CcdB	*fie* F	Ferrous-iron efflux pump
*fig*A, B, C, D, E, F, G, H, I, K, L, M, N	Flagella components	*cva*A	Colicin V secretion protein CvaA	*erp* A	Iron-sulfur cluster insertion protein
*fil*C, D, E, F, G, H, I, J, K, M, N, O, P, T, S	Flagella components	*ykfI*	Toxin YkfI	*ytf* E	Iron-sulfur cluster repair protein
pil E, T, Q	Type IV system (Twitching)	*tab A, tab*B	Toxin-antitoxin biofilm proteins	*feo* A, B, C	Fe(2+) transporter
yha K	Prin-like protein	*cir*A	Colicin I receptor	*fec* A	Fe(3+) dicitrate transport protein
cid A	Holin-like protein	*sym*E	Toxic protein SymE	*fhu*A, B, C, D, F	Ferrichrome uptake system
pil Q	Type IV pilus biogenesis			**Metal resistance**	
*yd i*V	Anti-FlhC/FlhD factor	**Iron acquisition, transport and metabolism**		czcA	Cobalt-zinc-cadmium resistance
ycg R	Flagellar brake protein	*fha* B, C	Filamentous hemagglutinin	*ars*B, C, R	Arsenate resistance
out O	Type 4 prepilin-like protein	*tiy* C	Hemolysin C	*cor*A	Cobalt/magnesium transport
Adhesin		*hmu* T, U	Hemin transport system	*teh*B	Tellurite methyltransferase
*mka*B, C	28.1 kDa virulence protein	*fes*	Enterochelin esterase	*nik*A, B	Nickel transport
*vat*D	Streptogramin A acetyltransferase	*hmp*	Flavohemoprotein	*rcn*A	Nickel/cobalt efflux system
*cdI*4	Immunity protein	*shl* B	Hemolysin transporter	*cso*R	Copper-sensing transcriptional repressor
*pho*Q	Virulence sensor histidine kinase	*fep* A, B, C, D, G	Ferrienterobactin system	*znu*A, B, C	Zinc transport
i*nl*A	Internalin-A	*ybd* Z	Enterobactin biosynthesis	*znt*B	Zinc transport
eae	Intimin	*ent* A, B, E, F, S	Enterobactin biosynthesis	*pco*C	Copper resistance
inv	Invasin	*ccm* B, C	Heme exporter	*zit*B	Zinc transport
*yee*J	Invasin	*hem* S	Hemin transport	*cop*A	Copper export
*yrf*F	IgaA homolog	frd B	Fumarate reductase iron-sulfur subunit	*mnt*B	Manganese transport
*esl*B	Immunoglobulin A binding protein	*hmu* T, U, V	Hemin-binding and transport	*zur*	Zinc uptake
		ftnA	Bacterial non-heme ferritin		

During infection, where iron is present at growth-inhibitory concentrations, bacteria have to be able to access iron from haemoglobin or iron proteins or ferritins. Both isolates contain multiple genes coding for hemolysin C (gene *tiy* C,) siderophores such as enterobactin (genes *ent* A, B, E, F, S) and aerobactin (gene *iuc* C) reference Table 12. However other siderophore genes are absent from these isolates (*iuc* A, B, and *iut* A), although its receptor *aer* is present. The potential use of enterobactin is clearer; where the Fep P outer membrane acceptor protein and the Fes esterase, necessary for cleavage of iron from the siderophore, are present (Table 12).

Table 12. Genes that code Virulence factors, adhesins, appendages and toxins in isolates *EwS1* and *EwB2*.

gene	function	*gene*	function	*gene*	function
mka B, C	28.1 kDa virulence protein, transcriptional activator	*flh*A, B, C, D, E	Flagellar assembly	*ccm*B, C	Heme exporter proteins
*vat*D	Streptogramin A acetyltransferase	*fig*A, B, C, D, E, F, G, H, I, K, L, M, N	Flagellar components	*hem* S	Hemin transport protein
*pho*Q	Virulence sensor histidine kinase	*fil*C, D, E, F, G, H, I, J, K, M, N, O, P, T, S	Flagella components	*hmu* T, U, V	Hemin-binding, transport protein
*inl*A	Internalin-A	*pil*E	Fimbrial protein	*cbt* A	Cytoskeleton-binding toxin CbtA
*cdl*4	Immunity protein CdiI-o11	*yha*K	Pirin-like protein YhaK	*cbe* A	Cytoskeleton bundling-enhancing protein CbeA
eae	Intimin	*cid*A	Holin-like protein CidA	*cir* A	Colicin I receptor
inv	Invasin	*pil*Q	Type IV pilus biogenesis and competence protein PilQ	*sym*E	Toxic protein SymE
yeeJ	Invasin	*ydi*V	Putative anti-FlhC(2)FlhD(4) factor YdiV	*apxl*B	Toxin RTX-I translocation ATP-binding protein
*esl*B	Secretory immunoglobulin A-binding protein EsiB	*fha*B, C	Filamentous hemagglutinin, Filamentous hemagglutinin transporter protein	*par*E, D	Toxin ParE1, Antitoxin ParD1
*tau*A, B	Taurine-binding periplasmic protein A, Taurine import ATP-binding protein TauB	*tiy*C	Hemolysin C	*hig*A, B	Antitoxin HigA1, Toxin HigB-2
*yrf*F	Putative membrane protein IgaA homolog	*hmu*U	Hemin transport system permease protein	*cdi*A	Toxin CdiA
*yra*I, H, K	putative fimbrial-like protein YraK and YraH	*fep*A, B, C, D, G	Ferric enterobactin transport proteins	*cmi*	Colicin-M immunity protein
smf	Major fimbrial subunit SMF-1	*fes*	Enterochelin esterase	*vgr*G	Actin cross-linking toxin VgrG1
*yad*V	putative fimbrial chaperone	*ybdZ*	Enterobactin biosynthesis protein	*ccd*B	Toxin CcdB
*csg*A, B, C, D, E	Curli production	*elf*A	Fimbrial subunit ElfA	*cva*A	Colicin V secretion protein CvaA
fik	Flagellar regulator	*ent* A, B, E, F, S	Enterobactin synthase	*ykf*I	Toxin YkfI
*lpf*A, B	major fimbrial subunit, fimbrial chaperone	*hmp*	Flavohemoprotein	*tab*B	Toxin-antitoxin biofilm protein TabA
*fim*D	OM usher protein FimD	*shl*B	Hemolysin transporter protein	*lic*B	glycolipid that is a necessary component and antigenic determinant of the outer membrane

To colonise host tissue, bacteria must exhibit invasiveness. *EwS1* and *EwB2* have genes such as surface-sensing type 1 and/or type IV pili or fimbriae. It is not known whether isolates *EwS1* and *EwB2* exhibit flagella impairment, but they have the genes for type III flagella include the core operon (*fhl* D, C) and the *fhl* A, B, E operon for type III flagella where the *fhl* E gene product is thought to be important for swarming (McDowall *et al*., 2014),. In addition to these operons, those responsible for flagella motor switch (*fig* G, M, N) and for general biogenesis of the flagella (Table 12) including gene *fil* C coding for a-type flagellin. The genomes do contain genes that potentially code for pili and fimbriae, some of which have been implemented in adhesion of bacterial cells to human cells. The *yra* H, I, J, K operon (Table 12) codes for a type 1 fimbria adhesin. Another type 1 fimbria involved in adhesion in enteropathogenic *E. coli* infections is the long polar fimbria, coded for by the *lpf* ABCDE operon (Ideses *et al*., 2005). Other genes present in the genomes that could be involved in adhesion include *smf*-1, a major fimbrial sub-unit that can mediate agglutination of red blood cells (de Oliveira-Garcia *et al*., 2003), *fim* D, an outer membrane protein, and *elf* A, the major fimbrial sub-unit of the *elf* ADCG-*ycb*UVF operon that is involved in adhesion to abiotic substrates (Korea *et al*., 2010). It is unknown whether any of these gene form active products. Within members of the *Enterobacterales*, the curli fimbriae are encoded into two divergent operons, *csg* C, A, B and *csg* D, E, F, and G. The genomes of isolates *EwS1* and *EwB2* have these operons in a similar arrangement.

Genes coding for tissue invasion are present in the genomes of *EwS1* and *EwB2*. Surprisingly, isolates *EwS1* and *EwB2* have more than one type of adhesion protein capable of being expressed in their genomes. Three adhesin genes including invasion genes (genes *inv* and *yee*J), intimin genes (*eae*A) or internalin genes (*inl* A) are present in the genomes (Table 12).

The invasin gene was present in both genomes several times while the intimin and internalin genes are commonly found in bacteria belonging to different taxonomic groups, suggesting that the presence of all these genes was due to horizontal transfer. This notion is borne out by the phylogenetic relationship shown by the different invasion genes (Figure 32), where the *inv* gene groups with members of the *Yersiniaceae* while the *yee* J gene is found with members of the *Enterobacteriaceae*.

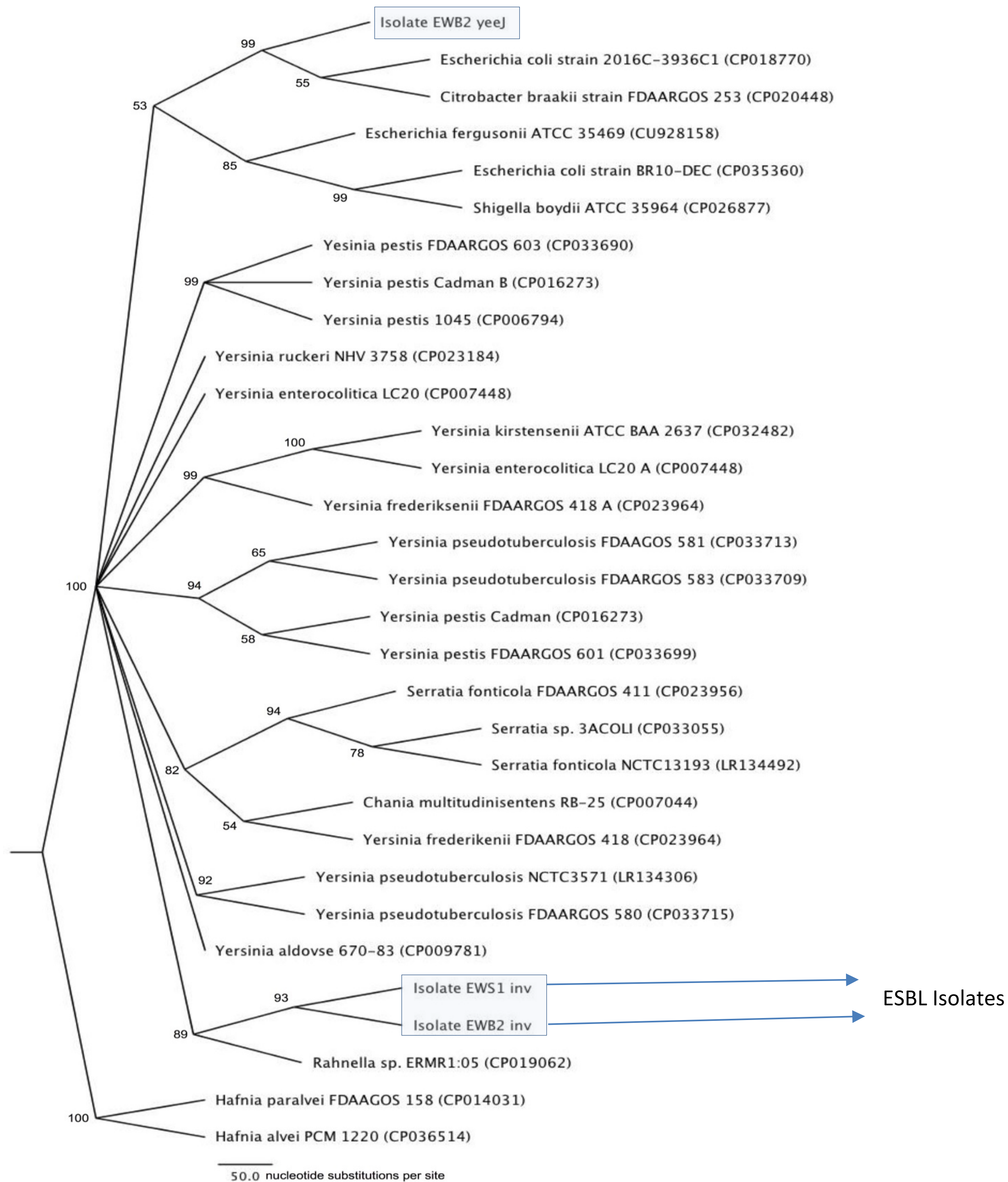

Figure 32. Maximum likelihood phylogenetic tree showing the relationship of the invasin gene sequences (Inv and yeeJ) from isolates *EwS1* and *EwB2* (Boxed) with those from other members of the Enterobacterales. The maximum likelihood model used to construct the tree was GTR+I+G with an estimated P-INV value of 0.089062, and this generated a tree of -ln L = 11655.63330 from 687 distinct data patterns.

