Norwegian University
of Life Sciences

Master's Thesis 2017 60 ECTS
Faculty of Chemistry, Biotechnology and Food Science

Shiga toxin-producing *E. coli* O26 – transduction of Stx bacteriophages

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Acknowledgments

This thesis has been written as part of the Master’s Degree Programme in Biotechnology (Microbiology as programme variant) at NMBU. It was carried out at the section for Food Safety and Emerging Threats in the department of Animal Health and Food Safety at the Norwegian Veterinary Institute (NVI). This thesis has been funded by the Research Council of Norway through the project “Pathogens in the food chain – persistence, elimination and risk management”.

I would like to thank my supervisors at NVI. I wish to express my deepest thanks and sincere appreciation to Camilla Sekse for her support and mentorship. Truly, no better motivation exists than the enthusiasm you spread. Who knew interest in phages could be so contagious! My gratitude also goes to Gro Johannessen and Roger Simm for their guidance. The three of you have been sources of endless knowledge and constant inspiration to this thesis.

I would like to extend my thanks to my family and friends. To mom & dad, thank you for your boundless encouragement and support. To my big sisters, Anja, Beth & Cora, thank you for not using up the “smart genes” so some (most, actually) was left for me. To momo, I wish you were here so I could share this journey with you. To my friends, thanks for sticking with me! I look forward to seeing you again soon.

Finally, I wish to thank my significant other. To Frank, I am sincerely grateful for your patience and understanding. Thank you for making our meals, cleaning the house, shopping for groceries (and whatever else we have needed), and all the other things you have single-handedly taken care of in this period. And thank you for always being positive and optimistic, which you manage even when I am the opposite. In short, thank you for your unconditional support in this journey. I am lucky I get to spend my days with you.

Skedsmokorset, May 2017

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Sammendrag

Shigatoxin-produserende *Escherichia coli* (STEC) har vært assosiert med flere tilfeller av sykdomsutbrudd forbundet med smitte via matprodukter. I alvorlige sykdomstilfeller kan STEC infeksjoner føre til livstruende tilstander som hemoragisk kolitt og hemolytisk uremisk syndrom. Den viktigste virulensfaktoren til STEC er Shigatoksinet (Stx). Genet for Stx sitter i Stx-bakteriofager (Stx-fag) og kan overføres til bakteriegenomet via transduksjon. Enteropatogene *E. coli* (EPEC) er en annen gruppe diaréfremkallende bakterier. Disse kan deles inn i typiske EPEC (tEPEC) og atypiske EPEC (aEPEC). Denne gruppen likner STEC, og de kan dele flere virulensfaktorer med unntak av *stx* genet. STEC O26 er den vanligste non-O157 serotypen som er assosiert med STEC-infeksjoner. Både aEPEC og STEC kan tilhøre serotype O26.

Målet med oppgaven var å undersøke og sammenligne *E. coli* O26 som var mottakelige for Stx-fag med ikke-mottakelige *E. coli* O26 via infeksjonsstudier, evne til fermentering av rhamnose og dulcitol, tilstedeværelse av *ehxA*, og for utvalgte isolater; undersøke mulige seter for Stx-fag i genomet hvor fagen kan inkorporeres.

Totalt ble 42 aEPEC O26, sju STEC O26, tre vel-karakteriserte *E. coli* stammer and en vel-karakterisert *Shigella sonnei* stamme brukt som mottakere i bakteriofagforsøkene. Stx-fagen ϕ731(*stx::cat*) (heretter kalt ϕ731) ble brukt til lytisk og lysogen infeksjon av alle mottakerne. To rekombinante fager ble laget: ϕC1-50(*stx2a::cat*) og ϕH1-43(*stx2a::cat*), som ble videre brukt til lysogen infeksjon av de vel-karakteriserte stammene, samt 13 aEPEC O26. *E. coli* O26 isolatene ble karakterisert etter tilstedeværelse av *ehxA* og fermentering av rhamnose og dulcitol. Hel-genom sekvensering ble gjennomført på flere isolater, og undersøkelse av tilgjengelighet på bakteriofag-seter ble utført for utvalgte isolater.

Ingen av *E. coli* O26 isolatene var mottagelige for lytisk infeksjon av ϕ731. Totalt var 27 av 42 aEPEC isolater og alle sju STEC isolater mottagelige for lysogen infeksjon av ϕ731. Ytterligere to aEPEC isolater var mottakelige for ϕC1-50(*stx2a::cat*). To ϕ731-mottakelige isolater hadde alle bakteriofag-setene tilgjengelig før infeksjon, og to isolater som ikke var mottakelige for denne fagen hadde et opptatt sete (*yehV*). Det ble ikke funnet noen klar forskjell mellom mottakelige og ikke-mottakelige *E. coli* O26 isolater i denne oppgaven. Undersøkelse av tilgjengelighet på bakteriofag-seter indikerte at aEPEC O26 isolater som kun har ledige seter med større sannsynlighet er mottagelig for inkorporering av en Stx-fag. Ingen aEPEC eller STEC isolater i denne oppgaven var mottakelig for lytisk infeksjon av ϕ731.

Summary

Shiga toxin-producing *Escherichia coli* (STEC) have been associated with several cases of foodborne disease outbreaks. In severe cases, infection with STEC has led to life-threatening conditions including haemorrhagic colitis and haemolytic uremic syndrome. The main virulence factor of STEC is Shiga toxin (Stx). The *stx* gene is carried on Stx-encoding phages (Stx phages) that can be transferred to the bacterial genome through lysogenic infection. Enteropathogenic *E. coli* (EPEC) is another group of diarrheagenic bacteria, which can be divided in typical EPEC (tEPEC) and atypical EPEC (aEPEC). This group is similar to STEC, they do not encode *stx*, but may share many other virulence factors. STEC O26 is the most common non-O157 serotype associated with STEC infections. Both aEPEC and STEC can belong to the O26 serogroup.

The aim of this study was to investigate and compare *E. coli* O26 susceptible to Stx2a phages with non-susceptible *E. coli* O26 by host infectivity studies, the ability to ferment rhamnose and dulcitol, the presence of *ehxA* and screening for phage insertion sites for selected isolates.

A total of 42 aEPEC O26, seven STEC O26, three well-characterized *E. coli* strains and one well-characterized *Shigella sonnei* strain were used as recipients. Stx phage ɸ731(*stx::cat*) (hereby called ɸ731) was used in lysogenic and lytic infection on all recipient strains. Two recombinant Stx phages were created in this study: ɸC1-50(*stx2a::cat*) and ɸH1-43(*stx2a::cat*), and were further used in lysogenic infection of the well-characterized strains and 13 aEPEC O26 isolates. The *E. coli* O26 strains were also characterized in a broader sense; presence of *ehxA*, fermentation of rhamnose and dulcitol, whole genome sequencing and screening for availability of phage insertion sites in selected isolates to investigate a possible connection between phage susceptibility and other host specific factors.

No *E. coli* O26 in the study were susceptible to lytic infection by ɸ731. A total of 27 of 42 aEPEC isolates and all seven STEC isolates were susceptible to lysogenic infection by ɸ731. Two additional isolates were susceptible to ɸC1-50(*stx2a::cat*). Two isolates susceptible to ɸ731 had all insertion sites vacant prior to lysogenic infection, and two isolates not susceptible to the phage had one insertion site (*yehV*) occupied.

This study found no distinct differences between susceptible and non-susceptible *E. coli* O26 isolates. Investigation of availability of the insertion sites on selected isolates indicated that aEPEC O26 isolates were more likely to incorporate a Stx2a phage if all insertion sites were available. No aEPEC or STEC isolate in this study was susceptible to lytic infection by ɸ731.

Table of contents

1 Introduction 1

1.1 *Escherichia coli* 1

1.2 Pathogenic *E. coli* 2

1.2.1 Shiga toxin-producing *E. coli* (STEC) 2

1.2.1.1 Shiga toxins 4

1.2.1.2 STEC identification 5

1.2.2 Enteropathogenic *E. coli* (EPEC) 6

1.2.3 *E. coli* O26 7

1.3 Bacteriophages 8

1.3.1 Shiga toxin-encoding bacteriophages (Stx phages) 10

1.4 Aim of the study 11

2 Materials and methods 12

2.1 Isolates 13

2.1.1 Freeze stock of isolates 17

2.2 DNA extraction 17

2.2.1 Boiling method 17

2.2.2 QIAamp DNA Mini Kit 18

2.3 PCR 18

2.3.1 Conventional PCR 19

2.3.1.1 Primer information and PCR programs 19

2.3.1.2 Visualization of PCR products on gel 21

2.3.2 Real-time PCR 21

2.3.2.1 Real-time PCR primers and probes 22

2.3.3 Designing primers for conventional PCR 23

2.3.3.1 Validation of new primers 25

2.4 Phage experiments 26

2.4.1 Phage filtrate 27

2.4.1.1 Phage filtrate quantification 28

2.4.1.1.1 Spot test to evaluate infectious capacity 28

2.4.1.2 Phage filtrate stability test 29

2.4.1.2.1 Phage survival with chloroform 29

2.4.1.3 Growth curves 29
2.4.2 Host infectivity 30
2.4.2.1 Lysogenic infection 30
2.4.2.2 Lytic infection 31
2.4.2.3 Plaque hybridization 31
2.4.3 Confirmation of stable lysogenic bacteria 33
2.4.3.1 Conventional PCR 33
2.4.3.2 Real-time PCR 34
2.4.4 Recombination experiment 34
2.4.4.1 Plasmid experiments 36
2.4.4.2 Amplimer construction and overlapping PCR 37
2.4.4.3 Recombination 38
2.4.4.4 Confirming phage recombination 39
2.4.4.4.1 Verification using PCR 39
2.4.4.4.2 Verification of viable phages 39
2.5 Characterization of *E. coli* O26 isolates 40
2.5.1 Rhamnose and dulcitol fermentation (RDF) test 40
2.5.2 Screening for virulence-associated gene *ehxA* 40
2.5.3 Preparation for whole genome sequencing 40
2.5.3.1 DNA concentration measurement with NanoDrop 2000 40
2.5.3.2 Normalisation of DNA using Qubit 41
2.5.3.3 Visualizing of DNA on gel 41
2.6 Data analysis 41
2.6.1 CLC Genomics Workbench 41
2.6.2 CGE VirulenceFinder 1.5 42

3 Results 43
3.1 Phage studies 43
3.1.1 Studies with phage ϕ731 43
3.1.1.1 Lytic infection 44
3.1.1.2 Lysogenic infection 45
3.1.2 Studies with native Stx2a phages 49
3.1.2.1 Lytic infection 49
3.1.2.2 Lysogenic infection 49
3.1.2.3 Growth curves 51
3.1.3 Phage survival in filtrate 52

3.1.4 Recombination experiment 53
3.1.4.1 Selection of strains 53
3.1.4.2 Generation of the long fragment 54
3.1.4.3 Recombination 57
3.1.4.4 Confirmation of recombinant phages 58
3.2 Characterization of *E. coli* O26 60
3.2.1 Rhamnose and dulcitol fermentation (RDF) 61
3.2.2 Characterization of virulence-associated gene *ehxA* 61
3.2.3 Whole genome sequencing of *E. coli* O26 62
3.2.3.1 Screening for possible Stx phage insertion sites 63
3.2.3.2 CGE VirulenceFinder 1.5 64
3.3 Validation of new primers 64

4 Discussion 68
4.1 Comparison of phage susceptibility by different *E. coli* O26 68
4.2 Limitations 75
4.3 Conclusion 76

5 Further work 77

References 78

Appendix A 82

Appendix B 85

Appendix C 88

Appendix D 89

Appendix E 91

1 Introduction

1.1 *Escherichia coli*

The *Enterobacteriaceae* family is a large group of gram negative, non-sporulating, facultative anaerobic bacteria with simple nutritional requirements. Several bacterial species belong to this group, including *Salmonella* spp, *Shigella* spp, *Yersinia* spp and *Escherichia* spp, all of which have been linked to gastrointestinal infection. For *Escherichia coli (E. coli)*, the primary habitat is the gastrointestinal tract of humans and most warm blooded animals (L'Abée-Lund and Wasteson, 2015). In the gut, *E. coli* is normally considered a harmless commensal, with helpful properties such as producing Vitamin K and aid in the absorption of nutrients (Blount, 2015). *E. coli* are in other words part of a healthy microbiota.

Depending on the species and strain, bacteria may be categorized as strictly pathogenic, opportunistic, commensal or non-pathogenic. *E. coli* can possess the properties to be described as all of the above (Leimbach *et al*., 2013). *E. coli* strains can range from being harmless commensals to lethal pathogens due to the high degree of phenotypic and genotypic diversity within the species. A high level of genome plasticity creates this great variation of *E. coli* strains (Gordo *et al*., 2014, Leimbach *et al*., 2013).

Several factors influence the chromosomal changes of bacterial DNA. The *E. coli* core genome consists of about 2000 conserved genes and the remaining ca. 3000 genes varies between different strains (Scheutz, 2014). Alterations of the genome are a result of horizontal gene transfer, DNA rearrangements, recombination, point mutations, gene loss and other genetic events. Of these, horizontal gene transfers and deletions is considered the main driving force of the *E. coli* continuous gene flux (Scheutz, 2014). Horizontal gene transfer includes conjugation, transformation and transduction. Constant alterations of the genome change the competitiveness and fitness of individual variants.

To classify *E. coli,* several approaches can be used. The species has been divided into seven phylogenetic groups related to *E. coli* sensu stricto based on genomic information, denoted A, B1, B2, C, D, E and F (Clermont *et al*., 2013). There is also an eighth phylo-group called Escherichia cryptic clade I (Clermont *et al*., 2013). *E. coli* can also be divided by serotyping. Combinations of the somatic (O), flagellar (H) and capsular polysaccharide antigens (K) presented on the surface have traditionally been used to determine a serotype (Orskov and

Orskov, 1992, Stenutz *et al*., 2006). In addition, an array of molecular methods may be used, including multilocus sequence typing (MLST), multiple-locus variable number tandem repeat analysis (MLVA), and whole genome sequencing (WGS) (Fratamico *et al*., 2016, Jenkins, 2015, Lindsey *et al*., 2016, Patel *et al*., 2016, Parsons *et al*., 2016).

1.2 Pathogenic *E. coli*

There are several different pathogenic variants of *E. coli* with the ability to cause illness. Both humans and animals are at risk, and the infection may be either intestinal or extraintestinal. While some *E. coli* are strictly pathogens, some of the commensal *E. coli* in the gut are opportunistic which may cause infection if they are introduced to other organs and tissues, such as the urinary tract (Blount, 2015). Other gut *E. coli* are harmless commensals in one species, but may cause severe disease in another (Ferens and Hovde, 2011).

Although most *E. coli* do not cause disease in humans, certain strains can cause severe illness and can even become deadly. Pathogenic *E. coli* which infects humans are divided in extraintestinal *E. coli* (ExPEC) and diarrheagenic *E. coli* (DEC) (Clements *et al*., 2012). ExPEC infections typically affect the urinary tract, the blood stream (sepsis) or the meninges. Infections with ExPEC are not the focus of this study, and will not be further discussed.

The severity of infection with DEC depends on the virulence and pathogenicity of the strain. DEC is divided into five main pathotypes. These pathotypes are divided according to clinical disease, virulence factors, and phylogenetic profile, collectively termed pathogenicity profiles (Clements *et al*., 2012). This study focuses on Shiga toxin-producing *E. coli* (STEC) and enteropathogenic *E. coli* (EPEC). The remaining DEC include enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC). These will not be discussed further in this thesis.

1.2.1 Shiga toxin-producing **E. coli** *(STEC)*

STEC, also known as verocytotoxin-producing *E. coli* (VTEC), can cause some of the most severe cases of gastroenteritis among the DECs. STECs are characterized by their ability to produce Shiga toxins, and can be classified into five seropathotypes (SPT) (Scheutz, 2014).

The SPTs are determined from an empirical classification scheme that divide the STEC by association of serotypes with human intestinal disease, outbreaks, and haemolytic-uremic syndrome (HUS) (Scheutz, 2014). The system is based on a gradient ranging from A-E, where SPT-A is graded “high risk” which causes severe disease and outbreaks, while SPT-E is graded “minimal risk” and have never been associated with human disease (Scheutz, 2014, Jafari *et al*., 2012).

As the seropathotype division reflect, STEC strains vary greatly in their pathogenicity. In fact, symptoms of STEC extends from asymptomatic carriage to life-threatening illness, with several in-between conditions such as uncomplicated diarrhoea (Jafari *et al*., 2012). A specific STEC pathotype may even be associated with both HUS and asymptomatic carriage (Scheutz, 2014). The patients typically affected by STEC are children under the age of five, elderly and immunocompromised patients. The primary site of infection is the colon, and the resulting diarrhoea is often watery. If the infection progress to include bloody diarrhoea, stomach cramps and abdominal pain, the condition is termed haemorrhagic colitis (HC) (L'Abée-Lund and Wasteson, 2015). Infection of STEC can also lead to HUS, a condition that affects the kidneys and blood, which may be life-threatening (Blount, 2015, L'Abée-Lund and Wasteson, 2015).

Human pathogenic variants of STEC that cause HC can be referred to as enterohaemorrhagic *E. coli* (EHEC) (Jafari *et al*., 2012). EHEC is a subgroup of STEC, comprised by certain serotypes. The EHEC serotypes most frequently implicated in severe clinical illness worldwide include O157:H7, O26:H11, O45:H2, O103:H2, O111:H8, O121:H19, O145:H28, and their non-motile (NM) derivatives (Delannoy *et al*., 2013). The O157:H7 and O157:NM serotype belong in the SPT-A group, being the most prevalent and virulent of the STEC strains (Scheutz, 2014). Even though many cases of EHEC are sporadic, several outbreaks of O157:H7 have been reported, some more severe than others (L'Abée-Lund and Wasteson, 2015). Most of these outbreaks are small and the source is not identified, whilst others inflict a large group of people such as in the outbreak in Japan of 1996 where more than 10 000 school children were infected from eating contaminated radishes served in the school lunch (L'Abée-Lund and Wasteson, 2015).

The disease severity depends on the combination of virulence genes that the individual STEC strain carries. The main virulence factor is the Shiga toxin. Other virulence factors include pathogenicity islands such as the locus of enterocyte effacement (LEE) which carries genes necessary for attachment and effacing (AE) lesion formation (including *eae* which encodes

the adhesin intimin), virulence plasmids which can carry genes such as enterohemolysin (*ehxA*) and toxin B (*toxB*), and fimbriae (Franzin and Sircili, 2015, Clements *et al*., 2012, Cookson *et al*., 2007).

The main reservoir for STEC is in asymptomatic cattle and other ruminants (Ferens and Hovde, 2011, L'Abée-Lund and Wasteson, 2015). Spread to humans include direct or indirect contact with infected humans or animals or their faeces. People usually get infected through non-host-associated sources such as faecally-contaminated food products and water. The meat industry is especially at risk of producing contaminated products if the slaughter hygiene is poor, as the chances of transferral of STEC from the animals' gut are high. However, contamination of other food products such as dairy products and vegetables is not uncommon (Bonanno *et al*., 2016, L'Abée-Lund and Wasteson, 2015). The infectious dose of STEC is very low. It is estimated that 100-1000 bacteria is enough to develop disease, which means that once in the population, person-to-person spread is common (L'Abée-Lund and Wasteson, 2015).

1.2.1.1 Shiga toxins

Shiga toxins are AB_5 holotoxins, which are proteins composed of one active A subunit associated with a pentamer of B subunits. The B subunits of the toxin can bind to glycolipid receptors (Gb3) located in the cell membrane of the host. The holotoxin is endocytosed and retrogradely transported to the Golgi apparatus and the endoplasmic reticulum where the A-subunit is released and translocated into the cytosol (Melton-Celsa, 2014). Here, the active A subunit inactivates the ribosomes, and protein synthesis in the cell is inhibited (Melton-Celsa, 2014).

Though STEC cells are typically non-invasive, the Shiga toxins (Stx) they produce may be absorbed through the epithelium and inflict systemic damage (L'Abée-Lund and Wasteson, 2015). The toxins translocate and follow the blood stream to target tissues rich in Gb3 receptors, such as endothelial cells and distal renal tubule cells, and causes cell death (Melton-Celsa, 2014, Meyers and Kaplan, 2000). The genes encoding Stx are carried on Shiga toxin-encoding bacteriophages called Stx phages (Kruger and Lucchesi, 2015).

The toxins produced by STEC were initially found to have a profound cytopathic effect on Vero cells and was then given the name verotoxins (VT) (reviewed in (Kruger and Lucchesi,

2015)). However, these toxins were found to be similar to Stx produced by *Shigella dysenteriae*, and so VT, Stx-like and Stx nomenclature have been used interchangeably (Melton-Celsa, 2014). The types of Shiga toxins found in *E. coli* are Stx1 and Stx2 (L'Abée-Lund and Wasteson, 2015). These are further divided into subtypes. The subtypes are designated Stx1a, Stx1c, Stx1d, Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f and Stx2g (Kruger and Lucchesi, 2015).

Subtyping of Stx is useful for STEC characterization and diagnosis. Clinical outcome after STEC infection varies and development of HUS is strongly correlated with the Stx subtype (Parsons *et al*., 2016). Stx2a is more likely to be involved in the development of HUS, while Stx2e, Stx2f and Stx2g are associated with low pathogenicity in humans (Kruger and Lucchesi, 2015).

1.2.1.2 STEC identification

To detect STEC, medical laboratory systems rely on a combination of culture and non-culture methods (Parsons *et al*., 2016). Even though faecal samples from ill patients can contain large numbers of the pathogen, some STEC serotypes are not part of the culture-based stool screening and can be overlooked (Rivas *et al*., 2015, Parsons *et al*., 2016). Assays to detect Stx or presence of the *stx* gene is used by many laboratories in conjunction with the culture-based method (Parsons *et al*., 2016). These tests may be performed using enzyme immune assay (EIA) or polymerase chain reaction (PCR)- based methods (Parsons *et al*., 2016). However, loss of the Stx phage can also occur, in which case the *stx* negative counterpart can be found (Bonanno *et al*., 2016). Detection of STEC is an even bigger challenge in the food industry. Some of the reasons for this is very low numbers of STEC, non-homogenous distribution and high background microflora in foods (Rivas *et al*., 2015).

When STEC is detected; isolation, characterization and typing is also needed. This is important for diagnostics, patient treatment, identification of potential sources of infection to prevent further spread and surveillance of epidemiology (Parsons *et al*., 2016, Scheutz, 2014). To isolate STEC, several differential and selective media has been developed, with various degrees of sensitivity and specificity (Parsons *et al*., 2016). For foods, pre-enrichment is recommended (Rivas *et al*., 2015). STEC can also be isolated using immunomagnetic separation (IMS), which can isolate O157 and other key serotypes (Rivas *et al*., 2015).

There is no single virulence marker or combination of markers that defines which STEC that are human pathogenic or not. However, many human pathogenic STEC possesses the LEE island and a virulence plasmid, but a diversity of other virulence-associated factors have also been described in human pathogenic strains (Parsons *et al*., 2016). Characterization and typing may include molecular methods (such as real-time PCR) and/or genomic pattern analyses to compare and classify STEC, using approaches such as pulse field gel electrophoresis (PFGE) and multiple-locus variable number tandem repeat analysis (MLVA) (Parsons *et al*., 2016). All methods however, have limitations, especially considering "non-typical" emerging STEC. The methods may be time-consuming or error-prone, and multiple methods are often used in conjunction to conclude the identification.

Whole genome sequencing (WGS) is a method which can be used as an in-depth characterization of isolated strains (Handelsman, 2004, Chen and Pachter, 2005, Lambert *et al*., 2015). WGS has superior discriminatory powers, and according to a review by Parsons *et al.* (2016) genomic typing through WGS may dramatically streamline the detection and typing workflow of STEC.

1.2.2 Enteropathogenic **E. coli** *(EPEC)*

EPEC infections are an important cause of high child morbidity and mortality rates in developing countries (L'Abée-Lund and Wasteson, 2015). The affected patients are usually below the age of two and living in areas with poor sanitation and limited access to clean drinking water. The main pathogenic property of the bacteria is creation of characteristic AE lesions, as some STEC strains. However, the *stx* gene specific for STEC is absent in EPEC (Bielaszewska *et al*., 2005). In both STEC and EPEC, *eae* present within the LEE pathogenicity island is necessary for formation of the AE lesions (Elliott *et al*., 2000, Bielaszewska *et al*., 2005). The localizations of the lesions differs. The infection site of EPEC is the small intestine, while STEC infection mainly manifests in the colon (L'Abée-Lund and Wasteson, 2015, Jafari *et al*., 2012).

AE causes the epithelium to rise, creating a pedestal-like formation were the bacteria can securely be rooted. The lesions are described as a focal loss of microvilli (Jafari *et al*., 2012, Gomes *et al*., 2016). This, together with the reduction of absorbance across the gut epithelium and disturbance in the electrolyte balance, leads to diarrhoea, which is often watery and

sometimes mucous. Vomiting and fever is not uncommon. Normally, the disease lasts from 12 hours to three days, but can become persistent. On rare events, severe cases of the infection may become deadly due to dehydration, acidosis and development of shock (L'Abée-Lund and Wasteson, 2015).

EPEC can be further divided into two subgroups: typical EPEC (tEPEC) and atypical EPEC (aEPEC). The tEPECs carry a plasmid called *E. coli* adherence factor (EAF). The EAF plasmid makes it possible for the bacteria to express bundle forming pili (bfp) that affects their ability of adherence. Infected humans are the only known carriers of tEPEC. aEPEC does not carry EAF, and can be carried by several animal species, including ruminants, cats and dogs (L'Abée-Lund and Wasteson, 2015).

1.2.3 **E. coli** *O26*

E. coli O26:H11/NM can belong to both STEC and aEPEC (Clements *et al*., 2012, Brandal *et al*., 2012). They may share specific virulence factors such as LEE and the Espl/NleA effector and also share many fitness genes, i.e. genes which contribute to adaptation and/or survival of the bacteria, and genotypic diagnostic markers (Bielaszewska *et al*., 2005, Scheutz, 2014). STEC O26:H11 is recognized as the most common non-O157 EHEC serogroup found in cases of human STEC infections, in addition to being the second-most frequent serogroup after O157 linked to registered cases of HUS (EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2016).

Ruminants are a major reservoir of *E. coli* O26, both STEC and EPEC. aEPEC O26 is one of the largest aEPEC serogroups present in sheep and can also regularly be found in cattle (Sekse *et al*., 2011, Ison *et al*., 2015). Acquisition of Stx phages can convert aEPEC to STEC, possibly creating new pathotypes (Bonanno *et al*., 2016, Muniesa and Schmidt, 2014). During infection, pathogenic aEPEC O26 may have an advantage to their STEC counterparts by avoiding lysis due to phage induction in the gastrointestinal tract. Furthermore, both aEPEC O26 and STEC O26 can coexist in the human intestine, and the human pathogenic aEPEC may convert to STEC leading to a more severe illness (Bielaszewska *et al*., 2007).