It is possible that the isolates *EwS1* and *EwB2* are capable of invading epithelial cells via a number of routes, although a gene for TIR was not identified suggesting that adhesion via the intimin route might not be feasible.

The acquisition of sulfur and nitrogen is essential for growth once colonisation has occurred. Both isolates have the genes coding for enzymes and proteins involved in ammonia, nitrate and nitrite assimilation and respiration (*nar* I, J, H, G, K, L, X, *nar* D, *nas* D), as well as those for acquiring glutathione (genes *gs*i A, B, C, D) and urease, but they did not appear to have genes coding for nitrogen fixation functions. Inorganic and organic sulfur is available in the human body, with levels of 215.5 μM sulfate, 100.8 μM thiosulfate, 37 μM glutathione, 62 μM cystine and 55.5 μM taurine present in the plasma (Lensmire *et al*., 2019). The isolates had approximately 32 identified genes involved in sulfur metabolism. These included genes for sulfate, thiosulfate and sulfite acquisition and metabolism (*cys* A, B, C, D, H, M, N, P, T, W, Z, *rhd* A, *dsb* A, B, E, *dbd* C, D genes) including cysteine synthesis, glutathione acquisition and metabolism (*gsi* A, B, C, D*, yei* G*, frm* A genes), and those responsible for the acquisition of taurine, 2-aminoethanesulfonic acid, (*tau* A,B,C, D genes) and other sulfonated substrates such as alkanesulfonates and substituted ethanesulfonic acid (*ssu* E, A, D, C, B genes) reference Table 10, 11 and 12.

4.0 Isolation of bacteriophages for specific bacteria isolated from wastewater.

4.1 Introduction

Viruses are the most abundant life forms on Earth, with estimated global number of 10^{31} particles or virions, which is 10 times greater than the number of bacterial cells (Willianson *et al*., 2005, Weitz *et al*., 2012, Güemes *et al*., 2016). Twort (1915) and D'Herelle (1917) discovered bacterial viruses independently, both of whom realised that an entity existed with the ability to lyse bacterial cells. It was D'Herelle that gave this entity the name *bacteriophage*, describing it as a virus, and developed methods for its enumeration. Also, he proposed the use of bacteriophages as therapeutic agents and as prophylactics, introducing the concept of phage therapy (D'Herelle, 1926). Commercial preparations of bacteriophages first became available in the nineteen thirties, but their consistency and quality were variable, and this, amongst other reasons, promoted sceptical reporting about the medical uses of phages (Summers, 2012). During this period, sulphonamides drugs were used to treat infections with success, which appeared to have a greater consistency and simplicity than viral treatments. Furthermore, medicine in Europe and the USA was about to enter the golden era of antibiotic discovery and use, with the development of penicillin as a broad-based anti-bacterial treatment. During this period, research into phage therapy was performed only in the Soviet Union, particularly at the Tbilisi Institute Georgia (now the George Eliava Institute), and, to a lesser extent, Germany (Summers, 2012). Eventually, world medicine, human and veterinary, became dependent on antibiotics as the main therapeutic and prophylactic agents against bacterial infections. This has been the case until now. The world-wide spread of resistances to most clinically useful antibiotics by pathogenic and environmental bacteria has led to a re-emergence of an interest in bacteriophage therapy (Kortright, 2019).

In the current study, the research focus was to isolate and characterise bacteriophages specific to ESBL-producing bacteria recovered from sewage - a rich source of enterobacteria and their viruses. The discovery of such viruses would be of particular interest because they are a potential replacement, or adjuvant, to antibiotics for the treatment of skin ulcers, wounds, burns, allergies, gastrointestinal ailments, methicillin-resistant *Staphylococcus aureus* infections (Abedon *et al*., 2011). In addition, they have also been used as biological control agents, reducing bacterial loads in foods, such as that of *Listeria monocytogenes* in food processing (Bai *et al*., 2016), and of zoonotic pathogens in livestock (Atterbury, 2009), or, in the treatment of crops to control the incidence of plant pathogenic bacteria (Buttimer *et al*., 2017). This chapter describes the isolation of phages

for ESBL-producing *Klebsiella pneumonia* ATCC 13883, *Pseudomonas* [Isolated from wastewater], *Escherichia coli* (strains 1-6 isolated from wastewater and *E. coli K802*) and the two novel bacteria related to the members of the genus *Rahnella*. Moreover, their specificity and transmission electron microscopy (TEM) study of selected isolates would be described.

4.2 Isolation of bacteriophages

As described in the previous chapters, different bacteria have been isolated, identified, and characterised. In the next step, bacteriophages that can kill these bacteria have been isolated and characterized. Phages were, isolated using the one-host enrichment method. In this approach wastewater is mixed with a culture of each isolated strain to obtain the lysate containing a population of bacteriophages capable of infecting these cells (Chapter 2.7.2). The resulting plaques observed were named according to their host bacterium, size, and morphology. In total, 5 different bacteriophages of *E. coli, Pseudomonas* and the cold tolerant bacterial strains were obtained. The isolated bacteriophages were named as EcC1, PsBT1 and PsST2 according to host bacterium *E. coli* (Ec) or *Pseudomonas* (Ps) and plaque type clear (C) or turbid (T) and size (B for big or S for small). Specifically: EcC1 produced round, clean edged clear plaques with an average size of 0.2 mm ± 0.2 mm. PsBT1 and PsST2 both produced a rough, haloed edged turbid plaques. They had an average size of 1 mm ± 0.3 mm for PsBT1 and 0.6 mm ± 0.2 mm for PsST2, while the 2 phages isolated for the cold tolerant bacteria were named EpC1 and EpT2 respectively for the clear and turbid varieties. The first had clear plaques of diameter 1.5 mm ± 0.5 mm with clean edges while the second were turbid plaques of diameter 1.0 mm ± 0.5 mm (Figure 33).

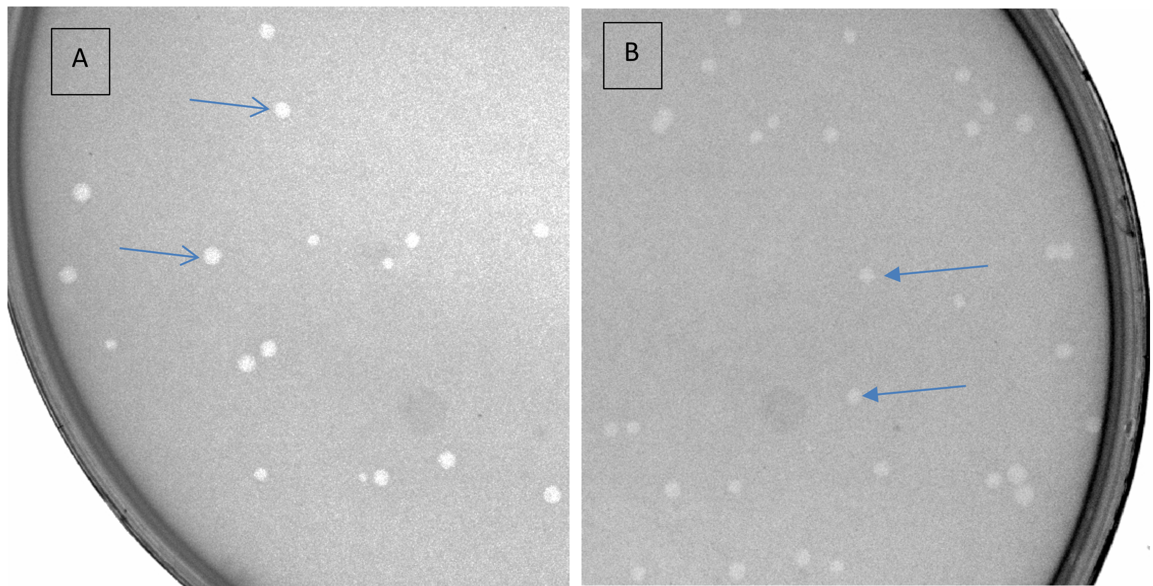

Figure 33. Images of EpC1 (A) and EpT2 (B) plaques on agar plates. Images obtained using Gel Doc™ XR system (Bio-Rad). The plaques represent zones of clearing of bacteriophage on bacteria lawn. Arrows indicate clear plaques and arrowheads the turbid variety.

4.2.1 Phage specificity

The 5 different bacteriophages were isolated as plaques formed on lawns of ESBL-producing bacteria. To determine their host range, bacteriophages EcC1, PsBT1 and PsST2 were tested against different bacterial strains at 37°C, whilst for the cold tolerant *EwS1* and *EwB2* strains, not able to grow at 37°C, their bacteriophages EpC1 and EpT2 were tested against different bacterial strains at 30°C. It was found that phages EpC1 and EpT2 propagated efficiently in both *EwS1* and *EwB2* strains and were not able to infect *Pseudomonas* and *E. coli* strains isolated from wastewater, nor the laboratory strains such as *Serratia marcescens (ATCC 17 AW)*, *Klebsiella pneumonia (ATCC 13883)*, *Proteus mirabilis (ATCC 7002)*, *E. coli K802* or *Hafnia alvei* (Table 13). Thus, these bacteriophages exhibited high specificity for their host. It was concluded that these bacteriophages are specific to only *EwS1* and *EwB2* strains newly isolated from treated wastewater. Moreover, it was observed that EcC1 efficiently propagated in all *E. coli* strains and PsBT1 and PsST2 bacteriophages on one of the *Pseudomonas* strains (strain 1). The three phages were unable to infect either of the strains of the cold tolerant bacteria.

Table 13. Propagation of all isolated bacteriophages on different bacterial strains

	Bacteriophages				
Bacterial Strains	EpC1	EpT2	EcC1	PsBT1	PsST2
**EwS1*	+	+	–	–	–
**EwB2*	+	+	–	–	–
**Pseudomonas strain 1*	–	–	–	+	+
**Pseudomonas strain 2*	–	–	–	–	–
**Pseudomonas strain 3*	–	–	–	–	–
**Pseudomonas strain 4*	–	–	–	–	–
**E. coli strain 1*	–	–	+	–	–
**E. coli strain 2*	–	–	+	–	–
**E. coli strain 3*	–	–	+	–	–
**E. coli strain 4*	–	–	+	–	–
**E. coli strain 5*	–	–	+	–	–
**E. coli strain 6*	–	–	+	–	–
E. coli K802	–	–	+	–	–
Serratia marcescens 17 AW	–	–	–	–	–
Hafnia alvei UoP culture collection	–	–	–	–	–
Klebsiella pneumonia ATCC 13883	–	–	–	–	–
Proteus mirabilis ATCC 7002	–	–	–	–	–

- (+) Growth
- (-) No-growth
- (*) Bacteria isolated from sewage.