The pathogenic potential of *E. coli* O26 depends on several virulence genes. Some aEPEC O26 that have several virulence genes in common with EHEC O26 may be described as EHEC-like (Bugarel *et al*., 2011). EHEC O26 and EHEC-like isolates can be divided in a

separate cluster from less pathogenic aEPEC O26, based on virulence genes and other markers, such as rhamnose and dulcitol fermentation (RDF) (Leomil *et al*., 2005). A study by Brandal *et al.* (2012) characterized aEPEC and STEC(/EHEC) O26:H11 from ovine and human sources by RDF and *ehxA* presence (amongst other markers), and found that RDF negative and *ehxA* positive isolates were grouped to pathotype EHEC or EHEC-like. The study suggests that markers such as no RDF and presence of *ehxA* potentially can be used to indicate EHEC-like O26 isolates, which makes these isolates interesting for further investigation in regards to phage susceptibility.

1.3 Bacteriophages

Viruses infecting bacteria are referred to as bacteriophages, or phages for short. Phages transfer genetic material to bacteria through a process called transduction. Numerous phages exist and the size and shape varies between the bacteriophage families. The bacteriophage Lambda (λ) from the *Siphoviridae* family and the bacteriophage T4 from the *Myoviridae* are examples of phages with different shape, as illustrated in figure 1.1 (Willey *et al.*, 2014). Even though their shape is different, these phages both have the ability to infect *E. coli* (Willey *et al.*, 2014). Some phages may specifically infect a bacterial species, while other have a broader range of potential hosts (Muniesa and Schmidt, 2014, Willey *et al*., 2014).

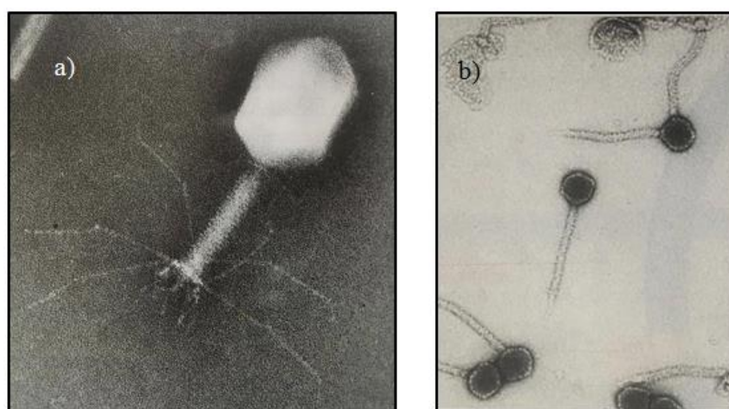


Figure 1.1: Illustration of different bacteriophage shapes. a) Micrograph of bacteriophage T4. b) Photomicrograph of bacteriophage Lambda (the icosahedral head not obvious).
(Images gathered from figure 6.7 and figure 27.9 in Willey et al. *(2014)).*

Not only may the shape, size and host range vary between phages, the mode infection varies too. Bacteriophages may be either virulent or temperate. While temperate phages can either lyse their host through multiplication upon entry of the cell or remain within the bacteria, virulent phages have only the former option; to exploit and destroy the host (Willey *et al.*, 2014). Virulent phages are a hot topic for those researching alternative therapeutic treatments to deal with antibiotic-resistant bacteria (Cisek *et al.*, 2017).

Temperate phages may either enter lytic or lysogenic cycles upon entry of the host cell. When lysogenic cycle is chosen, the phage integrates their viral DNA in the bacterial genome and remains within the host (Willey *et al.*, 2014). In this state, the phage is referred to as a prophage and the bacterial host carrying the prophage is called a lysogen, or lysogenic bacteria (Casjens, 2003). Lysogenic bacteria replicate as normal, and their progeny cells are also lysogens. The relationship between a temperate phage and its host is called lysogeny (Willey *et al.*, 2014).

An important possible outcome of lysogeny is lysogenic conversion, which is phenotypic change of the host (Willey *et al.*, 2014). This may cause the host to gain pathogenic properties, such as toxin production upon induction (Tyler *et al.*, 2005). Prophage induction is the initiation of synthesis to create new virions, which leads to the prophage leaving the lysogenic cycle and enter the lytic cycle. When the prophage enters the lytic cycle, the host is lysed which results in release of progeny phages (Willey *et al.*, 2014). Changes in growth condition, antibiotics or UV irradiation of the host cell are factors that can cause induction (Muniesa and Schmidt, 2014). Prophage induction results in free living phages that are capable of infecting new bacteria.

Prophage DNA are major contributors to large varieties of individual bacteria of the same species. As much as 10-20% of a bacterial genome may be prophage DNA (Casjens, 2003). For example, in the *E. coli* O157:H7 Sakai, as many as 18 prophages and six prophage-like features were found (Hayashi *et al.*, 2001). However, many integrated virus genomes are defective and will no longer produce viable virions (Casjens, 2003). Nevertheless, the vast number of bacterial genomes with prophage DNA depicts the great importance of transduction as a method for gene transfer. As this may lead to the evolution of pathogenic bacteria, it is important to study the mechanisms of phage-mediated gene transfer (Casjens, 2003).

1.3.1 Shiga toxin-encoding bacteriophages (Stx phages)

Stx phages are a heterogeneous group of temperate lambdoid phages that carry a Shiga toxin (*stx*) gene (Allison, 2007). These *stx* mobilizing phages are found free living in many environments and may infect and incorporate into several bacteria, potentially leading to new human pathogens (Muniesa and Schmidt, 2014). However, Stx phages are mostly associated with *E. coli* (Muniesa and Schmidt, 2014). Stx phages are highly variable due to their ability to acquire and mobilize foreign genes, including virulence genes (Muniesa and Schmidt, 2014). In fact, diversity in the genome of STEC strains can principally be attributed to the phage mediated horizontal transfer of DNA (Casjens, 2003, Kruger and Lucchesi, 2015).

The diverse Stx phages may impact the virulence of STEC strains and generate new pathogens by introducing virulence genes and Stx production to a host cell. As described in section 1.2.1.1, there are different Stx variants, whereas not all are associated with serious human disease. Stx phages can encode either Stx1 or Stx2, but separate STEC can carry different Stx phages (reviewed in: (Mauro and Koudelka, 2011, Scheutz, 2014, Allison, 2007)). The host range of Stx phages depends on both the phage and the host (Muniesa and Schmidt, 2014). A study by Gamage *et al.* (2004) have indicated that susceptibility of some Stx phages may be dependent on the phylogenetic group of the hosts.

Infection of phages is dependent on adsorption to the host. Phages can recognize different host cell receptors, and in gram negative bacteria such as *E. coli*, both protein and lipopolysaccharide receptors are present (Rakhuba *et al*., 2010). Porins such as OmpC, OmpF and LamB can be utilized by certain phages for adsorption (Rakhuba *et al*., 2010, Chatterjee and Rothenberg, 2012). Few receptors specific for Stx phages are described, but the surface molecule YaeT is possibly one such Stx phage recognition site (Smith *et al*., 2007). After phage adsorption, the Stx phages have several insertion sites to where they integrate their viral DNA in the bacterial genome, and usually a certain site is preferred (Kruger and Lucchesi, 2015). Several insertion sites for Stx phages have been described, including *argW*, *prfC*, *torST*, *sbcB*, *wrbA*, *yehV*, *yecE*, and *Z2577* (Bonanno *et al*., 2015, Kruger and Lucchesi, 2015). For the LEE-positive O157 strains, *yehV* is preferred by Stx1 phages, while insertion site *wrbA* or *argW* is preferentially selected for Stx2a phages (Scheutz, 2014). In STEC O26:H11, the insertion sites for Stx phages is mainly *wrbA* and *yehV* (Bonanno *et al*., 2015). However, if the primary insertion site is occupied, another may be chosen (Kruger and Lucchesi, 2015).

When STEC infects the gut, several agents produced by the host immune system try to damage the bacterial DNA leading to a bacterial SOS response. This response causes induction of the prophage which then enters the lytic cycle. The *stx* genes are transcribed during the lytic phase as they are upstream of the lysis cassette located in the late regions of the phage genome. This position seems to be conserved between Stx phages (Muniesa and Schmidt, 2014, Tyler *et al*., 2005). There is no specific transport of either Stx or the Stx phages out of the cell. When lytic cycle is entered, virions and Stx are created within the cell until it burst, releasing both phages and toxins to the surroundings. The release of Stx phages and toxins in the gut may influence the development of HC and HUS (Muniesa and Schmidt, 2014). In addition, free Stx phages may potentially enter new bacterial hosts, possibly creating further issues for the afflicted patient (Allison, 2007).

1.4 Aim of the study

The aim of this study was to investigate and compare *E. coli* O26 susceptible to Stx2a phages with non-susceptible *E. coli* O26 by host infectivity studies. Other characteristics such as the ability to ferment rhamnose and dulcitol, the presence of virulence-associated *ehxA* and screening for possible phage insertion sites for selected isolates were also included in the comparison.

2 Materials and methods

The main experiments are phage susceptibility experiments that involve transduction of Stx2a phages into bacteria, which were used to evaluate lysogenic and lytic abilities (collectively termed host infectivity) of the phages towards different *E. coli* O26. Both labelled and wild type phages have been used. The labelled phages were obtained either from a previous study or created in the recombination experiment and will be described further in the associated sections. The isolates of interest in this study are *E. coli* O26 isolates from human, ovine and bovine sources. These isolates consisted of both *stx* negative and *stx2a* positive *E. coli*, also referred to as aEPECs and STECs respectively.

Data analysis of WGS data was used to compare insertion sites occupancy and vacancy of representative isolates according to their phage susceptibility. WGS data was already available from earlier studies for some of the isolates, but others had to be prepared. Throughout the study, use of PCR techniques have been central for verification of results. New primers were necessary for some of the experiments in this thesis, and data analysis was performed to design these primers. The primers were subsequently validated for use through testing with well-characterized isolates. The data analysis part was done using CLC Workbench programs (CLC Bio/Qiagen, Denmark/Germany).

Many of the isolates used in this study had already been characterized regarding presence of the *ehxA* gene and fermentation abilities of rhamnose and dulcitol. For the isolates missing this information, tests were performed. In addition, the STEC isolates' WGS data were also analyzed with the online analysis tool VirulenceFinder 1.5 provided by the Center for Genomic Epidemiology (CGE).

Beyond these main parts of this thesis, several small experiments have been conducted and will be described in the following texts. This includes test of stability of phages in filtrate, test to improve the phage survival in filtrate, growth curves and antibiotic resistance test.

2.1 Isolates

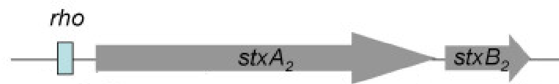
Throughout the experiments, well-characterized, non-virulent strains of *E.coli* and *Shigella* were used. These strains may be referred to as laboratory strains (lab strains), control strains or recipient/host strains. However, the term recipient/host strain may also be used regarding the aEPEC and STEC isolates. Recipient strain is used when the isolate is used to receive e.g. a phage from another isolate, while host strain (or donor strain) is used on an isolate which carry e.g. a phage. Control strain is used when the isolate is an established control for the method in question. In lack of better alternatives, the term "lab strains" is sometimes used. Table 2.1 provides an overview of these isolates and their role in the various experiments.

Table 2.1: Lab strains information and their role in the experiments conducted.

Name	Experiments with phage Φ731	Experiments with Stx2a phages (native and recombinant)	PCR	Reference	Stx Profile	Species
C600	Control recipient host infectivity and quantification,	Recipient strain host infectivity	Negative control *stx2a*, *stx2a::cat*	(Sambrook and Russell, 2001)	-	*Escherichia coli (E. coli)*
C600::ϕ731	Host (/donor) strain	-	Positive control *stx2a::cat*	(Solheim *et al.*, 2013)	*stx2a::cat*	*E.coli*
DH5α	Recipient strain host infectivity	-	Carries pKD3 used in the recombination experiment	(Sambrook and Russell, 2001)	-	*E.coli*
***Shigella sonnei* 866**	Recipient strain host infectivity	Recipient strain host infectivity	Negative control *stx2a*	(Muniesa *et al.* 2003)	-	*Shigella sonnei*
MG1655	Recipient strain host infectivity	Recipient strain host infectivity	-	ATCC 700926	-	*E.coli*
EDL933	-	-	Positive control for *stx2a* PCR	CCUG 29197-B	*stx2a*	*E.coli*

E. coli C600::ϕ731 contain an altered Stx2a phage where the gene chloramphenicol acetyltransferase (*cat*) is placed within the *stx2a* gene, illustrated in figure 2.1. The phage is called ϕ731(*stx::cat*) or simply ϕ731. The *cat* gene is a marker gene that makes the isolate carrying the phage resistant to the antibiotic Chloramphenicol (Cm). An altered phage as such may also be referred to as labelled and permits the use of selective media.

a) Original structure of the stx operon.



b) Stx operon with inserted cat gene.

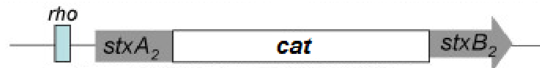


Figure 2.1: a) a normal structure for a stx2a *operon. b)* stx2a *operon interrupted by an inserted* cat *gene.*

In this study, a total of 49 *E. coli* O26 isolates were used in various experiments. Of these, 42 were characterized as aEPEC and seven as STEC. All of the *E. coli* O26 isolates were used as recipients in the host infectivity experiments regarding phage ϕ731. Many of the isolates were whole genome sequenced, and some were screened for presence of the *ehxA* gene and tested for RDF. Some isolates were used in the recombination experiment and a few selected were used in the data analysis of insertion sites.

In addition to the above-mentioned experiments, sequence data from all STEC isolates were analysed using the CGE VirulenceFinder 1.5. and the H1-46 isolate was tested for antibiotic resistance towards ampicillin. In the host infectivity experiments, STEC isolates have a dual role. Regarding phage ϕ731, STEC isolates are treated as recipients, but they are host (donor) of the native Stx2a phages used in another part of the host infectivity experiments. STEC isolate information and overview of other experiments than host infectivity performed on the STEC isolates is listed in table 2.2.

Table 2.2: Overview of STEC O26 isolates used in this study: Isolate name used in this study and original, source, a reference for where it is isolated and other experiments than host infectivity experiments performed.

Isolate name (this thesis)	Original isolate name	Source	Reference	Other experiments*
H1-43	*1110-1361 (HUS) (FHI-79)*	*Human*	*Norwegian Institute of Public Health (NIPH)*	REH
H1-44	*1108-0073 (HUS) (FHI-27)*	*Human*	*NIPH*	-
H1-45	*1107-2514 (HUS) (FHI-24)*	*Human*	*NIPH*	-
H1-46	*102-09818 (HUS) (FHI-4)*	*Human*	*NIPH*	CLC, ABR
C1-47	*2014-22-142-1-3*	*Bovine*	*Norwegian Veterinary Institute (NVI)*	RDF, *ehxA*, DEN
C1-50	*2014-22-162-1-2*	*Bovine*	*NVI*	RDF, *ehxA*, REH
S1-51	*2007-60-10067-51-2*	*Ovine*	*NVI*	RDF, *ehxA*

** Abbreviations are used for the different experiments: RDF = test for rhamnose and dulcitol fermentation,* ehxA *= real-time PCR screening for presence of the* ehxA *gene, CLC = data analysis of insertion sites, REH = recombination experiment (host/donor of recombinant phage), ABR=antibiotic resistance test (regarding ampicillin), DEN=* de novo *assembled.*

aEPEC isolate information and overview of other experiments than host infectivity with phage ф731 performed on the aEPEC isolates is listed in table 2.3.

Table 2.3: Overview of aEPEC O26 isolates used in this study: isolate name used in this study and original, source, a reference for where it is isolated and other experiments than host infectivity experiments with phage ф731 performed.

Isolate name (this thesis)	Original isolate name	Source	Reference	Other experiments*
H0-1	*1110-1777*	*Human*	*Norwegian Institute of Public Health (NIPH)*	*REC*
H0-2	*1108-3552*	*Human*	*NIPH*	*WGS, REC, PLH*
H0-3	*102-11754*	*Human*	*NIPH*	*WGS, CLC, REC*
H0-4	*1109-0914*	*Human*	*NIPH*	*WGS, REC*
H0-5	*1106-1338*	*Human*	*NIPH*	*WGS, REC*
H0-6	*1103-0574*	*Human*	*NIPH*	*WGS, REC, PLH*
S0-7	*2007-60-10180-53-2*	*Ovine*	*Norwegian Veterinary Institute (NVI)*	*WGS*
S0-8	*2007-60-10246-55-2*	*Ovine*	*NVI*	*WGS*
S0-9	*2007-60-10384-55-7*	*Ovine*	*NVI*	*WGS*
S0-10	*2007-60-10389-51-2*	*Ovine*	*NVI*	*WGS, PLH*
S0-11	*2007-60-10473-55-2*	*Ovine*	*NVI*	*WGS*

S0-12	*2007-60-10710-54-4*	*Ovine*	*NVI*	-
S0-13	*2007-60-10714-52-2*	*Ovine*	*NVI*	*WGS, REC*
S0-14	*2007-60-11082-53-2*	*Ovine*	*NVI*	*WGS, REC, ECT-N*
S0-15	*2007-60-11115-54-2*	*Ovine*	*NVI*	*WGS, ECT-P*
S0-16	*2007-60-11806-51-2*	*Ovine*	*NVI*	*WGS*
S0-17	*2007-60-12312-52-3*	*Ovine*	*NVI*	*WGS, REC*
S0-18	*2007-60-12552-52-7*	*Ovine*	*NVI*	*WGS*
S0-19	*2007-60-12872-52-2*	*Ovine*	*NVI*	*WGS, REC*
C0-20	*2014-22-153-1-2*	*Bovine*	*NVI*	*RDF,* ehxA
C0-21	*2014-22-160-1-3*	*Bovine*	*NVI*	*RDF,* ehxA
C0-22	*2014-22-169-1-3*	*Bovine*	*NVI*	*RDF,* ehxA
C0-23	*2014-22-175-1-2*	*Bovine*	*NVI*	*RDF,* ehxA
C0-24	*2014-22-232-1-2*	*Bovine*	*NVI*	*RDF,* ehxA
C0-25	*2014-22-239-1-3*	*Bovine*	*NVI*	*RDF,* ehxA
C0-26	*2014-22-252-1-2*	*Bovine*	*NVI*	*RDF,* ehxA
C0-27	*2014-22-261-1-2*	*Bovine*	*NVI*	*RDF,* ehxA
C0-28	*12_BKT025087*	*Bovine*	*Swedish National Veterinary Institute (SNVI)*	*WGS, RDF,* ehxA
C0-29	*12_BKT063891*	*Bovine*	*SNVI*	*WGS, RDF,* ehxA
C0-30	*11_BKT066101*	*Bovine*	*SNVI*	*RDF,* ehxA
C0-31	*12_BKT062255*	*Bovine*	*SNVI*	*WGS, RDF,* ehxA
C0-32	*11_BKT084287*	*Bovine*	*SNVI*	*WGS, RDF,* ehxA
C0-33	*11_BKT086372*	*Bovine*	*SNVI*	*WGS, RDF,* ehxA
C0-34	*11_BKT086159*	*Bovine*	*SNVI*	*WGS, RDF,* ehxA
C0-35	*12_BKT060916*	*Bovine*	*SNVI*	*RDF,* ehxA
C0-36	*12_BKT035266*	*Bovine*	*SNVI*	*WGS, RDF,* ehxA
C0-37	*12_BKT053292*	*Bovine*	*SNVI*	*WGS, RDF,* ehxA
C0-38	*12_BKT040099*	*Bovine*	*SNVI*	*WGS, RDF,* ehxA
C0-39	*11_BKT084291*	*Bovine*	*SNVI*	*WGS, RDF,* ehxA, *REC*
C0-40	*12_BKT038336*	*Bovine*	*SNVI*	*WGS, RDF,* ehxA, *REC*
C0-41	*11_BKT064249*	*Bovine*	*SNVI*	*WGS, RDF,* ehxA, *REC*
C0-42	*11_BKT066085*	*Bovine*	*SNVI*	*WGS, RDF,* ehxA

** Abbreviations used for the different experiments: WGS = whole genome sequencing, RDF = test for rhamnose and dulcitol fermentation,* ehxA *= real-time PCR screening for presence of the* ehxA *gene, CLC =* de novo *assembly and subsequent data analysis of insertion sites, REC = recombination experiment (recipient of recombinant phage) PLH= plaque hybridization, ECT-N = negative control for* ehxA *PCR, ECT-P= positive control for* ehxA *PCR.*

In the recombination experiment, three plasmids were used. The names and their function in the experiment are described in table 2.4.

Table 2.4: Plasmids used in the recombination experiment.

Plasmid name	Relevant properties	Reference
pKD46	Carries the λ red gene, temperature sensitive, ampicillin resistant.	(Datsenko and Wanner, 2000)
pUC19	High copy number *E. coli* plasmid. Ampicillin resistant.	(Norrander *et al.*, 1983)
pKD3	Contains *cat* cassette.	(Datsenko and Wanner, 2000)

2.1.1 Freeze stock of isolates

To conserve the lysogenic bacteria listed in table a.1-a.3 in appendix A which were created in the experiments, freeze stocks of the isolates were made. For each lysogenic isolate, a loopful of colony material were deposited in tubes with 1 ml of Heart Infusion Broth (Difco, MG Scientific, USA) and Glycerol 85% (Merck Millipore, Germany) solution (with ca. 17% glycerol), and frozen temporarily at –20°C and later at -80°C for longer storage.

2.2 DNA extraction

DNA is used as template for the PCRs and whole genome sequencing. DNA extraction was either done by boiling or with commercial kit. The latter method provided purer DNA, and was used when this was necessary.

2.2.1 Boiling method

A loopful of colony material was suspended in 20-500 µl MilliQ water in Eppendorf tubes and boiled for 5-10 minutes. The samples were centrifuged at max speed (13200 x *g*) for 1 minute and the supernatant was transferred to a new tube. The new tube containing the supernatant was stored in either refrigerator or freezer (-20°C) until use.

2.2.2 QIAamp DNA Mini Kit

QIAamp DNA Mini Kit (Qiagen, Germany) was used to extract DNA for methods which have higher requirements for DNA purity. The procedure was gathered from: http://emerald.tufts.edu/~mcourt01/Documents/QIAGEN_protocol.pdf (Date: 11.11.2016) and protocol C for isolation of genomic DNA from bacterial cultures on page 52 was followed. Several of the steps have alternatives. To standardize the method, fixed adjustments were set. This includes: Adding 170 µl of ATL to each pellet, incubating 1 hour at 56°C and vortexing every 15 minutes, using freshly made 96% ethanol, and including the optional step of RNase treatment and the additional centrifugation. For the extractions made for WGS, the elution buffer was 10 mM TRIS pH 8 (Merck, Germany) instead of the kit buffer EB.

2.3 PCR

PCR is a method to amplify a specific sequence in the DNA. If the sequence is present, fluorescent dye that bind to DNA is used to visualize the amplified product. This study use both conventional PCR and real-time PCR, which will be described in the following sections. The genes of interest and PCR method used to detect their presence in the DNA are listed in table 2.5.

Table 2.5: PCR methods used for confirming presence of genes, and the function of the genes.

Gene	Function	PCR method
ehxA	Enterohaemolysin	Real-time PCR
stx2a	Shiga toxin 2a (subunit Stx2A and Stx2B)	Real-time PCR
stx2a::cat	Chloramphenicol acetyl transferase (*cat*) placed inside *stx2a*. Chloramphenicol resistance.	Conventional PCR and real-time PCR

The PCR runs included controls, both positive and negative. Real-time PCR always included a no template control, and conventional PCR included H_2O as negative controls to assess if the master mix had been contaminated. The isolates used as control is listed in table 2.1 and 2.3. If more than one negative control sample could be used the control chosen for the individual run varied.

Some of the PCRs run in the recombination experiment were to create certain short and long fragments, described in section 2.4.4.2. These were run without including control.

2.3.1 Conventional PCR

Conventional PCR is an end-point analysis. A master mix containing necessary reagents to amplify a specific segment of DNA is made and the DNA of interest is added. The master mix must contain DNA polymerase, primers (forward and reverse), nucleotide triphosphates (dNTPs), $MgCl_2$ and buffer. The master mixes used in this study is described in appendix B, table b.1-b.4.

The samples are placed in thermocyclers (in this study the conventional PCRs were run on either T100^{TM} Thermal cycler, Bio-Rad, USA or SureCycler 8800, Agilent Technologies, USA). First the DNA is denatured by high temperature, creating single stranded DNA. Then, the primers anneal to the single stranded DNA at an appropriate temperature for the specific primer pair. The temperature is then raised to 72°C which is the optimal temperature for the DNA polymerase to work, and double stranded DNA is synthesized from the forward and the reverse primer using the dNTPs. This process of varying temperatures is repeated for several times, and depending on the efficiency, the PCR product increase nearly logarithmically for each cycle. After amplification, the PCR products are usually visualized using gel electrophoresis. Visualization is described in section 2.3.1.2.

2.3.1.1 Primer information and PCR programs

The primers used in this study, name, sequence and target gene are presented in table 2.6.

Table 2.6: Information regarding Primers used in conventional PCRs in this study.

Primer	Sequence	Target gene	Reference
GK4	TCAGTCATTATTAAACTG	*stx2a (subunit B)*	*(Serra-Moreno* et al.*, 2006)*
Cm5	TGTGTAGGCTGGAGCTGCTTC	*cat*	*(Serra-Moreno* et al.*, 2006)*
Rho	ATATCTGCGCCGGGTCTG	*rho*	*(Serra-Moreno* et al.*, 2006)*
Cm3	CATATGAATATCCTCCTTAG	*cat*	*(Serra-Moreno* et al.*, 2006)*

StxCM_END_fwd	ACAACTCAAAAAATACGCCC	*cat*	*This study*
StxCM_END_rev	TCTTTCCCGTCAACCTTC	*stx2a (subunit B)*	*This study*
StxCM_START_fwd	GTGGATATACGAGGGCTT	*stx2a (subunit A)*	*This study*
StxCM_START_rev	TGAGCTGGTGATATGGGA	*cat*	*This study*
S2Aup	ATGAAGTGTATATTATTTA	*stx*	(*Serra-Moreno* et al., *2006)*
Cm 5-stx	GAAGCAGCTCCAGCCTACACA ACGAAGATGGTCAAAACGCG	*stx*	(*Serra-Moreno* et al., *2006)*
Cm3-stx	CTAAGGAGGATATTCATATGA GGAGTTAAG**C**ATGAAGAAG	*stx*	(*Serra-Moreno* et al., *2006)*
Alternative (GK4)	ACCCACATACCACGAATCA	Downstream *stx2a (subunit B)*	*This study*

Some of the PCR programs needed to be optimized (described in section 2.3.3 and 3.3). A temperature gradient in the thermocycler made it possible to evaluate several annealing temperatures from one PCR run. The elongation time was also experimented with. Table 2.7 describes the optimal PCR programs found in this study used for the different primer pairs, in addition to the polymerase used.

Table 2.7: Primers pairs, associated polymerase and PCR programs used in this study.