4.2.2 Effect of divalent cations on phage growth

The formation of plaques by phages EpC1 and EpT2 on complete media without the addition of divalent cations was observed, and therefore the following experiments done in triplicates were designed to test whether phage propagation could be improved by cations addition. Phage growth was investigated in the presence of different concentrations of Ca^{2+} and Mg^{2+}: 0 mM, 2 mM, 5 mM and 10 mM. In the absence of Ca^{2+} and Mg^{2+} ions the titers of phage EpC1 and EpT2 stocks were 5.0 X 10^6 and 1.3 x 10^6 pfu/ml (Table 14). Variation in calcium and magnesium ion concentrations influenced phage propagation significantly. Increasing the Ca^{2+} concentration to 10 mM resulted in a 34-44 times increased plaque formation for both phages (Figure 34a), whereas increasing the Mg^{2+} to 10 mM improved plaque formation by 5-7 times (Figure 34b). Given that inclusion of calcium had the greatest

effect on plaque formation, it was included at (10 mM) as a supplement in all subsequent experiments.

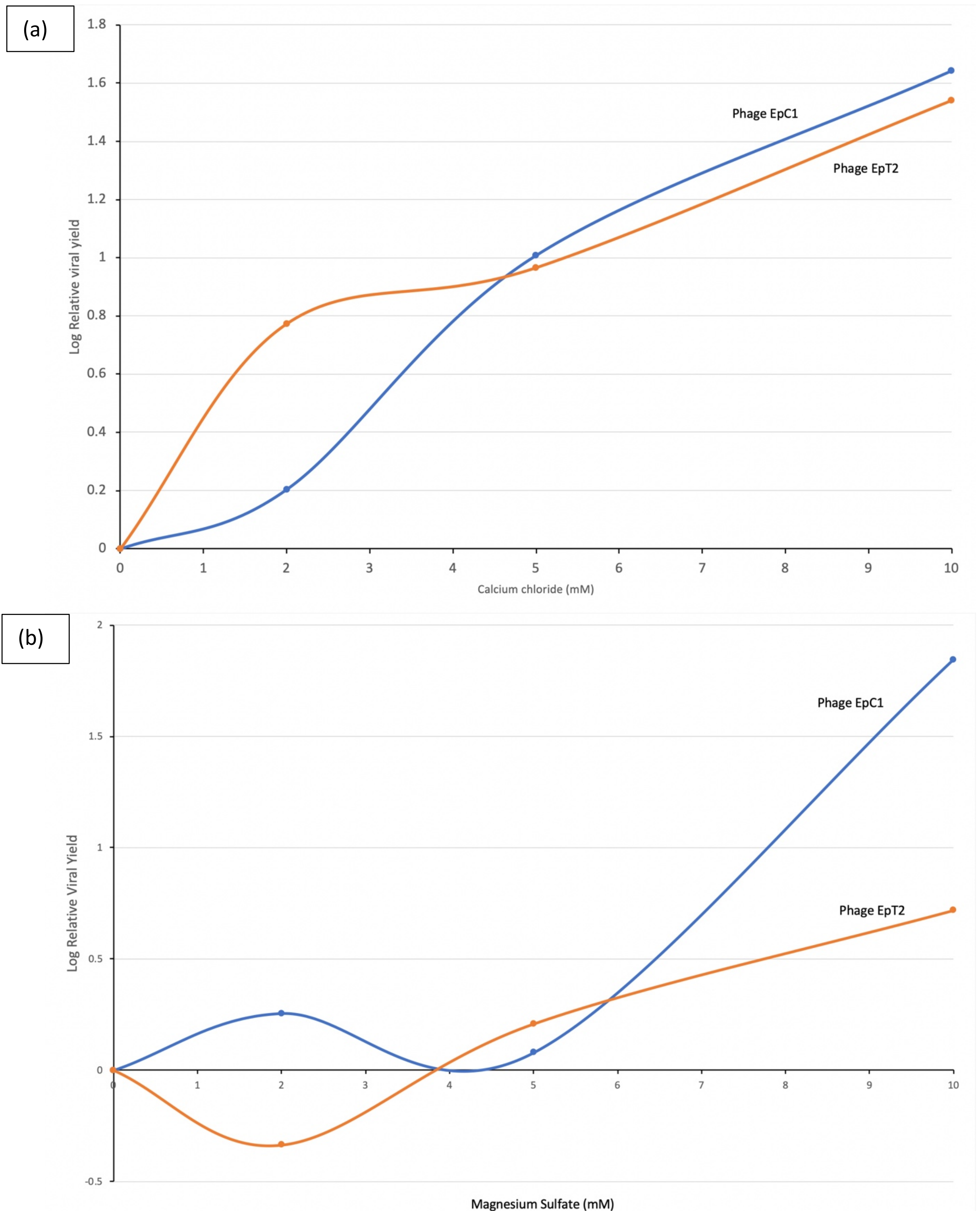

Figure 34. Influence of divalent cations on the growth of phages EpC1 and EpT2 done in triplicate. (a) Calcium chloride concentration and (b) Magnesium sulfate concentration. The viral yield was plotted against the concentration of the cations. the graphs show that calcium cation is the better propagator for bacteriophage EpC1 and EpT2, with very significant increases in viral yields at 5- AND 10-MM concentration.

Table 14. Phage progeny production in liquid medium containing different concentration of divalent cations.

$CaCl_2$ and $MgSO_4$ concentration, mM	Phage titer (pfu/ml)			
	EpC1	Standard Error	EpT2	Standard Error
2 mM $CaCl_2$	8.0×10^6	1.15×10^5	7.7×10^6	$\pm 10^5$
5 mM $CaCl_2$	5.1×10^7	1.73×10^6	1.2×10^7	$\pm 10^6$
10 mM $CaCl_2$	2.2×10^8	1.73×10^7	4.5×10^7	$\pm 10^6$
2 mM $MgSO_4$	9.0×10^6	3.53×10^5	6.0×10^5	$\pm 10^4$
5 mM $MgSO_4$	6.0×10^6	1.53×10^5	2.1×10^6	$\pm 10^5$
10 mM $MgSO_4$	3.5×10^7	2.89×10^6	6.8×10^6	$\pm 10^5$
Control (No $CaCl_2$ or $MgSO_4$)	5.0×10^6	1.15×10^5	1.3×10^6	$\pm 10^5$

4.2.3 High titer lysate preparation of bacteriophages

In order to further study the bacteriophages of the cold tolerant bacteria *EwS1* and *EwB2* it was necessary to improve their working stock titers. This can be achieved by incorporating a concentration stage during their isolation. Traditional concentration methods include ultracentrifugation, density centrifugation, or phase separation using polyethylene glycol (PEG). The titer of bacteriophage stocks prepared directly from agar plates ranged from 7 to 8 log PFU/ml. To improve this recovery, a PEG phase separation was employed where PEG 6000 (10 %) and NaCl (0.5 M) were added to the lysate followed by precipitation of the virions by centrifugation. This resulted in a 100-fold increase in the viral titer after the virions were resuspended in a buffer (Table 15).

Table 15. Comparison of bacteriophage titer before and after PEG6000 precipitation.

Phage	Phage titer (pfu/ml)	
	before precipitation	after precipitation
EpC1	2.34×10^8	4.12×10^{10}
EpC1	2.2×10^8	5.6×10^{10}
	3.59×10^7	1.73×10^9
EpT2 EpT2	3.7×10^7	6.0×10^9

4.2.4 Restriction profile analysis of the bacteriophages

Restriction analysis of EpC1 and EpT2 bacteriophages was carried out to determine whether they are different from each other. DNA was extracted from high titer viral stocks

(as described in chapter 2.7.10) and subjected to digestion with Bgl II. The restriction fragments were separated by agarose gel electrophoresis. The results presented on Figure 35 showed that the patterns of Bgl II restriction digestion profiles for EpC1 and EpT2 bacteriophages were different, indicating that these phages were distinctive isolates. Furthermore, restriction endonucleases cut only double-stranded DNA, consequently restriction digestion of EpC1 and EpT2 phage DNA by Bgl II confirm that both phages were dsDNA viruses.

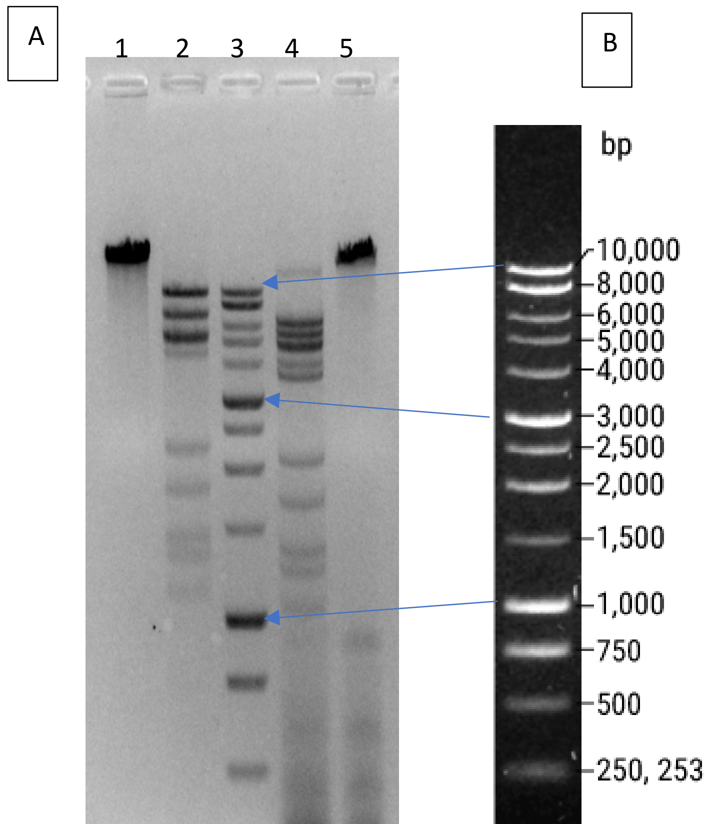

Figure 35. (A) An example of agarose gel electrophoresis of Bgl II digested bacteriophage DNA EpC1 and EpT2, (B) Promega provided image of DNA ladder band pattern. Lane 1: native DNA EpC1 before digestion, Lane 2: DNA EpC1 digested by Bgl II enzyme, Lane 3: 1 kb DNA-ladder (Promega), Lane 4: DNA EpT2 digested by Bgl II enzyme, Lane 5: native DNA EpT2 before digest.

4.2.5 Transmission Electron Microscopy

The morphology of the EpC1 phage was determined by transmission electron microscopy analysis (Figure 36) using 2 independently isolated stocks. Electron microscopy analysis of EpC1 showed an icosahedral head measuring 64 nm and a long, flexible, noncontractile tail that is 167 nm in length and 18 nm in width, similar to the average size of noncontractile tails (Appendix 8). No tail fibers were observed. The phage head is supposed to be filled with double stranded DNA based on DNA digest data (chapter 4.2.4). Based on the obtained TEM images this bacterial virus could belong to the *Siphoviridae* Family.