Primer pairs	**DNA polymerase**	**PCR program**
GK4 + Cm5 Rho + Cm3	Taq DNA polymerase (Qiagen, Germany)	95°C 3 min – 35 cycles (95°C 1 min – 41°C 30 sec - 72°C 1.5 min) - 72°C 7 min - 4°C ∞
StxCM_END_fwd + StxCM_END_rev StxCM_START_fwd + StxCM_START_rev	Taq DNA polymerase	95°C 5 min – 35 cycles (95°C 30 sec – 56°C 30 sec - 72°C 30 sec) - 72°C 7 min - 4°C ∞
Cm3 + Cm5	Phusion DNA Polymerase (2 U/µL) (Thermo Fisher Scientific, USA)	98°C 3 min – 35 cycles (98°C 15 sec – 52°C 15 sec - 72°C 30 sec) - 72°C 5 min - 4°C ∞
Cm5-stx + stx2aup Cm3-stx + GK4	Phusion DNA Polymerase (2 U/µL)	98°C 3 min – 35 cycles (98°C 15 sec – 41°C 15 sec - 72°C 5 sec) - 72°C 5 min - 4°C ∞
GK4 + S2Aup	Phusion DNA Polymerase (2 U/µL)	98°C 3 min – 35 cycles (98°C 15 sec – 41°C 15 sec - 72°C 30 sec) - 72°C 5 min - 4°C ∞
Rho + Alternative GK4	Taq DNA polymerase	95°C 3 min – 35 cycles (95°C 30 sec – 53°C 30 sec - 72°C 2 min) - 72°C 5 min - 4°C ∞

2.3.1.2 Visualization of PCR products on gel

The PCR products are mixed with loading dye before applied to the gel. The gel itself contains fluorescent dye that binds DNA and is placed in an electrophoresis chamber with running buffer. The gel is then subjected to electric current for a given time and the negatively charged DNA (e.g. PCR products) travels towards the positive end. The length travelled depends on the size of the fragments. After the run, the gel is then placed in UV-light so that the bands of PCR products become visible.

In this study, 1% gels were made using agarose and either 1xTAE or 1xTBE buffer. GelRed (NucleoAcid Gel Stain, Biotium, USA) was used as fluorescent DNA dye and added to the gel. The gel was placed in an Electrophoresis chamber, where electric current was applied. The running buffer was 1xTAE or 1xTBE depending on the gel. The samples (PCR products) were mixed with 6x loading dye (LD) (Thermo-Scientific, USA) before application on the gel. A standard DNA ladder of 50 bp and/or 1 kb (O'GeneRuler/Generuler, Thermo-Scientific, USA) was always added for comparison. The gel was then run on 80-90 V for 40-45 minutes.

After electrophoresis, the gel was placed in Molecular imager ChemiDocTM XRS Imaging System (Bio-Rad, USA). This machine use ultraviolet (UV) light to make the fluorescent component of GelRed bound to DNA visible, and take a picture. The associated computer program is called ImageLab (version 5.1, build 8) and was used to process the image file. If there are PCR products in the samples they will appear as bands on the gel. The ladder can be used to evaluate the band size.

2.3.2 Real-time PCR

Real-time PCR collects data as they are produced, i.e. in real-time. The technique is based on linking amplification of DNA to generation of fluorescence, which can be detected with a camera for each PCR cycle. The fluorescence increase as the number of copies increases with every reaction (cycle). How many cycles necessary for the fluorescent signal to cross a certain threshold is used to evaluate the real-time PCR result for any given sample.

In this study, fluorescence in the real-time PCRs is linked to a sequence-specific probe, but non-specific double-stranded DNA binding dyes can also be used. The master mixes prepared for real-time PCR in this study is shown in table b.5-b.7 in appendix B.

Another name for real-time PCR is quantitative PCR (qPCR), as the method may be used quantitatively. This is unlike conventional PCR, which at best may be semi-quantitative. However, in this study, real-time PCR has been used as an end-point analysis for detection of the genes described in table 2.5. Samples were regarded as positive for presence of the gene in question for each run if the signal crossed the cycle threshold (CT) before 25 cycles. Samples were regarded as negative if there was no CT or the signal was late (CT after 30 cycles). Samples with values in-between these were evaluated and usually re-run unless otherwise described.

The main benefit of using real-time PCR as opposed to conventional PCR for this study is time efficiency. The run itself is shorter, and there is less hands-on time as there is no need for an additional step for visualization.

2.3.2.1 Real-time PCR primers and probes

Primers and probe for detection of *stx2a* were already designed and available. For the real-time PCR for *ehxA,* stock of Prime Time Std qPCR assay (Integrated DNA Technologies (IDT), USA) was used. Newly developed in-house primers and probes for *stx2a::cat* confirmation were tested during this thesis. This method could replace the conventional PCR for verification of presence of *stx2a::cat*. Information of the primers and probe is listed in table 2.8.

Table 2.8: Information regarding Primers and probes used in this study.

Primer/Probe	Sequence	Target gene	Reference
PROBE VT2a-Qp	HEXCRCAATCCGCCGCCATTGCA TTAACAGAA-BHQ1	*Stx2a*	Unpublished primers SVA
PRIMER VT2a-QfLNA 1	GGCGG+TTTT+ATT+TGCATTA+G		
PRIMER VT2a-QrLNA 2	CG+TC+AAC+CTT+CACTGT+A		

EhxA-Fwd	GTGTCAGTAGGGAAGCGAACA	*EhxA*	*Bugarel AEM 2010. With modified probe to include all ehxA variants*
EhxA-Rev	ATCATGTTTTCCGCCAATG		
EhxA-Probe	FAM-CGTGATTTTGAATTCAGARC CGGTGG-BHQ		
StxCAT_F1	CGAAGTGATCTTCCGTCACA	*Stx2a::cat*	*In house – not published**
StxCAT_R2	CCGCCATAAACATCTTCTTCA		
StxCAT_probe1	[6FAM]AGGAACTTCGGCGCGCCT AC[BHQ1]		

** Real-time PCR primers and probes for* stx2a::cat *were designed by Camilla Sekse using the sequencing data described in section 2.3.3.*

This study used the 2xBrilliant III Ultra fast QPCR Mastermix (Agilent technologies, USA). DNA templates and controls were added and the PCR was run on Strategene Mx3005P machines (Agilent Technologies, USA). The results were analyzed with the corresponding software program, which created amplification plots based on the fluorescence of the products. PCR program used for the primers and probes is listed in table 2.9.

Table 2.9: Real-time PCR programs and controls used for the primers and probes.

Primer and probes	**PCR program**
PROBE VT2a-Qp + PRIMER VT2a-QfLNA 1 + PRIMER VT2a-QrLNA 2	95°C 3 min – 40 cycles (95°C 3 sek – 60°C 30 sek)
EhxA-Fwd + EhxA-Rev EhxA-Probe *(20x qPCR assay, IDT*)*	95°C 3 min – 35 cycles (95°C 10 sek – 60°C 30 sek)
StxCAT_F1 + StxCAT_R2 + StxCAT_probe1	95°C 5 min – 40 cycles (95°C 20 sek – 60°C 30 sek)

** Integrated DNA Technologies*

2.3.3 Designing primers for conventional PCR

Designing primers was done using the CLC Main Workbench 6 program (CLC Bio/Qiagen, Denmark/Germany). To be able to design primers, a relevant sequence must be available.

Primers for verification of *stx2a::cat* presence were made using Sanger sequence obtained from *E. coli* C600::ϕ731. Conventional PCR with primer pairs GK4 and Cm5, and Rho and Cm3 was run using *E. coli* C600::ϕ731 as template, primer and PCR program information is listed in table 2.6 and 2.7. The PCR product was purified using ExoSAP-IT (Thermo Fisher Scientific, USA). A total of 5 µl PCR product was mixed with 2 µl ExoSAP-IT and placed in a thermocycler. The mix was heat treated with 15 min of 37°C and then 15 min of 80°C, before it was stored at 4°C, and sent to Sanger sequencing (in house).

The sequences were trimmed by manually evaluating the electrogram and aligned in the CLC Main Workbench program. The *cat* gene was then localized on the sequence. To make usable primers which confirm the *cat* gene inside the *stx2a* gene, one area within the *cat* gene and one area within the *stx2a* gene was chosen. Primer pair suggestions were then created by the program within these positions by choosing the option "Design primers" on the nucleotide sequences. Each primer pair would get a score and a suggested annealing temperature. Two pairs were chosen, one pair that annealed to the area of the *stx* gene upstream from the *cat* gene and to the beginning of the *cat* gene and another pair that annealed to the end of the *cat* gene and the *stx2a* gene area downstream of the *cat* gene. These are the primers named StxCM_END_fwd, StxCM_END_rev, StxCM_START_fwd, and StxCM_START_rev in table 2.6. Figure 2.2 illustrates the primer sites, both original and created in this thesis.

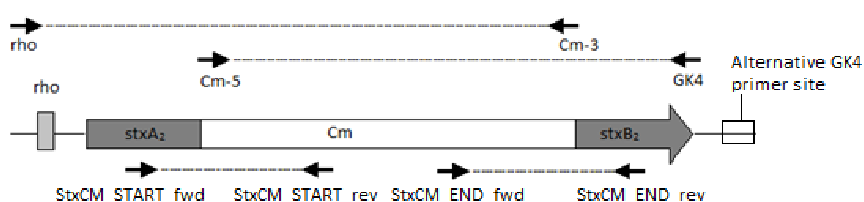


Figure 2.2: Primer sites on stx2a::cat *gene, both designed in this study (both beneath and to the right of the illustration of gene area) and previously designed (above the illustration of gene area).*

For the recombination experiment (described in section 2.4), it was important to evaluate the available sequences of the STEC isolates to see if the primers described in Serra-Moreno *et al.* (2006) were applicable. CLC Genomics Workbench 8, version 8.0.3, (CLC bio/Qiagen, Germany) was used to assemble the genome of the isolate where this was missing, using the *de novo* assembly function. The sequence for the *stx2a* gene was found using the NCBI nucleotide database searching for *E. coli* EDL933 (accession number CP015855).

The *stx2a* gene were localized in the isolates' sequence data, and a subset of the sequences including an area of ca. 800 bp upstream and downstream of the gene was extracted. These were then aligned together with the sequence for the *stx2a* gene gathered from EDL933. The primer sequences were then also aligned to find were, and if, the primers match. Changes in the primers were made before ordering, if necessary. In addition, an alternative primer was created from sequence downstream of the GK4 primer as shown in figure 2.2. Figure 2.3 is a screenshot from the program when the sequences are aligned with some primers.

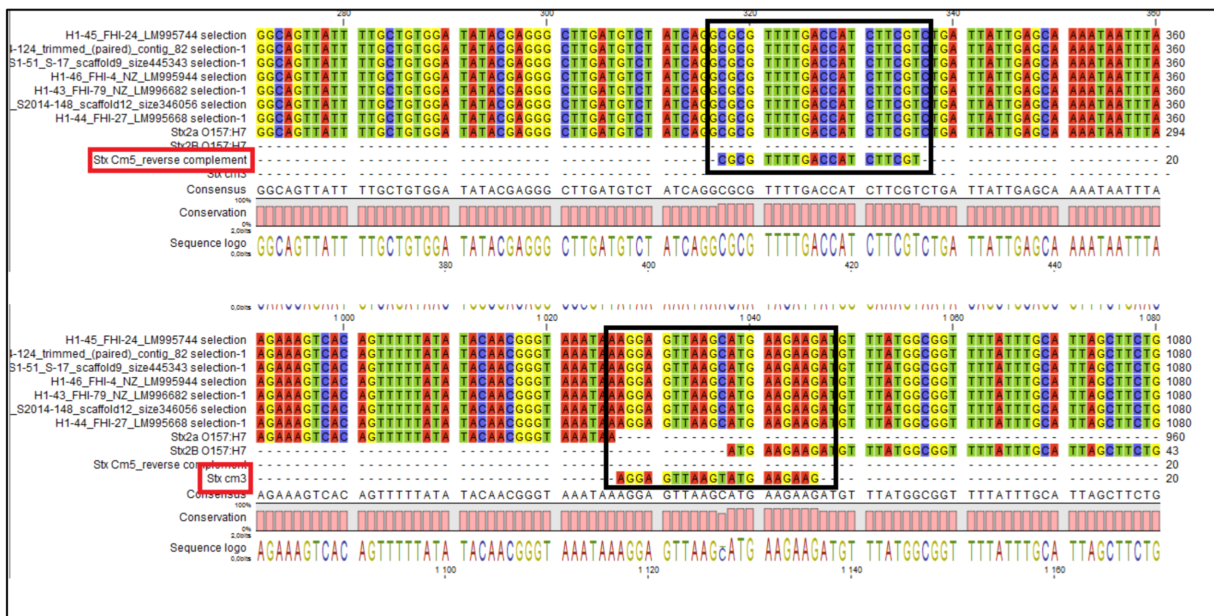


Figure 2.3: Screenshot from the CLC Genomics Workbench program of aligned STEC sequences, stx2a gene sequence of EDL933 and two primers.

2.3.3.1 Validation of new primers

Conventional PCR with the new primers for *stx2a::cat* verification were carried out on the lysogenic *E. coli* strains C600::ϕ731, DH5α::ϕ731, MG1655::ϕ731 and *Shigella sonnei* 866::ϕ731. *E. coli* C600::ϕ731 was used as positive control, while *E. coli* C600 was used as negative control.

For the new alternative primer designed for the recombination experiment, *in silico* searches of the ϕ731 phage and the *stx2a* positive isolates were made to predict the length of PCR product

with *stx2a,* and with *stx2a::cat*. Conventional PCR was run on isolates with original STEC isolates, and their recombinant derives.