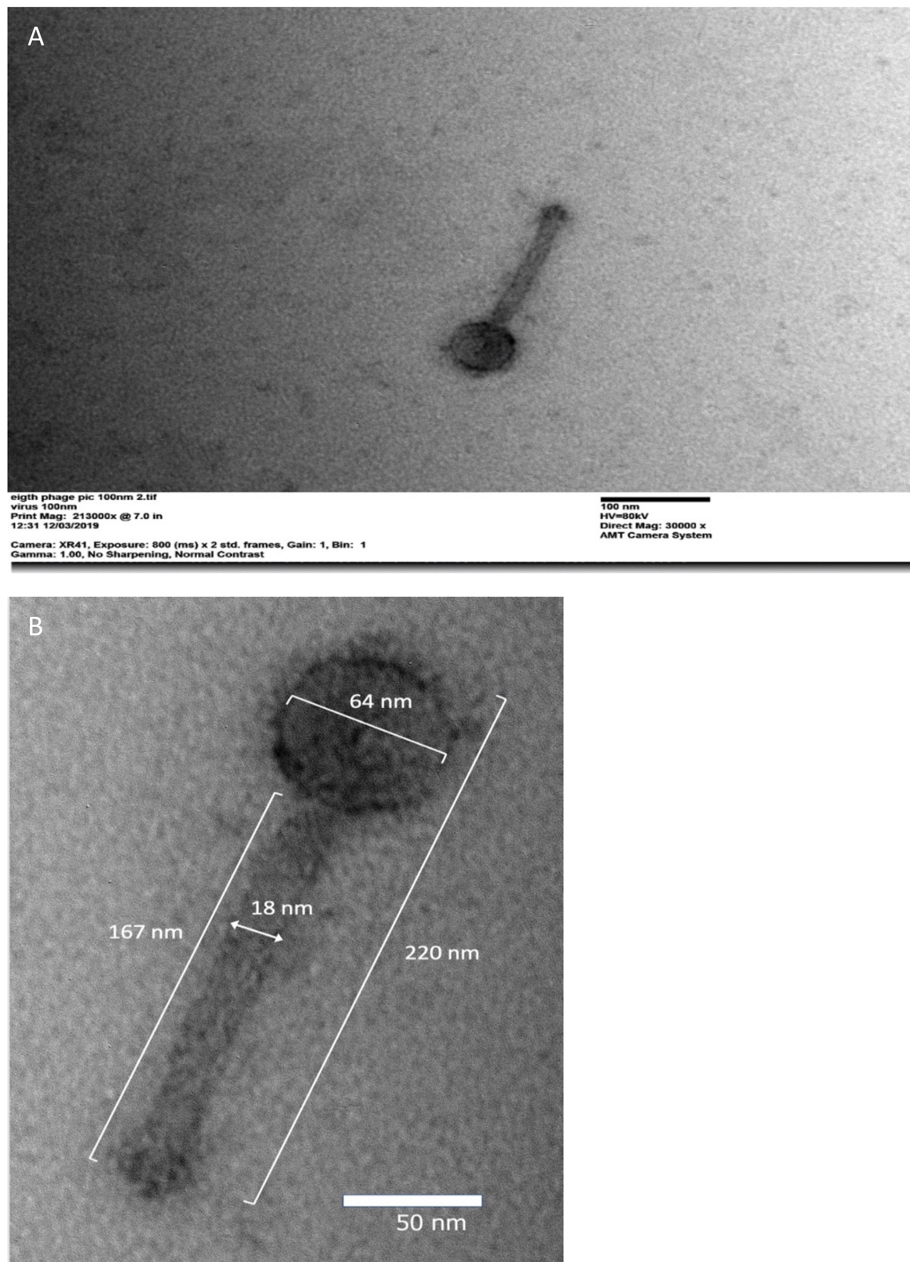

Figure 36. A. Transmission Electron Microscopy image of a bacteriophage, which exhibits an icosahedral head, long non-contractile tail. This morphology corresponds to the *Siphoviridae* Family. Bar, 100 nm. Magnification, x30000. B. Enlarged image of phage EpC1 showing its dimensions.

In summary, 12 bacteria were isolated from wastewater. Six of them carried genes responsible for antibiotic resistance (CTX-M and TEM genes). Two of the isolates (*EwS1* and *EwB2*) were characterised extensively and the results suggested that these bacteria were novel and belonging to a new genus of the *Enterobacteriaceae* family.

Five bacteriophages were also isolated from the wastewater. Two of these were strain specific to *EwS1* and *EwB2* and were found to be belonging to *Siphoviridae* family.

5.0 Discussion and conclusions

The present study describes the discovery of two novel cold tolerant environmental bacteria *EwS1* and *EwB2* containing ESBL genes (CTX-M and TEM) and virulence factors, thus posing potential environmental and human health risks. They belong to *Enterobacteriaceae* family and have close phylogenetic matches to *Ewingella americana* and *Rahnella* species. Also, 10 more different bacteria were isolated from wastewater sampled from the Southern water treatment site at Petersfield. Six isolates were identified as belonging to *E. coli* species and 4 belonging to Genus *Pseudomonas*.

These two novel cold tolerant bacteria (*EwS1* and *EwB2*) are environmental species that could be cohabitants with plants but have acquired multi-drug resistance to 5 out of 13 tested antibiotics. This is of interest as such bacteria can resist some treatments used in water treatment plants and serve as an exposure route to humans. ESBL genes in these bacteria are encoded on plasmid and can easily be transferred to other species that can be pathogenic to humans. It is known that genes associated with mobile elements facilitate their spread in the population and horizontal transfer to other organisms (Cornejova *et al*., 2015).

It was observed that CTX is the predominant gene compared with TEM (ratio 6:2) among all 12 isolated strains. SHV and OXA groups genes were also analysed but no gene presence was seen. There is a possibility that the primers used for identification of these genes did not perform adequately and therefore that this is a false negative result. However, it is more likely that no amplification was seen because these genes are rarely present, as literature suggests (Bonnet, 2003). The molecular biological and microbiological analyses of these two strains suggest that they constitute a new bacteria taxon that could not be assigned to any known genus.

It was considered necessary to classify the unknown bacteria because of its unique molecular, physiological, and biochemical characteristics. These bacteria which are mesophilic but could grow at low temperature looked to be a plant symbiont but contained several genes that allowed it to be pathogenic. It also had antibiotic resistance genes, inavsin genes, all which are associated with pathogens. This identification and characterization have become increasingly important because it was discovered in sewage treatment plant, where this treated effluent is expected to be free of microbes. This reveals that this effluent can serve as infection route to humans and water recycled to the environment containing such microbes can produce biofilms and survive on plants and

because the antibiotic resistance genes are encoded on plasmids, they can easily be transferred to other human pathogenic bacteria.

Identifying virulence genes from genome sequence information can be risky and should be accompanied by experimental work (Wassenaar and Gaastra, 2001). Considering this, it is difficult to conclude whether isolates *EwS1* and *EwB2* represent pathogens, capable of surviving temperatures above and below their growth optimum and producing thermoresistant proteins (chapter 3, Table 10), and they have acquired genes coding for functions that would enable them to survive in human tissue.

One of the great challenges for a bacterium colonising animal tissues is the acquisition of iron, which is essential for growth. The concentration of free iron in the human body is estimated to be 10^{-18} M, which is several orders below that required (10^{-6} M) for bacterial infection (Braun *et al*., 1998). In natural, pH-neutral environments iron is present in its ferric form as insoluble hydroxide or oxyhydroxide precipitates. Mammalian cells have high binding affinity glycoproteins, transferrin and lactoferrin, to help solubilise and deliver iron, which helps protect the cell from the toxic effects of iron overload. Over one half of the iron in human bodies is locked up in haemoglobin present in blood cells, with the rest being stored in the ferritins. It is unsurprising to find that infecting bacteria target these for their iron needs.

All bacteria need to have a number of strategies to handle iron, whether they are pathogens or not, in order to avoid the toxic effects of the metal (Frawley and Fang, 2014). The cell has to maintain a balance of iron within the cytoplasm so that it does not reach a level that can cause damage from the Fenton reaction. The expression and exportation of siderophores, and other iron import systems, allow iron to be acquired from the environment. Iron transported into the cell is incorporated into iron-sulfur cluster and mononuclear iron proteins as well as being sequestered by ferritin. The storage of iron in this way prevents its build up within the cytoplasm. Additional iron can be further sequestered by the DNA-binding protein Dps. When stressed, iron can be mobilised from these storage units and exported by iron efflux systems to balance the metal concentration within the cell. The genomes of isolates *EwS1* and *EwB2* have conserved genes that code for all of these functions (Chapter 3, Table 12; Figure 37), allowing survival in an external environment.

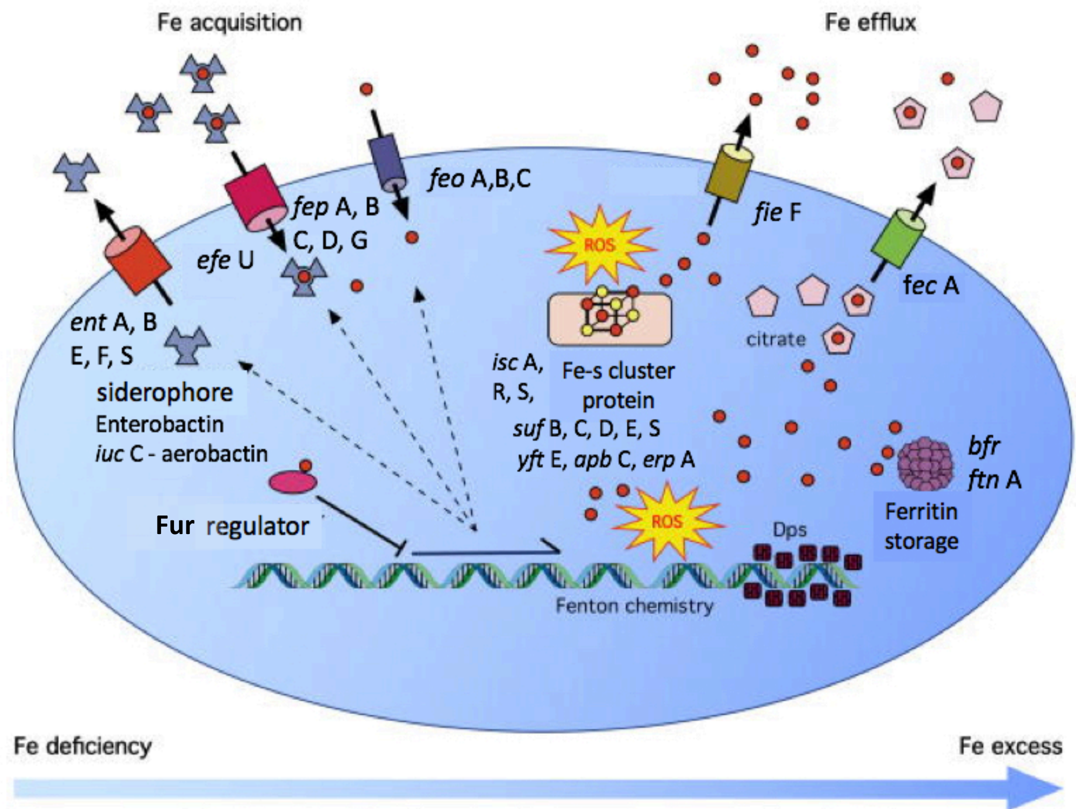

Figure 37. Iron transport in isolates *EwS1* and *EwB2*. When intracellular iron levels are low (left), cells express siderophores and other iron import systems to acquire iron. Under iron replete conditions, the iron-sensing transcriptional regulator Fur represses the expression of iron acquisition genes. Available iron is incorporated into iron-sulfur cluster and mononuclear iron proteins, and excess iron is sequestered by ferritin (right). Additional iron can be sequestered by the DNA-binding protein Dps. Under stress conditions in which iron is mobilized from proteins, iron exporters, predicted to be localized in the inner membrane, promote iron efflux to mitigate DNA damage from Fenton chemistry. Redrawn from Frawley and Fang (2014).