2.4 Phage experiments

The phage experiments include preparation of phage filtrates and subsequent quantification and/or spot test, host infectivity tests, and the recombination experiment. Different phages are used in this study, including phage ϕ731, native Stx2a phages and recombinant Stx2a phages created in the recombination experiment part of this study.

The ϕ731 phage is obtained from *E. coli* C600::ϕ731 described in section 2.1. This phage was transduced to aEPEC and STEC isolates through lytic and lysogenic infection. For the lysogenic infections, the isolates were spread on LB agar plates supplemented with 25 mg/L Cm (25 mg/L Cm LB agar plates). Figure 2.4 illustrates the workflow for this phage.

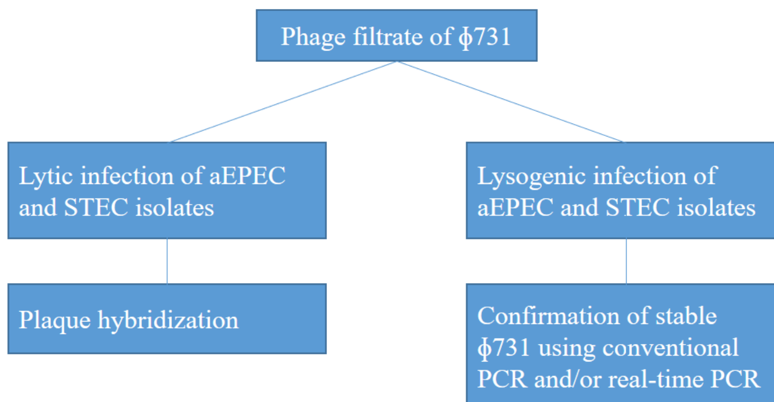


Figure 2.4: Workflow for experiments with phage ϕ731.

Experiments with the Stx2a phages from the STEC isolates also include lytic and lysogenic infection. The strains *E. coli* C600, *E. coli* MG1655 and *S. sonnei* 866 were used as recipient cells. However, since the native Stx2a phages do not contain marker genes which facilitates growth on selective media, the lysogenic infection protocol was modified and subsequent screening of *Stx2a* on single colonies using real-time PCR was required. Experimental overview is illustrated in figure 2.5.

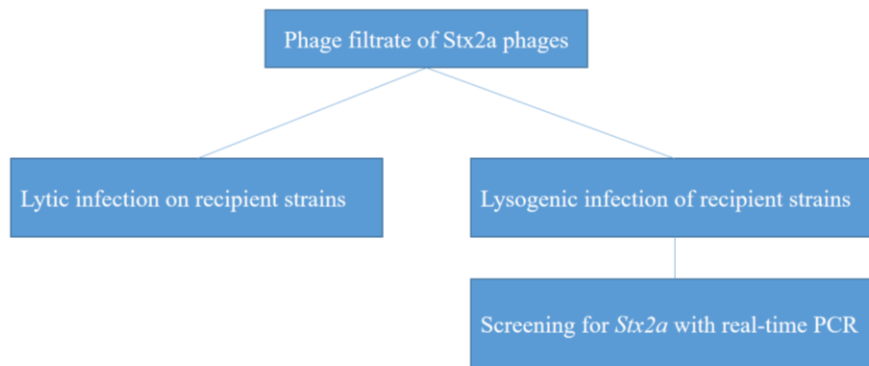


Figure 2.5: Workflow of experiments with Stx2a phages.

The recombinant phages created in the recombination experiment were evaluated for lytic infectivity abilities using certain recipient strains and different phage filtrate dilutions, including spot test. Lysogenic infectivity abilities were also evaluated using several recipient strains, including selected aEPECs.

2.4.1 Phage filtrate

Phage filtrate was prepared by induction of phages with the antibiotic Mitomycin C (MitC) and subsequent filtration as described by Bonanno *et al.* (2016).

Overnight (ON) cultures were made by transferring one colony of the chosen isolate to a tube with 5 ml Luria-Bertoni (LB) broth (Merck, Germany) and incubated ON at 37°C and 150-180 rpm. A total of 1 ml of ON culture of a lysogenic isolate was transferred to a 250 ml Erlenmeyer flask with 100 ml of LB broth. This was incubated at 37°C and 150-180 rpm to an OD of 0.3-0.5 was reached. OD was measured with Ultrospec 10 (Amersham biosciences/GE healthcare Bio-sciences AB, England). When target OD was reached, 50 ml of the culture was transferred to a 100 ml Erlenmeyer flask and this was added 50 µl of 0,5 mg/µl MitC (Sigma-Aldrich, USA). The new flask was covered in aluminums foil to keep light from penetrating as MitC is sensitive to prolonged exposure to light. Both flasks were incubated ON at 37°C and 150-180 rpm. To evaluate if the MitC have had effect, OD was measured from both flasks after the incubation. From the culture with MitC, 15 ml (or 2x15 ml) was transferred to a 15

ml Falcon tube and centrifuged at 4.4 x 1000 rpm for 15 min at 4°C. This was then filtered into a 50 ml falcon tube through a ~0.2 µm pore filter (Minisart Syringe Filter, Sartorious, Germany).

The phage filtrates were also tested for stability, and adjustments to the method to improve phage survival were tried. To calculate/estimate the concentration of phages in the filtrates, phage filtrate quantification and/or spot test was carried out.

2.4.1.1 Phage filtrate quantification

Quantification of phages in filtrate was done by making a dilution series (ten-fold dilutions) of the filtrate from undiluted (UD) to 10^{-6} in PBS de Boer and infecting a control isolate with the respective dilutions. After ON incubation, the plaque forming units (PFUs) on the plates with the different dilutions were counted and the phage concentration in the undiluted filtrate calculated: Number of plaques x 10 x the inverse of the dilution factor value (Sambrook *et al*. 2001).

A total of 50 µl of ON culture of recipient strain was added to 5 ml of LB broth and grown to an OD of 0.3-0.5 by incubating at 37°C and 150-180 rpm. Soft agars (0.7 % LB agar) were used as top agar and prepared by boiling for 5 minutes and placed in a water bath at 50°C until further use. LB agar plates were prewarmed to 37°C. Eppendorf tubes were prepared by mixing 100 µl of phage filtrate (of the respective dilutions) and 100 µl of 0.1M $CaCl_2$. When the cultures hit their target OD, 900 µl of culture were added to the prepared Eppendorf tube. The Eppendorf tubes were incubated at 37°C for 30 minutes. After incubating, the content of the Eppendorf tube was transferred to the molten LB top agar and mixed well. This was then immediately poured onto a LB agar plate, distributed evenly and allowed to solidify. The plates were incubated at 37°C ON.

2.4.1.1.1 Spot test to evaluate infectious capacity

Spot test of phages in filtrate was done by making a dilution series of the filtrate from UD to 10^{-5} in PBS de Boer and infecting a control isolate with the respective dilutions on the same agar plate, divided in six zones.

This protocol is similar to the protocol in section 2.4.1.1, in regards to the preparation of cultures, soft agars and LB agar plates. When the cultures hit their target OD, 900 µl of culture was added directly to the molten top agarose and mixed. The soft agar was then immediately added 100 µl 1M $CaCl_2$ and further mixed, before poured onto a LB agar plate and allowed to solidify. When the agar was firm, 10 µl of each dilution was pipetted within its respective zone of dilution. This was let dry and incubated ON at 37°C.

After ON incubation, the PFUs on the plates with the different dilutions were evaluated to analyze the lytic abilities of the phages.

2.4.1.2 Phage filtrate stability test

Phage ϕ731 was used in the tests to evaluate the stability of phage survival in storage and possible improvements. By repeating the phage filtrate quantification after keeping the filtrate in refrigerator for several days, phage filtrate stability was established.

2.4.1.2.1 Phage survival with chloroform

Chloroform was used to test if the phages survival in filtrate could be improved. After phage filtrate was made as described in section 2.4.1, 6 ml was transferred to a new 15 ml Falcon tube and added 1 ml chloroform. This was vortexed and then centrifuged at 3000 x *g* for 5 minutes. Then, 5 ml of the supernatant was transferred to a new falcon tube without transferring the chloroform. Both filtrates were stored in refrigerator at 2-8°C. Phage filtrate quantification was set up one week later using both filtrates to compare and evaluate if chloroform have effect on phage survival.

2.4.1.3 Growth curves

When preparing phage filtrate, growth curves for STEC isolates and the host *E. coli* C600::ϕ731 with and without MitC was established. As the cultures were grown in shaking incubator, OD was regularly measured.

2.4.2 Host infectivity

Host infectivity include lytic and lysogenic infection by the phages to their hosts/recipients, which reflect the phage susceptibility of these hosts. The lysogenic infection experiments have three slightly varying protocols. The potential lysogens were confirmed using PCR (except the lysogens listed in table a.3 in appendix A). The lytic infection was evaluated. Three plates from the lytic infection with ф731 were chosen for plaque hybridization.

2.4.2.1 Lysogenic infection

The protocol used to create lysogenic bacteria was gathered from Bonanno *et al.* (2016). Some modifications were made, and two additional methods were developed. When phage ф731 or the recombinant phages have been used, the potential lysogenic bacteria are spread onto selective media i.e. LB agar plates with 25 mg/L Cm. For the experiments with Stx2a phages, LB agar plates were used.

Protocol 1

A total of 50 µl of ON culture of recipient isolate was added to 5 ml of LB broth and grown to an OD of 0.3-0.5 by incubating at 37°C and 150-180 rpm. Then, 100 µl was added to Eppendorf tubes containing 800 µl of LB and 100 µl of phage filtrate and incubated ON at 37°C. After incubation, the Eppendorf tubes were centrifuged at max speed for 5 minutes. The supernatant was removed and the pellet was resuspended in 100 µl of LB. This material was spread onto LB agar plates with or without 25 mg/L Cm. The plates were placed in incubator ON at 37°C.

Protocol 2

A total of 50 µl of ON culture of isolate was added to 5 ml of LB broth and grown to an OD of 0.3-0.5 by incubating at 37°C and 150-180 rpm. Then, 100 µl was added to Eppendorf tubes containing 800 µl of LB, 100 µl 0.1M $CaCl_2$ and 100 µl of phage filtrate and incubated for 2 hours at 37°C, at 150-180 rpm. 100 µl from each tube was then spread onto LB agar plates with or without 25 mg/L Cm. The plates were placed in incubator ON at 37°C.

Protocol 3

This protocol is similar to protocol 1, but with the addition of 100 µl of 0.1M $CaCl_2$ to the Eppendorf tubes before ON incubation.

2.4.2.2 Lytic infection

Lytic infection was performed using the same protocol as described in 2.4.1.1 except a dilution series was not always made. Mainly UD phage filtrate was used, but in a few cases the phage filtrate was diluted to 10^{-1}.

For the lytic infection by the ϕ731 on STEC as recipient strains, a control was set up for each isolate. This was done because the isolates may be affected by their own Stx2a phages. The procedure for the control was the same as described in 2.4.1.1, but without the addition of ϕ731 phage filtrate. This way, it was possible to evaluate if lysis on the plate maybe were a result of lytic abilities of the Stx2a phages.

The phage filtrate quantification used *E. coli* C600 as control isolate, which was used to verify the experiment conditions for the lytic infection.

2.4.2.3 Plaque hybridization

Plaque hybridization was done to verify lytic infection on host strain. The DIG DNA labelling and detection kit from SigmaAldrich (USA) was used, and the method was based on the manufacturer's protocol and Muniesa and coworkers (Muniesa *et al.*, 2003).

Probe preparation

A probe that targeted the ϕ731 phage was made. First, DNA extraction of control isolate C600::ϕ731 was done using the boiling method. Then, two PCRs were run using the primer pairs StxCM_END_fwd and StxCM_END_rev, and StxCM_START_fwd and StxCM_START_rev (see table 2.6 and 2.7). The PCR product (probe) was purified using the Nucleospin Extract II kit (Macherey-Nagel, Germany). After the purification, the probe was then labelled using the DIG DNA labelling and detection kit. The probe was stored in refrigerator until use.

Plaque hybridization

Buffer information is listed table c.1 in appendix C.

Plaque transfer onto nylon membrane: A Nylon Membrane for Colony and Plaque Hybridization (Roche, USA) were placed onto one plate from phage quantification test and three different plates from the lytic infection experiment, respectively, for approximately 2 minutes and marked. With the side of the membrane with the plaque facing upwards, the membrane was transferred to a Whatman filter (Munktell, Sweden) wetted with Denaturalization buffer for 5 minutes. The membrane was then transferred to a Whatman filter wetted with Neutralization buffer for 5 min. Empty petri dishes were added 2xSSC and the membranes were washed in these for 5 minutes. After the wash, the membranes were dried and a positive control of 1 µl of C600::ϕ731 DNA was spotted on the membrane. For DNA fixation, UV light was used for 2 minutes on each side. The membranes were kept in Falcon tubes (50 ml) in the fridge until hybridization.

Pre hybridization: The membranes were pre hybridized in hybridization buffer in the hybridization oven at 68˚C for ca. one hour. The hybridization buffer was discarded and hybridization buffer with probe added (approximately 10 ml for each blot). The membranes were then hybridized at 55˚C for two days (should have been ON, but was extended).

Washing: Washing of the membranes was done by first placing the membranes in 2xSSC, 0.1% SDS at room temperature for 2 x 5 min, using petri dishes on shaking apparatus. Next, they were washed with 0.04% SSC, 0.1% SDS at 60˚C in the hybridization oven for 2x 15 min using pre-warmed solution.