During infection, where iron is present at growth-inhibitory concentrations, bacteria have to be able to access iron from haemoglobin or iron proteins or ferritins. Siderophores such as enterobactin and aerobactin are capable of binding ferric ion with great efficiencies (Carrano and Raymond, 1979; Meyier, 1999), and aerobactin has been associated with urinary tract infections.

One of the most important virulence factors of pathogenic bacteria is the formation of biofilms. Biofilms are aggregations of cells or microbial communities attached to a surface and encased in an extracellular matrix mainly composed by extracellular polymeric substances (EPS) (Harper *et al*., 2014, Guo *et al*., 2014). Around 15 % of bacteria can survive in biofilms after treatment with chemicals, antibiotics or host immune system,

reproducing potentially harmful toxins after treatment. An example of bacteria surviving after treatment with chemicals was reported in Luna-Guevara *et al*., 2019 where they reported 531 cases of illness, including one death, related to the consumption of contaminated fruits and vegetables between 2008 and 2010. *EwS1* and *EwB2* have genes responsible for EPS production. Therefore, these bacteria can produce biofilms that can stick to growing plants even after treatment. Also, because these bacteria are cold tolerant and can produce biofilms and toxins, thus food kept in fridge can still be at risk of spreading infections, if not properly washed. This ability of bacteria to form biofilms represents a serious medical challenge. Therefore, there is a need to identify and develop therapeutic strategies to eradicate bacteria producing biofilms.

The curli fimbriae form the major proteinaceous part of the extracellular matrix, which may also comprise cellulose and/or polysaccharides to give the characteristic colony morphology. Curli are also important in the formation of biofilms, specifically allowing *Salmonella enteriditis* to adhere to Teflon and steel (Barnhart and Chapman, 2006).

Marginal evidence exists in *EwS1* and *EwB2* for the presence of type IV pili involved in twitching mobility (Mattick, 2002) with the presence of the genes *out* O (pre-pillin), *pil* Q (secretion protein for pili extension) and *pil* T (mobility protein). There is also some evidence to suggest that the genomes contain genes coding for a type II section system (Korotkov *et al*., 2012), important in the translocation of proteins across the periplasm in gram negative bacteria. It is unsurprising to find genes for these systems within these genomes as they are present in all members of the *Enterobacterales*, which may or may not be involved in tissue invasion.

Within the genomes of isolates *EwS1* and *EwB2* there are multiple copies of *lpf* A and *ipf* B, which code for the major fimbrial subunit and the chaperone but not genes lpf C (usher protein), D (minor subunit) or E (adhesin). This suggests that this operon has been introduced by horizontal transfer and lost many times during the evolution of these bacteria.

Other genes such as the *yra* H, I, J, K operons exported by the chaperone/usher pathway is implemented in the binding to environmental abiotic factors as well as improving bladder and epithelial cells attachment in the absence of the *flm* operon (Korea *et al*., 2010). Genes coding for either invasion (genes *inv* and *yee*J) or intimin (*eae*A), are commonly associated with members of the *Yersiniaceae* or the *Enterobacterales* (Isberg *et al.,* 2000; while internalin (*inl* A) is associated with *Listeria monocytogenes*, a gram-positive bacterium, infections (Braun and Cossart, 2000).

Pathogenic bacteria belonging to the *Yersiniaceae* or *Enterobacteriaceae* target specialised epithelial cells, known as M cells, that are located in lymphoid tissue known as Peyer's

patches. The function of M cells includes endocytic and phagocytic uptake of particles that are then transferred to intraepithelial pockets for the attention of T and B cells and macrophages. Pathogenic bacteria can use this uptake to gain access to the lymphoid system from where they can be distributed around the body (Palumbo and Wang, 2006). The three proteins present for tissue invasion attach to target cell by different mechanisms (Figure 38), although all three proteins promote the accumulation of actin beneath the attached cell that initiates phagocytosis.

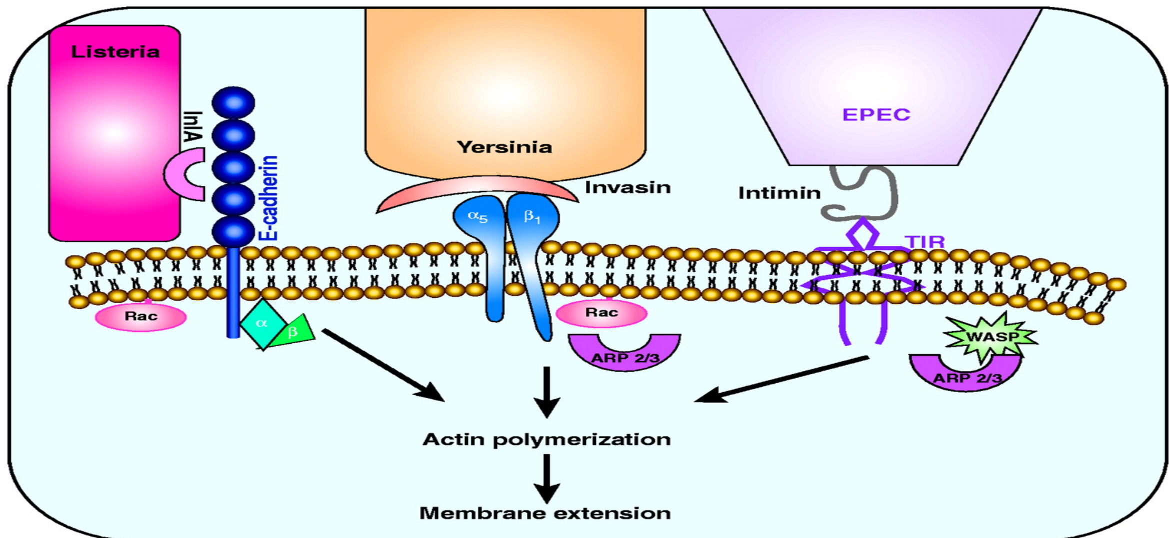

Figure 38. Various bacterial pathogens and their mechanisms for stimulating membrane protrusion. *Listeria monocytogenes* adheres to host cells via the binding of a bacterial surface protein, internalin A (IlnA) to E-cadherin. E-cadherin, via Rac activation, can trigger dynamic events of actin polymerization and membrane extension, culminating in bacterial uptake. *Yersinia expresses* invasin on its surface, which binds with high affinity toα 5β1 integrins. *Yersinia* uptake requires Rac1 and the Arp2/3 complex. EPEC attach to host cells through translocated intimin receptor (TIR), a receptor secreted by EPEC and inserted into the host cell plasma membrane where it acts as a receptor for intimin. WASP and the Arp2/3 complex are recruited to sites of attachment and stimulate actin polymerization required for pedestal formation (DeMali and Burridge, 2006).

Internalin is a bacterial surface protein that binds to E-cadherin on the surface of the M cell initiating actin polymerisation and phagocytosis via the zippering mechanism (DeMali and Burridge, 2006). Invasin is another bacterial surface protein that has a high binding affinity to β1 integrins (Isberg *et al*., 2000), where uptake of the cell requires the host protein RAC1 and the ARP2/3 complex (Figure 38). In contrast, intimin is an adhesin protein that binds to a receptor (translocated intimin receptor, TIR) on the host membrane that has been inserted by the bacterial cell after it has been expressed and translocated (Figure 37). The binding of intimin at the receptor stimulates actin reorganisation to form a pedestal structure that can extend up to 10 μm beneath the pathogen (Goosney *et al*., 2001).

Interestingly, in *Escherichia coli* the expression of genes for sulfonate metabolism, is depressed in the absence of sulfate or cysteine, and the deletion of *tau* A, B or C genes results in the cell losing the ability to grow on minimal media supplemented with taurine, but not other sulfonates (Eichhorn *et al*., 2000). Taurine is a major constituent of bile and it is present in the large intestine, amongst other organs, and accounts for up to 0.1% of total human body weight (Schuller-Levis and Park, 2003). It is, therefore, a potential source of carbon, nitrogen and sulfur for microbes (Cook and Denger, 2006) infecting animal bodies. The presence of the *tau* A, B, C and D genes in *EwS1* and *EwB2*, however, does not necessarily reflect that the organism has a pathogenic life strategy. Taurine is a common molecule released into the environment by mesozooplankton (Webb and Johannes, 1967) and algae (Tevatia *et al*., 2015), and it is a major source of carbon and energy in marine environments (Clifford *et al*., 2019). This would be unsurprising given the linkage of *EwS1* and *EwB2* to the genus *Rahnella*, which contains known symbionts of plants that have the ability to fix atmospheric nitrogen, a diagnostic feature of the genus. *Rahnella aquatilis* has a 38-kDa major outer membrane protein that functions as a root adhesin and porin (Achouak *et al*., 1998). This protein shares a high sequence similarity with those (*omp*C and *omp*F) from gram-negative pathogens, leading to the suggestion that this adhesin may mediate adhesion to epithelial cells during bacteria invasion.

Given that *EwS1* and *EwB2* have several virulence genes, these bacteria could potentially be risky to human health. Moreover, the presence of ESBLs and multi-drug resistant genes in *EwS1* and *EwB2,* which can be easily transferred to different pathogenic species, is another source of danger to humans. Importantly, the fact that the EpC1 and EpT2 viruses were found in sewage suggests that the quantity of the discovered *EwS1* and *EwB2* bacteria live there is enough for populations of viruses to build up. These newly identified phages could be used for treatment or for adjuvant treatment of infection caused by *EwS1* and *EwB2* ESBL producing multi-drug resistant bacteria. Therefore, these bacteriophages specific to *EwS1* and *EwB2* were characterized further.

The diversity of bacteriophages in wastewater is great and this environment is a rich source for discovering novel viruses (Jurczak-Kurek *et al*., 2016). In this study, 5 different bacteriophages were isolated from wastewater samples. Two isolates infected *Pseudomonas* species, one infected *Escherichia coli*, and two infected a cold tolerant bacterial strain that had been isolated from the same wastewater treatment plant. These bacteria belong within the *Enterobacteriaceae* (see Chapter 1) but are novel with their closest phylogenetic matches to *Ewingella americana* and *Rahnella* species. These bacteria are primarily environmental species interacting with plants; however, they can be pathogenic to humans and they have acquired multi-drug resistance. There are no reports

of viruses infecting these bacteria, but the DSMZ microbiology culture collection offers phages that infect *Rahella variigena* (DSM 108139) and *Ewingella americ*ana (DSM 4580). Both of these phages, *Rahnella* phage vB RvaM-O238 (DSM 108187) and *Ewingella* phage PT-JD18 (DSM 27304), belong to the *Mycoviridae*.

These identified phages EpC1 and EpT2 are novel viruses that specifically infect novel cold tolerant bacteria. Appendix 8 lists 52 genera belonging to the *Siphoviridae*, describing the basic characteristics such as their morphology, genome size and main host. The morphology of phage EpC1 falls within the range for head and tail dimensions observed in Appendix 8, these phages are distinctive isolates as shown by different restriction digestion patterns. But, for these genera, there is no exact match, particularly for a host. Care should be taken here, however. The genera listed represent only about 20% of those described for the *Siphoviridae*, which currently total 265 genera. Furthermore, the majority of these genera comprise a single or two species, with only 36 that have been classified into higher orders and 9 that contain more than 10 species (https://talk.ictvonline.org/taxonomy/). The novelty of phages EpC1 and EpT2 lies in their limited host range, and the observation that these are the only described species likely to belong to the *Siphoviridiae* that infect bacteria belonging to the *Ewingella* & *Rahnella* group.

These bacteriophages can be used in controlling bacterial plant pathogens. The main determining factor whether a phage can be appropriate for biocontrol is whether a phage is exclusively lytic (virulent) or temperate in nature. Preferably a phage for biocontrol applications should be exclusively lytic and possess a host range which allows productive infection on all strains of the pathogen genus/species being targeted (Buttimer *et al*., 2017). The current opinion is that phages should produce high numbers of new phages quickly while lysing the host cell and diffusing easily though the environment to which they are being applied. Although in invitro studies, a phage may show great potential for phage infection, this is not always translated into biocontrol potential in the field (Buttimer *et al*., 2017). An example is one study by Balogh (2006) where 2 out of 3 phages of *X. citri* pv *citri* showing lytic activity invitro were unable to lyse their host bacterium on grapefruit leaves. In deciding which phage will be applicable for biocontrol, care should be taken to the receptors that a given phage recognizes on a bacterial target as this can assist in the making of phage cocktails with a reduced possibility of bacterial resistance. The individual members of this phage cocktail can work in synergy to eliminate the target bacterium (Frampton *et al*., 2014; Born *et al*., 2011).

However, with temperate phages, the phage genome replicates as part of the bacterial genome of its host until a trigger switches it into the lytic cycle. These triggers can be

chemical or physical (UV light or heat) in nature (Buttimer *et al*., 2017; Muller *et al*., 2012). Sato (1983) also suggested that certain plant extracts can also trigger these events. In some cases, the presence of some genes on phage genomes can increase the fitness of their bacterial host. One example was reported by Evans *et al*., 2010 where the *P. atrosepticum* prophages ECA41 and ECA29 both improved the motility of their bacterial host. However, there are concerns with using temperate phages because these prophages can also harbor genes for toxins, examples are shiga, and cholera toxins Another concern is in the spread of virulence genes by transduction, where these phages can remove themselves from their host genomes incorporating host DNA into their own genomes facilitating horizontal transfer of genetic material among bacteria (Abedon and Lejeune, 2005; Griffiths *et al*., 2000).

Bacteriophages have evolved various virion-associated carbohydrate active enzymes, termed EPS depolymerases, that recognize, bind, and degrade the EPS to gain access to bacterial cell surface receptors. The clear phages appeared to be typical of virulent phages, while turbid phages may be temperate or a virulent phage that can produce plaque halos by the enzymatic activity of a phage-encoded EPS depolymerase. Such phages can produce cloudy clearings not as a direct consequence of phage population growth, but by extra-virion diffusion along with virion-free enzymatic degradation of bacteria. These enzymes do not degrade the whole bacterium but instead just exterior layers that are bacterium produced and associated (Abedon, 2011). According to Drulis-Kawa *et al*., 2015 phage depolymerases appear in two forms: (i) as integral components of the phage particles or (ii) as soluble enzymes generated during host cell lysis. Association with virion particles allows for EPS depolymerase utility during the adsorption process, that is, as moving towards bacteria, whereas soluble enzymes are useful particularly in the moving away from bacteria. The depolymerase activity is commonly identified by a constantly increasing halo surrounding the phage plaques (Pires *et al*., 2016). Plaques produced by EPS depolymerase can be turbid. EpT2 was observed to have similarities to plaques produced as a result of EPS depolymerase activity. A mixture of bacteriophages can be used on the novel bacteria, where one phage encoding EPS depolymerases destroys the biofilm produced by bacteria while the other with lytic characteristics attacks and lyses the bacteria.

Divalent cations are needed for bacteriophage growth of EpC1 and EpT2. They are required for viron stability, for phage adsorption and, for viral genome penetration (Marks and Sharp, 2000; Karnik and Gopinathan, 1980), as well as for intracellular growth. The presence of the optimal concentration of divalent cations in adsorption buffers and media will determine the successful handling of the virus. Calcium and, to a lesser extent, magnesium are the cations universally required by phage for their growth (Jończyk *et al*., 2011, Kutter and

Goldman, 2008), and it was found that increased concentrations of these cations are indeed required for the peak phage propagation. Typical concentration of calcium in sewage is 20 to 120 mg/L. The concentration at any one tank will vary, particularly with pH, but it will also vary within a tank depending on the level of electrochemical phosphorus recovery. To reconcile the shortage of mined phosphorus and the overabundance of it in wastewater, the phosphorus is removed and reused by formation of amorphous calcium phosphate (Lei *et al*., 2018).

Generation of progeny for EpC1 and EpT2 bacteriophages at low temperatures could be of particular applicability, for example in wastewater treatment. Host specificity is central to selection of suitable phages for wastewater treatment applications. Success would depend on accurate identification of problem, effective isolation and unbiased enrichment of phage and ability of phage to penetrate and remain infective in *in situ* condition (Periasamy and Sundaram, 2013). Biological hazard in water resources in the form of pathogenic organisms are responsible for major outbreak in most of the developing countries. Therefore, every effort leading to reduction in sewage pollution and pathogenic microbes must be promoted and implemented. This will not only safeguard the interest of the people but also help to maintain healthy and sustainable environment. Entry of antibiotic resistant pathogens into the sewage is inevitable as survival is the key for existence. Development of multidrug resistant bacteria and exit of many antibiotic companies necessitates to search for novel approaches to tackle the multidrug resistant bacteria. Phage therapy is an alternate to overcome these menacing organisms.

Furthermore, foodborne illnesses remain a major cause of hospitalization and death worldwide despite many advances in food sanitation techniques and pathogen surveillance. Such was the case as British scientist discovered that bagged salad could fuel the growth of food-poisoning bugs like salmonella and make them more dangerous (Moye *et al*., 2018). Traditional antimicrobial methods, such as pasteurization and autoclaving processing are capable of reducing microbial populations in foods to varying degrees, but they also have negative impacts by reducing nutritional value of foods, killing many beneficial bacteria that are naturally present in foods. So, the interest for natural antimicrobial compounds has increased also due to alterations in consumer attitudes towards the use of chemical preservatives in foodstuff and food processing surfaces (Moye *et al*., 2018). Bacteriophages fit in the class of natural antimicrobials and their effectiveness in controlling bacterial pathogens in agro-food industry has led to the development of different phage products already approved by USFDA and USDA (Sarhan and Azzazy, 2014). Treatment with specific phages in the food industry (Leverentz *et al*., 2003, Bren, 2007) can prevent the decay of products and the spread of bacterial diseases and ultimately promote safe

environments in animal and plant food production, processing, and handling (Sillankorva *et al.*, 2012). Work presented in this thesis makes novel contribution to this endeavour as it potentially offers new tools for environmentally safer treatment to overcome the alarming threat of antibiotic resistant bacteria.

Although significant findings were produced, limitations within this study must be considered. The first is the average utilization (average well colour development) of the carbon sources in the plates was not obtained using optical density as a measure. The colour development estimates in this study were based on perspective/observational study and images were sent to the BIOLOG company for interpretation. This test was done with more emphasis in trying to identify the bacteria isolated than profile the physiological characteristics of the isolate. The second limitation is that the gel image of the restriction digest showed DNA partial digest. Despite this image, the DNA structure of these two bacteriophages appeared different, suggesting they are different but for final identification, sequence analysis should be done. The third limitation concerns time constraint and mechanical failure of the machine as the TEM imaging of the second bacteriophage could not be achieved. Also, the novelty of the bacteria and bacteriophages made the designing of protocols more complicated, as model protocols had to be slightly adjusted to accommodate the characteristics of the isolated bacteria and bacteriophages.

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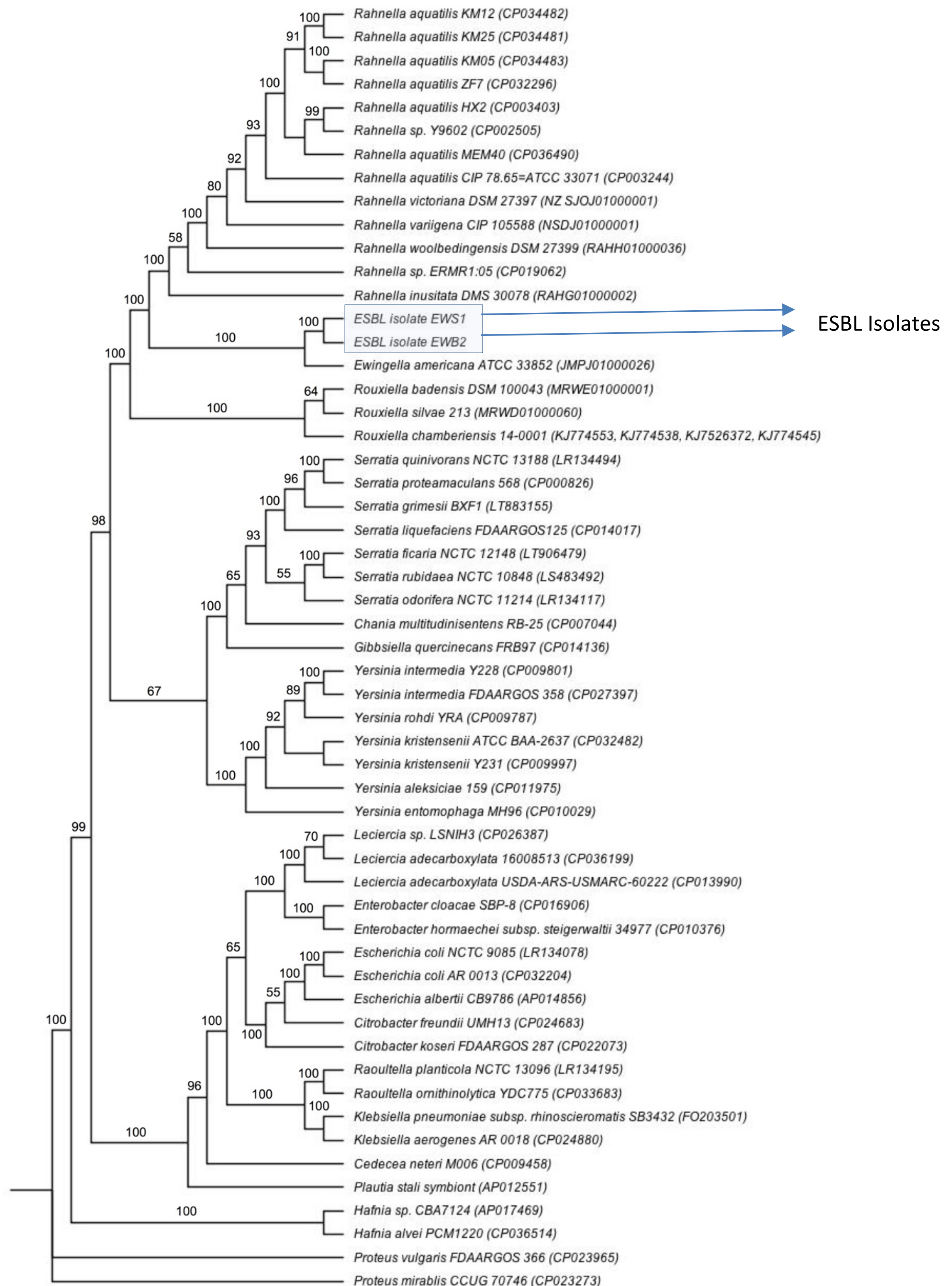
Appendices

Appendix 1. Four-gene phylogeny (sucA, pyrG, rpoB and rplB). 4 rate classes. showing the relationship between the ESBL isolates *EwS1* and *EwB2* (boxed) within the *Yersiniaceae*. Maximum likelihood analysis of 9394 nuelotide sites using model TN93 with 4 rate classes generated 3303 patterns, 59.52% of which were monomorphic. Using estimated values of Ts/ts ratio = 5.384303, Alpha = 0.871231, maximal likelihood analysis using nearest neighbour interchange produced a tree ln(L) = -86239.2. P-inv= 0.517210. Bootstrap values above 50% are shown above the branches. the ESBL isolates are the ones boxed**.**



Appendix 2. Four-gene phylogeny (sucA, pyrG, rpoB and rplB) showing the relationship between isolates *EwS1* and *EwB2* within the *Enterobacterales*. Maximum likelihood using GTR model and non-parametric bootstrap analyses produced a tree of L (ln) -96031.597630, with alpha = 0.885352, P-inv = 0.522864. Bootstrap values are shown above the branches and sequence accession numbers are shown in brackets after the OTU names.