Colour reaction: The membranes were washed in Buffer 1 for 1 minute. Then they were further washed in Buffer 2 for 30 minutes. Again, the membranes were washed in Buffer 1 for 1 minute. The membranes were then incubated with antibody solution for 30 minutes at room temperature, before wash in Washing buffer 2 x 15 minutes. The washing was done in petri dishes placed on shaking apparatus. The membranes were then equilibrated in Buffer 3 for 2 minutes at room temperature. The membranes were placed in a hybridization bag (Roche, Germany) and added ca. 3 ml colouring solution per membrane. This was wrapped in aluminum foil and kept dark. When the colour appeared after ca. 6 hours, the membranes were washed in distilled water and dried.

2.4.3 Confirmation of stable lysogenic bacteria

To confirm stable lysogenic bacteria from lysogenic infectivity experiments with the ϕ731 phage, 1-2 single colonies from each isolate with growth on the 25 mg/L Cm LB agar plates were replated several times. Replating involves picking a single colony and spreading this onto a new LB agar plate, in this case also containing 25 mg/L Cm. This process was repeated a minimum of two times for each single colony from a lysogenic isolate. DNA was extracted with the boiling method from colonies which had been replated at least twice, and both conventional and real-time PCR was used for confirmation of the *stx::cat* gene.

For the confirmation of stable lysogenic bacteria from the lab strains with Stx2a phages, a screening was first conducted: Single colonies from the LB agar plates described in section 2.4.2.1, were picked with a loop and point inoculated onto new LB agar plates with a numbered grid. The left-over material in the loop was deposited in an Eppendorf tube with 500 µl MilliQ water. Material from ca. 10 single colonies was placed in the same Eppendorf tube, and DNA was extracted using the boiling method. The LB agar plate was incubated ON at 37°C.

Real-time PCR was run on the pooled samples. If the analysis gave positive result for presence of *stx2a* in a pooled sample, the associated isolates in the LB agar plate with grid was individually extracted for DNA with the boiling method and replated onto a new LB agar plate with grid which was incubated ON at 37°C. When the individual *stx2a* positive strains from the pooled samples were identified by PCR, yet another replating was performed. The isolate could be replated several times before the final confirmation of stable Stx2a phage.

2.4.3.1 Conventional PCR

To confirm stable ϕ731 lysogenic bacteria, conventional PCR using the primer pairs GK4 and Cm5, and Rho and Cm3 were initially run, but were later replaced by primer pairs StxCM_END_fwd and StxCM_END_rev, and StxCM_START_fwd and StxCM_START_rev. Primer information is listed in table 2.6. The PCR program run with the different primer pairs is listed in table 2.7. The products were visualized as described in section 2.3.1.2.

2.4.3.2 Real-time PCR

Real-time PCR was run to confirm presence of *stx2a::cat* and *stx2a.* The primers and probes are described in table 2.8, and the PCR program is described in table 2.9.

2.4.4 Recombination experiment

The recombination experiment in this study used a modified version of the protocol described by Serra-Moreno *et al.*, 2006. Homologous recombination was used to alter Stx2a phages from STEC isolates. The alteration introduced an antibiotic resistance cassette carrying the *cat* gene, replacing part of the *stx2a* gene. The workflow of the recombination experiment is illustrated in figure 2.6.

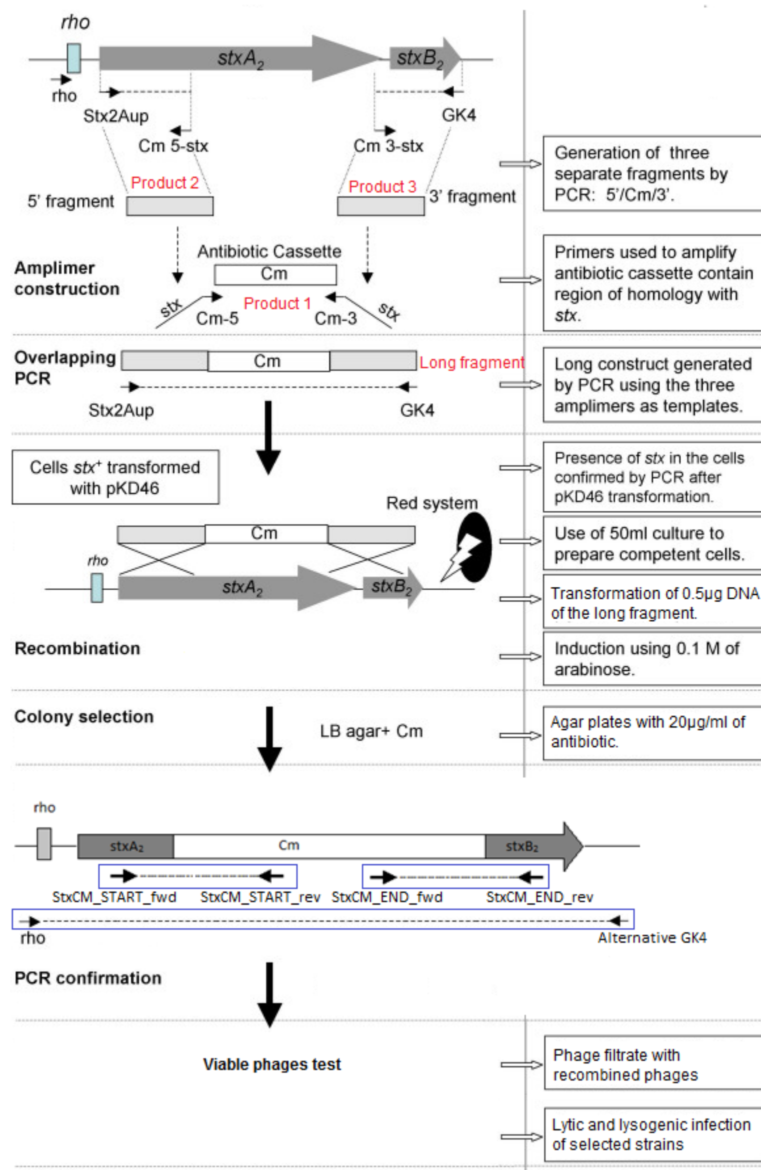


Figure 2.6: Recombination experiment workflow. This figure is a modified version of Figure 1 in Serra-Moreno et al*, 2006. Amplimer construction: Fragments of the antibiotic cassette is generated (product 1), in addition to fragments from the start and end of the* stx *gene (product 2 and 3). Overlapping PCR: The fragments of product 1, 2 and 3 are run together as templates in one PCR to create a long construct (long fragment). Recombination: The long fragment is introduced to competent cells using electroporation. Colony selection: Colonies from LB agar plates with 20 µg/ml antibiotic were replated two times. PCR confirmation: Three conventional PCRs and one real-time PCR was run to validate presence of* stx2a::cat*. The areas marked in blue represent the conventional PCR products. Viable phages test: Phage filtrate of the recombinant phages were made and lysogenic infection was performed on selected strains.*

Before the strains could be subjected to the recombination, plasmid experiments with pUC19 and pKD46 were performed on strains listed in table 2.2 and in table a.2 in appendix A.

2.4.4.1 Plasmid experiments

The plasmids pUC19 and pKD46 were used in this part of the experiment. pUC19 was used to determine the host cell transformation efficiency, while introduction of pKD46 in the bacteria was essential for recombination later in the experiment.

The recipient cells were treated similarly until the plasmids were added. A total of 100 µl of the respective ON cultures was added to 10 ml of LB broth. The cultures were grown in a shaking incubator at 37°C and 150-180 rpm. When the cultures reached an OD value of 0.4-0.6 they were placed on ice for ten minutes. From this point on, all solutions, cuvettes (Bio-Rad, Gene Pulser Cuvette, 0.1 cm, USA), Eppendorf tubes and samples were kept ice-cold, unless otherwise stated. After cooling, the samples were centrifuged at 4°C and 3000 x *g* for five minutes. After centrifugation, a pellet was formed at the bottom of the tube. The supernatant was poured off and the pellet was resuspended in 10 ml ice-cold, sterile MilliQ water and centrifuged at 4°C and 3000 x *g* for five minutes. This step was repeated twice, achieving a total number of three MilliQ wash steps. After the last wash step with MilliQ, the pellet was resuspended in what was left of the MilliQ water after the supernatant was poured off (ca. 100 µl).

From each sample, 50 µl was added to two Eppendorf tubes. A total of 3 µl (ca. 13 ng/µl) pKD46 was added to one of the tubes. The equivalent of 10 pg pUC19 DNA (1 µl) was added to the other tube. The samples were then transferred to the chilled cuvettes which have a 1mm space between the electrodes, and underwent electroporation using GenePulser Xcell (Bio-Rad, USA) with set specifications: voltage =1800 V, capacitance = 25 µF, and resistance = 200Ω. After electroporation, 1 ml LB was added to the cuvette containing the sample and mixed, and transferred back to its Eppendorf tube and kept at room temperature.

Bacterial cells treated with pUC19: The cells were incubated for one hour at 37°C. After incubation, 100 µl from each sample was spread on to LB agar plates with 50 mg/L Ampicillin, and incubated ON at 37°C. The next day, the number of colonies on the plates was counted. The competence of the bacteria, also called transformation efficiency, is calculated using this formula: (#of colonies /10 pg pUC19 DNA)·(10^6 pg/µg)·(Dilution factor 1050 µl total volume/100 µl plated)

Bacterial cells treated with pKD46: The cells were incubated for one hour at 28°C. After incubation, the samples were spun for one minute at 5000 x *g*. After centrifugation, 800 µl

supernatant was removed from each sample, and the pellet was resuspended in the remaining solution. This (ca. 200 µl) was then spread onto LB agar with 50 mg/L Ampicillin, and incubated ON at 28°C. Only the isolates that incorporated pKD46 and subsequently grew on LB with Ampicillin, were used further.

2.4.4.2 Amplimer construction and overlapping PCR

The construction of the DNA fragment used for the recombination experiment (referred to as "the long fragment") consists of several PCRs, gel runs and purifications. First, PCR products of the smaller fragments were created, and then the long fragment was created by combining the small fragments, see figure 2.6. After every PCR, the products were purified using QIAquick gel extraction kit (Qiagen, Germany) following the manufacturer's protocol. All centrifugation steps were carried out at 17900 x *g* (13000 rpm) in a conventional table-top microcentrifuge (Eppendorf centrifuge 5415D, Eppendorf AG, Germany). For the PCR products that were purified directly and not via gel, 40-45 µl PCR product was added to 150 µl Buffer QG. The size, amount and purity of the DNA was subsequently analysed on a gel as described in section 2.3.1.2 and/or the concentration and purity of the DNA was analysed using a NanoDrop 2000c spectrophotometer (Thermo Scientific, USA).

DNA was extracted from *E. coli* DH5α with pKD3 and the isolate C1-50 with pKD46 as described in section 2.2.2. DNA from DH5α with pKD3 and C1-50 with pKD46 was used as template, and three different PCRs were run. Primer pairs used for the different templates are described in table 2.10. The table also include the PCR product name after the run. Further information about the primers and PCR runs is described in table 2.6 and 2.7.

Table 2.10: Template, associated primer pairs and PCR product name used in the recombinant experiment.

Template	Primers	PCR product name
DNA from DH5α with pKD3	Cm-5 / Cm-3	Product 1
DNA from C1-50 w. pKD46	Cm5-stx / stx2aup	Product 2
	Cm3-stx / GK4	Product 3

Product 1 was purified from gel, Product 2 and 3 from PCR product. The next PCR had Product 1-3 as templates and GK4 and Stx2Aup as primer pairs. Table 2.6 and 2.7 describes the primers and PCR runs. The final PCR product is the long fragment, which was purified from a gel.

2.4.4.3 Recombination

To confirm the pKD46 carrying isolates still also carried the Stx2a phage, DNA was extracted as described in section 2.2.1, and real-time PCR was run to confirm presence of the Stx2a gene (primers, probe and PCR program is described in table 2.8 and 2.9).

This part of the experiment is similar to the plasmid experiment, but several small adjustments are included. Using 250 ml Erlenmeyer flasks, 50 ml of LB broth, 500 µl of the respective ON cultures, 50 µl Ampicillin (final concentration of 50 mg/L) and 7.5 ml L(+) Arabinose(10%) were mixed. The cultures were grown in a shaking incubator at 30°C and 200 rpm. When the culture hit an OD value of 0.4-0.6 the samples were transferred to 2x50 ml Falcon tubes and placed on ice for 10 minutes. From this point on, all solutions, cuvettes and samples were placed on ice, unless otherwise stated. After cooling, the samples were centrifuged at 4°C and 3000 x *g* for five minutes. After centrifugation, a pellet was formed at the bottom of the tube. The supernatant was poured off and the pellet was resuspended in 10 ml ice-cold, sterile MilliQ water and centrifuged at 4°C and 3000 x *g* for five minutes. This step was repeated twice, achieving a total number of three MilliQ wash steps, but before the last centrifugation the samples were pooled respectively. The last centrifugation was 4°C and 5000 x *g* for five minutes. After the last wash step with MilliQ, the pellet was resuspended in what was left of the MilliQ water after the supernatant was poured off (ca. 120 µl).

For each sample, 50 µl were transferred to two Eppendorf tubes. A total of 3.5 µl (142.4 ng/µl) of the long fragment was added to each tube. The Electroporation was performed as in 2.4.4.1.

The samples were incubated at 37°C for three hours. After incubation, the samples were centrifuged for 1 minute at 5000 x *g*. After centrifugation, 800 µl of supernatant was removed and the pellet was resuspended in the remaining solution. A total of 100 µl was spread onto LB agar plates with 5 mg/L and 20 mg/L Cm for each sample, and incubated ON at 37°C.

2.4.4.4 Confirming phage recombination

To confirm the recombinant Stx2a phages, PCRs were run as described in figure 2.6. However, PCRs are not a definite test of viable phages. Therefore, phage filtrate was prepared and lysogenic infection test was performed. Phage filtrate quantification and lytic infection tests were performed simultaneously with the lysogenic infection test.