Appendix 3. Percentage sequence similarity for the genes fusA, sucA, pyrG, rplB and rpoB of the isolates *EwS1* and *EwB2* with *Rahnella* species.

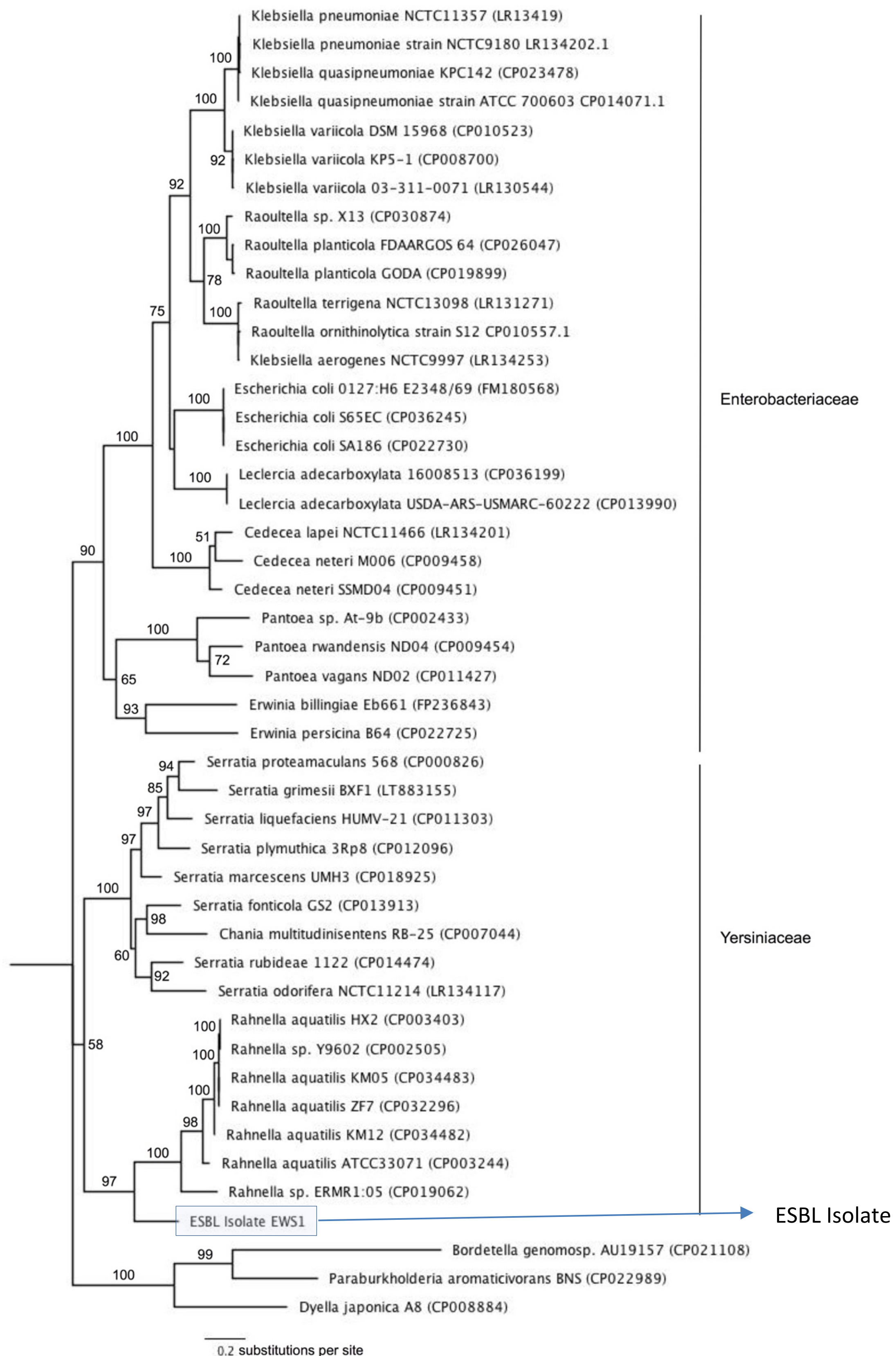
Gene	fusA		sucA		pyrG		rplB		rpoB	
Organism	*EwS1*	*EwB2*	*EwS1*	*EwB2*	*EwS1*	*EwB2*	*EwS1*	*EwB2*	*EwS1*	*EwB2*
Rahnella sp. ERMR1:05 (CP019062)	85.01	95.37	88.81	89.03	89.33	89.44	96.12	96.12	94.99	94.99
Rahnella aquatilis ATCC 33071 (CP003244)	85.55	93.24	88.49	88.71	90.05	90.05	96.24	96.24	94.09	94.09
*Rahnella aquatil*is MEM40 (CP036490)	84.79	93.10	88.24	88.46	90.48	90.48	96.24	96.24	93.89	93.89
Rahnella aquatilis HX2 (CP003403)	84.78	93.05	88.49	88.71	90.54	90.54	96.24	96.24	93.89	93.89

Appendix 4. Iron acquisition, chemotaxis, antibiotic resistance genes of isolates *EwS1* and *EwB2*

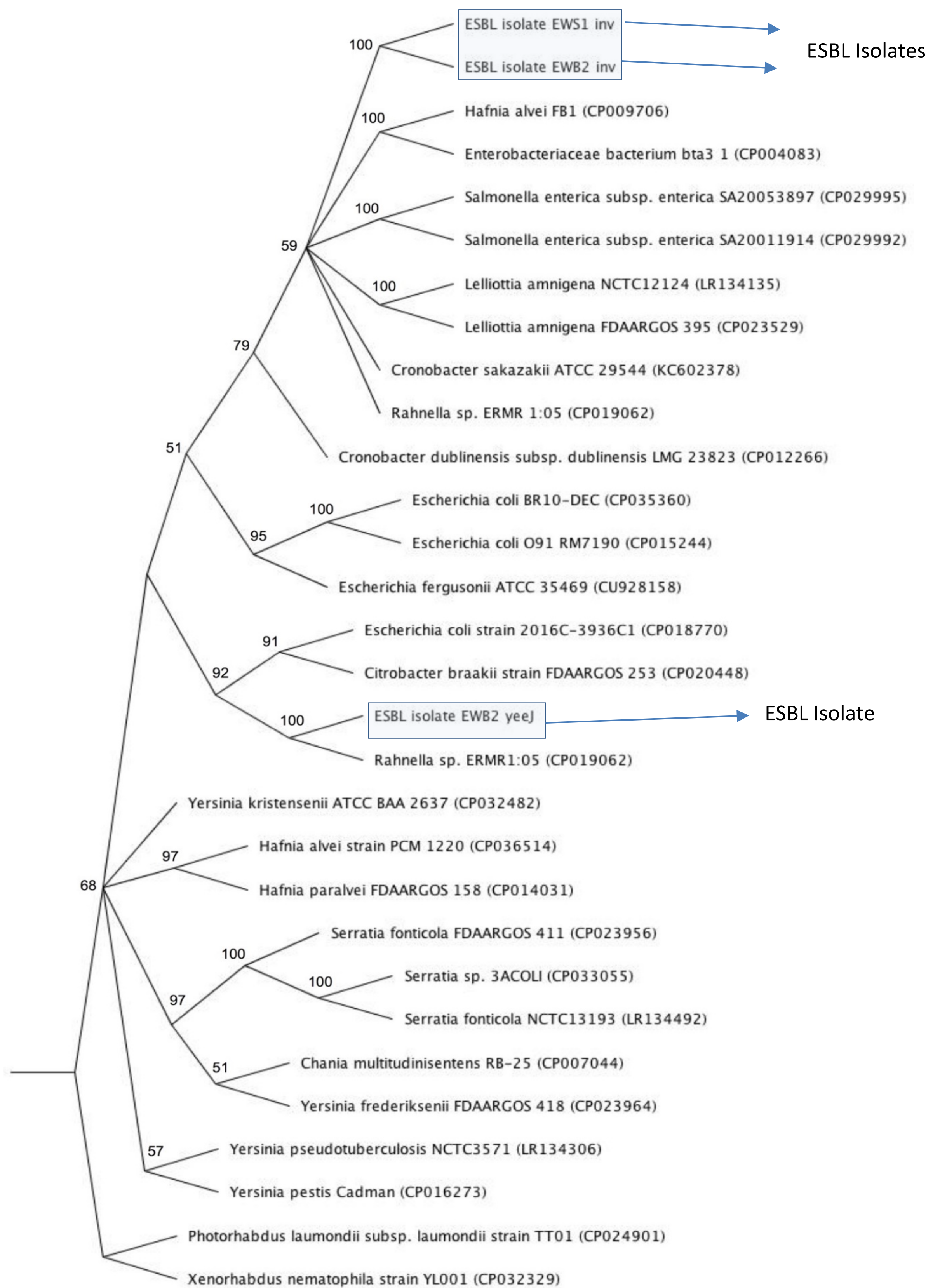
Activity	Gene	Function	Activity	Gene	Function
Iron acquisition	fbpA	Fe(3+) binding periplasmic	heavy metal	terZ	tellurium resistance
	fbpB	Fe(3+) transport permease		terA	
	fbpC	Fe(3+) ion import		terB	
	entF	enterobactin synthase		terC	
	entS	enterobactin exporter		arsH	arsenic resistance
	entE	Enterobactin synthase		tehB	
	entB	isochorismatase	Antibiotic resistance	macA	
	entC	isochrismate synthase			
Virulence	srfB	Virulence protein		mdtA	Multidrug transporter. confers resistance against novobiocin and deoxycholate. MdtABC requires TolC for its function
		virulence effector protein		acrD	aminoglycoside efflux pump
		virulence factor		mmr	methylneomycin resistance
	yeeJ	invasion		bla	Beta-lactamase CTX-M-2
	sprA	virulence protein		tcmA	tetracenomycin resistance protein
	sprB	mono ADP-ribosyltransferase		bla	
	shlA	hemolysin		bla TEM	Beta-lactamase TAM
	hpmB	hemolysin transporter		smvA	viologen resistance
	fepA	hemoglobin binding	Chemotaxis	trg	methyl accpeting chemotaxis protein III
	fhaB	filamentous hemagglutin		tcp	methyl accepting chemotaxis citrate transducer
	fhaC	filamentous hemagglutin transporter protein	Secretion	clp VI	VI protein
	hrtE	OMP usher protein		icmF	type VI secretion protein
	fimB	chaperone protein		datL	typw IV/VI secretion system
	ipfA	long polar fimbria protein A		impA	type VI secretion associated protein
	ipfB	chaperone		vgr	type vi secretion system
	ipfC	outer membrane protein			
	fimG			pqiA	paraquate-inducible protein A
	ydeR	fimbrial subunit		pqiB	paraquate-inducible protein B

Appendix 5. Virulence factors, toxin genes and drug resistance genes of isolates *EwS1* and *EwB2*

Toxins and virulence factors		Drug resistance	
int	Intimin	*emr*K	erythromycin
inv	Invasin	*emr*B	multidrug export protein
*cbt*A	cytoskeleton-binding toxin	*mfs*	spectomycin/tetracycline
RTX-1	RTX toxin	*mdt*L	chloramphenicol
antigen LP4	O antigen	*bcr*	bicyclomycin
*fli*C	H antigen: flagella component	*mdt*A	
Haemolysin C	haemolysin C	*stp*	
*p*IIT	twitching mobility protein	*mda*B	Adriamycin and etoposide
*ipf*A	Major fimbriae subunit	*mdt*H	
*ipf*B	Major fimbriae chaperone	*bmr*3	
*hlg*A	Class-S component: AB toxin	*mrc*A	penicillin binding protein
*hlg*B	γ-hemolysin: AB toxin	*mdt*G	forfomycin, deoxycholate
*p*IIQ	Type IV pilus biogenesis	*stt*H	streptothricin
*shl*B	outer membrane protein for shlA (haemolysin) export	*yhe*I	ABC transporter
*hum*T	hemin-binding periplasmic protein	*arc*A	multidrug efflux pump
hha	toxicity modulator protein	*arc*B	
*vgr*G1	actin crosslinking toxin	*mac*A	macrolide export protein
*vir*S		*toho*1	beta-lactamase
*cyo*E	protoheme IX famesyltransferase	acrD	aminoglycoside/multidrug transporter subunit
*tom*B	Attenuates Hha toxicity and regulates biofilm formation		
*yrf*K			
*yrf*J			
*yrf*I			
*yrf*H	Heat shock protein 15		colicin V
loc 311110	taurine binding protein	*cml*	colicin M
*cafl*A	F1 capsule anchoring protein	*mcc*F	microcin C7
csgA	major curlin subunit		barstar
CsgB	curlin minor subunit	*rat*A	ribosome association toxin
csg C	aggregative fimbriae protein	srpA	srpABC pump
ShlB/FhaC/HecB	hemolysin secretion/activation protein	yeeV	
cdi A1	adhesin/hemagglutinin/hemolysin	yeeU	
cdi A2	adhesin/hemagglutinin/hemolysin		
CsgG	Curli production assembly/transport component		
CsgF	Curli production assembly/transport component		
CsgE	curli assembly protein		
CtsH1	hemolysin coregulated protein 1		
ivy	lysozyme inhibitor		
*ykf*I	Toxin YKFI		



Appendix 6. Maximum likelihood phylogenetic tree showing the relationship of the invasin gene sequences (Inv) from isolate *EwS1* (Boxed) with those from other members of the *Enterobacterales* and *Yersiniaceae*. The maximum likelihood model used to construct the tree was GTR+I+G with an estimated P-INV value of 0.057042, and this generated a tree of -ln L = 11923.56370 from 687 distinct data patterns.



Appendix 7. Parsimony consensus tree of invasin sequences from ESBL isolates *EwS1* and *EwB2* (Boxed). Tree lenth – 3916, CI = 0.539, RI = 0.562, RC = 0.303, HI = 0.461

Datset contained 2320 sites, 934 of which were PI.

Heuristic search of a dataset containing 30 OTU generated one tree of length 3756, and 2 tree islands. CI = 0.562. RI = 0.601, RC = 0.338, HI = 0.438.

Maximum Likelihood

Model: GTR + I + G

Gamma: 4 categories

Distinct number of patterns: 1028

-In L = 16047.477

Base frequencies

A	0.268851
C	0.225373
G	0.266597
T	0.239180

Rate matrix R:

AC	2.05445
AG	3.62070
AT	0.84593
CG	2.27538
CT	7.22025
GT	1.00000
Shape	0.951152
P-inv	0.079318

Appendix 8. Properties of some members in the *Siphoviridae* family.

Genus	Head	T	Tail	fibres	Genome	Host	Example
Amigovirus	approx. 50nm		approx. 200 nm	No	59 kbp	Arthrobacter	Amigo
Anatolevirus	50 nm diam		160 – 190 nm		35 kbp	Propionibacterium	Anatole
Arquatrovirinae/Camvirus	60 nm diam		230 nm		49 kbp	Streptomyces	Amelia
Arquatrovirinae/Likavirus					49.5 kbp	Streptomyces	Aaronocolus
Attisvirus					47.8 kbp	Gordonia	Attis
Triavirus	100 x 45 nm		370 nm	Yes	43 kpb	Staphylococcus	Phage 3a
Biseptimavirus	60 nm diam		250 nm	No	41 kpb	Staphylococcus	Phage 77
Andromedavirus	55 nm diam		115 x 21 nm	No	49 kbp	Bacillus	Andromeda
Barnyardvirus	60 nm diam		360 nm	terminal	69-71 kbp	Mycobacterium	Barnyard
Bignuzvirus	80 nm		350 nm	No	69-71 kbp	Mycobacterium	bignuz
Bronvirus	80 nm		350 nm	No	69-71 kbp	Mycobacterium	bron
Ceduovirus	56 x 41 nm	T=4	86 – 111 nm	Short	22 kbp	Lactococcus	Phage c2
Cequinquevirus	60 nm diam		120 nm, large base	No	31 kbp	Lactobacillus	Phage c5
Charlievirus	65 x 50 nm		150 nm	No	43 kbp	Mycobacterium	charlie
Cheoctovirus	60 nm		120 nm, large base	No	56-58kbp	Mycobacterium	Che8
Chenonavirus	150 x 50 nm		250 nm	No	47-57 kbp	Mycobacterium	Che9c
Chivirus	66 nm diam		220-230 nm x 13 nm	200-220 nm	59 kbp	Salmonella	Chi
Kostyavirus	60 nm diam		170 nm	No	74-76 kbp	Mycobacterium	CJW1
Corndogvirus	173 x 43 nm		258 nm	No	70 kbp	Mycobacterium	corndog
Casadabanvirus	40 nm diam		190 nm	Yes?	37 kbp	Pseudomonas	D3112
Detrevirus	55 nm diam		113 x 7 nm	6 with knob	98 kbp	Pseudomonas	D3
Liefievirus	70 nm diam		220 nm	No	42 kbp	Mycobacterium	Phage halo
Dhillonvirus	60 nm diam		140 nm		40-45 kbp	Escherichia	HK578
Cecivirus	55 nm diam		150 nm transverse tail	discs	53 kbp	Bacillus	IEBH
Jerseyvirus	62-64 nm diam		116-120 nm spikes on	Base 20 nm	40-43 kbp	Salmonella	jersey
Fromanvirus	60 nm diam		135 x 8 nm	Short fibre	52 kbp	Mycobacterium	L5
Lambdavirus	60 nm diam	T=7	120-130 nm	Yes	48 kbp	Escherichia	Lambda

Genus	Head	T	Tail	Fibres	Genome	Hosts	Example
Ravinvirus	60 nm diam		140 x 8 nm	Yes, short	46 kbp	Enterobacter	N15
Omegavirus	100 nm diam		230 nm	No	106-112 kbp	Mycobacterium	omega
Oshimavirus	100 nm diam	T=7	1000 nm	No	84 kbp	Thermus	P2345
PBI-like	60 nm		360 nm	single	64 kbp	Mycobacterium	PBI1
Pegunavirus	60 nm diam		360 nm	single	67-71 kbp	Mycobacterium	Pg1
Lomovskayavirus	53 nm		100 5 nm; 15nm base	4 tails	43 kbp	Streptomyces	phiC31
Phicbkvirus	205x60 nm		350 nm	No	205-13 kbp TR	Caulobacter	phicbk
Stanholtvirus	63 nm diam		203 x 8 nm	No	48-53 kbp	Burkholderia	phie125
Phietavirus	50 nm diam		175 nm	No	43 kbp	Staphylococcus	phieta
Phifelvirus					36-40 kbp	Enterobacter	Phi FL1
Coetzeevirus	59 nm diam		182 x 11 nm	Spikes	37 kbp	Lactobacillus	Phi JL1
Psimunavirus	55 nm diam		210x10 nm term knob	Spike/fib	30 kbp	Methanobacteriu m	Phi M1
Reyvirus	75 nm diam		300 nm	No	83 kbp	Mycobacterium	rey
Saphexavirus	120x45 nm		160 nm	No	54-59 kbp	Enterococcus	VD13
Brussowvirus	100 nm		400 nm	No	40 kbp	Streptococcus	Sfi 11
Moineauvirus	60 nm diam		260x8 nm		35 kbp	Streptococcus	DT1
Skunavirus	45 nm diam		120 nm	No	28 kbp	Lactococcus	SK1
Spbetavirus	81 nm diam		335x10 nm	6 tail	134 kbp	Bacillus	SPbeta
Tequintavirus	90 nm diam	T=13	160x11nm – 50 nm fiber	3 tail (120n	121 kbp	Enterobacteria	T5
Timquatrovirus	60 nm diam		600 nm	No	52-62 kbp	Mycobacterium	TM4
Lwoffvirus	58 nm diam		145 x 11 nm	single	37 kbp	Bacillus	TP21-L
Tunavirus	60 nm diam		151 x 8 nm	4 tail	50 kbp	Enterobacteria	T1
Wbetavirus	50 nm diam		200 nm	Cent fibre	36-40 kbp	Bacillus	Wbeta
Xipdecavirus	53 nm diam		173 nm	2-6 tail	44 kbp	Xanthomonas	Xp10
Yuavirus	72x51 nm		145 nm	4 tail	60 kbp	Pseudomonas	YuA

FORM UPR16

Research Ethics Review Checklist

Please include this completed form as an appendix to your thesis (see the Research Degrees Operational Handbook for more information

Postgraduate Research Student (PGRS) Information			**Student ID:**	714651
PGRS Name:	Nanpon Miri			
Department:	PHBM	**First Supervisor:**	Prof. Darek Gorecki	
Start Date: (or progression date for Prof Doc students)		01/10/2016		
Study Mode and Route:	Part-time ☐ Full-time ☐	MPhil ☐ PhD ☒	MD ☐ Professional Doctorate ☐	

Title of Thesis:	Discovery of the novel Extended Spectrum Beta-Lactamase (ESBL) producing psychrotrophic bacteria and their bacteriophages
Thesis Word Count: (excluding ancillary data)	30534

If you are unsure about any of the following, please contact the local representative on your Faculty Ethics Committee for advice. Please note that it is your responsibility to follow the University's Ethics Policy and any relevant University, academic or professional guidelines in the conduct of your study

Although the Ethics Committee may have given your study a favourable opinion, the final responsibility for the ethical conduct of this work lies with the researcher(s).

UKRIO Finished Research Checklist: (If you would like to know more about the checklist, please see your Faculty or Departmental Ethics Committee rep or see the online version of the full checklist at: http://www.ukrio.org/what-we-do/code-of-practice-for-research/)	
a) Have all of your research and findings been reported accurately, honestly and within a reasonable time frame?	YES ☒ NO ☐
b) Have all contributions to knowledge been acknowledged?	YES ☒ NO ☐
c) Have you complied with all agreements relating to intellectual property, publication and authorship?	YES ☒ NO ☐
d) Has your research data been retained in a secure and accessible form and will it remain so for the required duration?	YES ☒ NO ☐
e) Does your research comply with all legal, ethical, and contractual requirements?	YES ☒ NO ☐

Candidate Statement:	
I have considered the ethical dimensions of the above named research project, and have successfully obtained the necessary ethical approval(s)	
Ethical review number(s) from Faculty Ethics Committee (or from NRES/SCREC):	ETHICS-10165
If you have *not* submitted your work for ethical review, and/or you have answered 'No' to one or more of questions a) to e), please explain below why this is so:	
Signed ***(PGRS):*** [signature]	**Date:** 24/09/2020

Appendix 10. Completed UPR16 form