2.4.4.4.1 Verification using PCR

Confirmation of the recombinant phages was done using conventional PCR and real-time PCR targeting the *stx2a::cat* gene. The colonies that grew on the 20 mg/L Cm plates were tested. Selected colonies were replated two times, and DNA was extracted as described in 2.2.1. Table 2.6-2.9 describes the primers and PCR programs used in the conventional PCRs and the real-time PCR run.

2.4.4.4.2 Verification of viable phages

The host strains containing the recombinant phages were spread onto LB agar plates with 25 mg/L Cm and incubated ON at 37°C to confirm growth at this concentration, which is the concentration used in the lysogenic infection experiments.

To see if the recombinant phages still had infective abilities, phage filtrate was prepared and host infectivity experiments were run on selected strains described in table 2.1 and 2.3. Both lysogenic and lytic infective abilities were tested. The lab strains were subjected to lysogenic infection using all three protocols described in 2.4.2.1. The phage filtrates were also quantified with lab strains *E. coli* C600 and *S. sonnei* 866 using the spot test method and plates with UD filtrate (and dilution 10^{-1} for *S. sonnei* 866), described in 2.4.1.1 and 2.4.1.1.1.

2.5 Characterization of *E. coli* O26 isolates

2.5.1 Rhamnose and dulcitol fermentation (RDF) test

In order to determine the ability of an isolate to ferment rhamnose and dulcitol, a RDF test was carried out as described in Brandal *et al.* (2012). Tubes containing L-Rhamnose (Sigma-Aldrich, USA) and Dulcitol (Fluka, England), respectively, and BBL Phenol Red Broth Base (BD, USA) were each added one colony of the isolate in question. This was then incubated ON at 37°C. The tubes were inspected for colour change. The solution is originally red. If the colour has turned to yellow, this indicates fermentation.

2.5.2 Screening for virulence-associated gene ehxA

The samples tested for presence of e*hxA* is described in table 2.2 and 2.3. Real-time PCR was used, which is described in section 2.3.2. The primers and probes are described in table 2.8, and the PCR program is described in table 2.9.

2.5.3 Preparation for whole genome sequencing

Several of the *E. coli* O26 isolates chosen for this study had been sequenced earlier. For those missing whole genome sequencing (described in table 2.3), DNA samples were prepared and sent to Oslo University Hospital, Ullevål to be sequenced with NextSeq mid-output run with Nextera XT Library prep. Some lysogenic isolates described in table a.1 in appendix A were also prepared for whole genome sequencing. DNA was extracted as described in section 2.2.2.

2.5.3.1 DNA concentration measurement with NanoDrop 2000

Using TRIS (10 mM, pH 8) as blank, all the samples were measured on the NanoDrop 2000c spectrophotometer (Thermo Scientific, USA) to evaluate the purity of the samples. The 260/280 ratio had to be at least 1.8.

2.5.3.2 Normalisation of DNA using Qubit

The concentration of the DNA samples was normalized to lie between 8.5 and 11.5 ng/µl. Qubit 3.0 Fluorometer (ThermoFisher Scientific, USA) was used. Instructions on the Qubit Assays quick reference card were followed.

2.5.3.3 Visualizing of DNA on gel

This part was necessary for the samples to be accepted by the sequencing lab. The gel was prepared and run as described in section 2.3.1.2. All samples were applied to the gel, and the gel picture was sent along with the sample data to the sequencing lab.

2.6 Data analysis

2.6.1 CLC Genomics Workbench

CLC Genomics Workbench program (version 9.5.4) was used to evaluate the insertion sites. WGS data from selected isolates were BLAST searched for primer binding sites associated with insertion sites. Information on the insertion sites, and associated primers was gathered from a published paper by Bonanno *et al*. (2015). The isolates chosen for inspection are described in tables 2.2 and 2.3, in addition to table a.1 in appendix A.

Before performing BLAST searches, raw data from the WGS should be assembled. The STEC isolate without ϕ731 was already assembled. To assemble the other isolates, the tutorial presented on Qiagen bioinformatics web page named "De novo assembly and BLAST" from September 2016 was used. Using the information in this tutorial, a work flow was created to automatically assemble all the genomes in a similar fashion. The work flow and details to assembly is illustrated in figure 2.7 below.

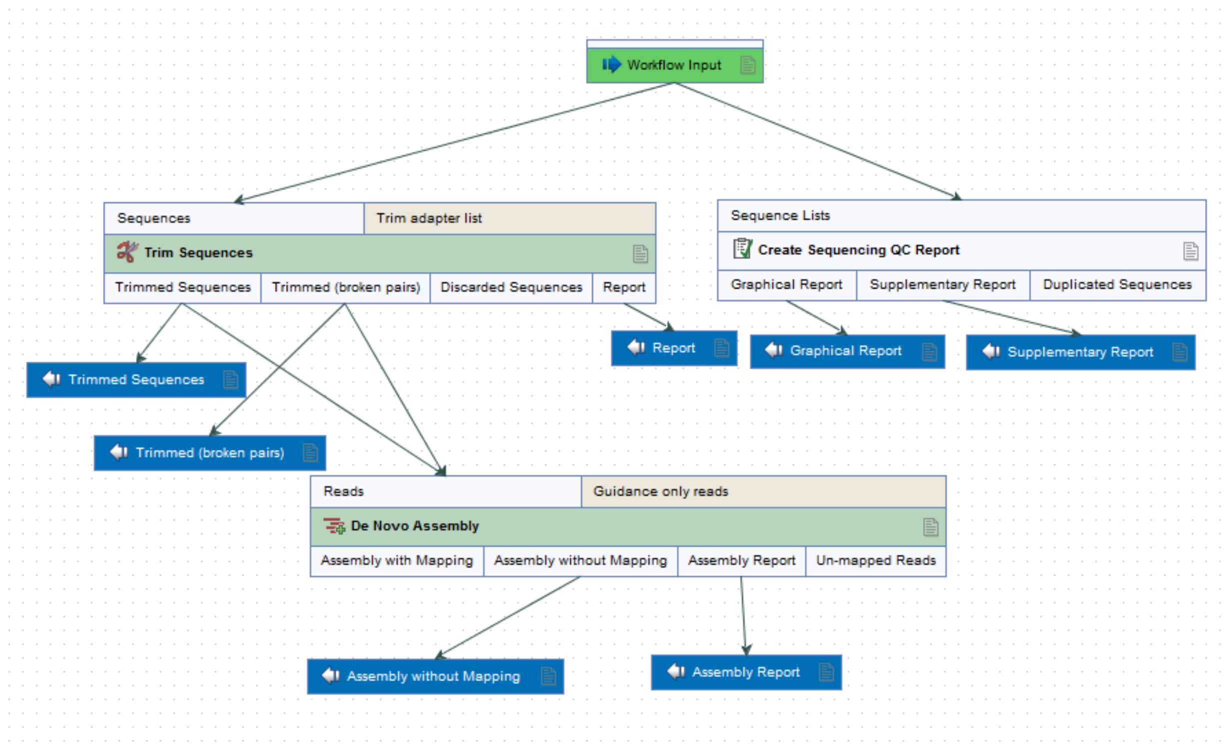


Figure 2.7: Screen shot of work flow used to assemble genomes. The boxes explain the specifications made in the command.

Several alternative pathways were tried and evaluated, including using longer and shorter contigs, mapping reads back to contigs, etc.

2.6.2 CGE VirulenceFinder 1.5

The assembled WGS data from all the STEC isolates were uploaded to the CGE VirulenceFinder 1.5 database, and results were returned.

3 Results

3.1 Phage studies

The phage studies include the phage susceptibility experiments with both native Stx2a phages and phage ɸ731, phage survival in filtrate tests and recombination experiment. The phage susceptibility experiments involve evaluation of both lytic and lysogenic infective abilities of the phages, and which is also referred to as host infectivity experiments. When describing the lytic infection, the comment "complete lysis" is used when the phages have lysed most of the cells and the plaques are impossible to count. "Almost complete lysis" is used when there was thin growth of the bacteria on the plates which indicates some lysis; enough to hinder bacterial growth, but not enough to create areas of complete lysis. Other descriptions include estimates of PFU or "no lysis" (more than thin bacterial growth covering the plate).

The lysogenic infectivity experiments showed that it was possible to create lysogenic bacteria, both with the ɸ731 phage and the native Stx2a phages (including the recombinant phages). Several lysogenic isolates were created, and an overview of these lysogenic isolates is provided in appendix A, table a.1-a.3. However, lysogenic infection of recipient strains by native Stx2a phages were far more troublesome and lysogenic bacteria were found in much lower numbers.

3.1.1 Studies with phage ɸ731

Phage ɸ731 was obtained from *E. coli* C600::ɸ731, described in section 2.1 and listed in table 2.1. In each experimental set up, the concentration of phages in filtrate was quantified using *E. coli* C600 as recipient strain and a dilution series of the phage filtrate. The phage filtrate concentration varied from ca. $1.0 \cdot 10^6$ PFU/ml to $5.0 \cdot 10^7$ PFU/ml. The recipient strains for the host infectivity experiments with phage ɸ731 are listed in table 2.1-2.3. Some of the lysogens with phage ɸ731 were whole genome sequenced, and two were used in the data analysis of insertion sites, this is described in table a.1 in appendix A.

3.1.1.1 Lytic infection

In the first lytic infection experiment performed with phage ϕ731, the recipients as described in table 2.1 were used. A total of 100 µl of UD phage filtrate (ca. $6.0 \cdot 10^6$ PFU/ml) was used and the result was complete lysis of all the isolates.

The subsequent experiments were performed using the *E. coli* O26 isolates (table 2.2 and 2.3) as recipients. Only one replicate of the experiment was performed per isolate. The phage filtrate concentration varied as described in section 3.1.1. The results showed no lysis or visible PFUs in any of the samples. To verify the lack of PFU and lysis, plaque hybridization was performed for three samples. The plates with isolates H0-2, H0-6 and S0-10 and 100 µl UD phage filtrate (ca. $1.0 \cdot 10^6$ PFU/ml) from the lytic infection experiment was chosen. *E. coli* C600 with 10^{-3} dilution of phage filtrate (ca. 1000 PFU/ml) was used as control. Picture of the hybridization filters of the control and S0-10 is shown in figure 3.1.

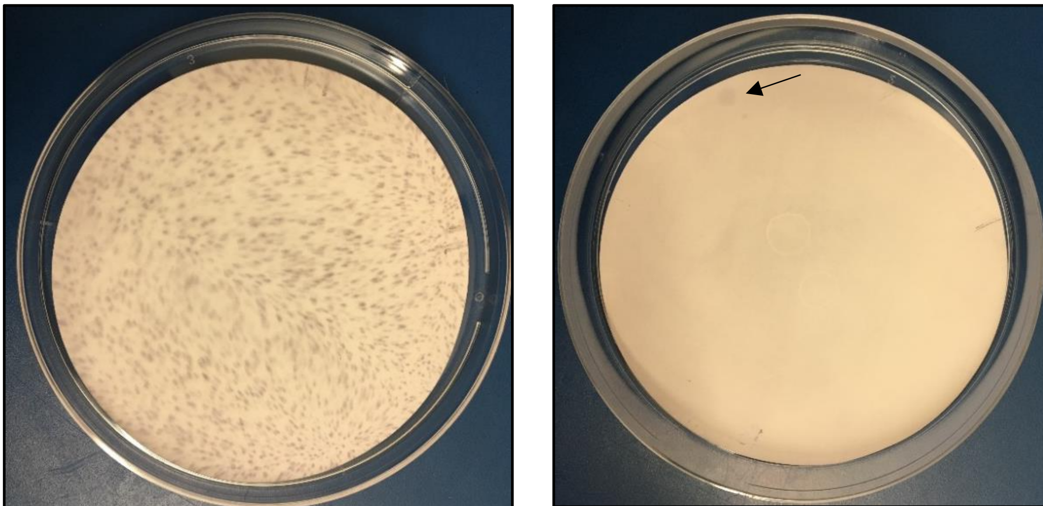


Figure 3.1: Plaque hybridization filters. The left picture is of the filter from E. coli C600 treated with 10^{-3} diluted phage filtrate. The purple spots are plaque forming units created by lytic infection of phage ϕ731. The picture on the right is of the filter with isolate S0-10 treated with undiluted phage filtrate of phage ϕ731. At the top left of the filter (arrow), a purple control spot is visible where 1 µl of phage DNA had been applied.

As figure 3.1 shows, the control for the plaque hybridization had rather high numbers of PFU, while S0-10 did not have any. The control DNA spotted on the S0-10 filter verified the experiment conditions. The result for H0-2 and H0-6 is similar to that of S0-10, and the lack of PFU was verified.

3.1.1.2 Lysogenic infection

Lysogenic infection with ϕ731 was performed using protocol 1 as described in 2.4.2.1 using LB agar plates supplemented with 25 mg/L Cm. In the first lysogenic infection experiment with phage ϕ731, recipients as described in table 2.1 were used. These strains were all successfully infected by the phage. *E. coli* DH5α however had only a few microcolonies, whereas the other strains had single colonies covering the plate and even overgrowth on the LB agar plates supplemented with 25 mg/L Cm.

Lysogenic infection of the *E. coli* O26 isolates was then performed in multiple set ups of the experiment. Two replicates were performed on all the isolates. In each experiment performed, *E. coli* C600 was used as a control to validate the experiment conditions.

The lysogenic infection of aEPEC O26 isolates with phage ϕ731 is illustrated in figure 3.2 and 3.3 with the approximate colony count from both experiments run on each isolate. Some isolates seem to be far more susceptible to the phage than others, which are visualized in figure 3.2. These isolates include H0-1, S0-13, S0-14, S0-17 and S0-19.

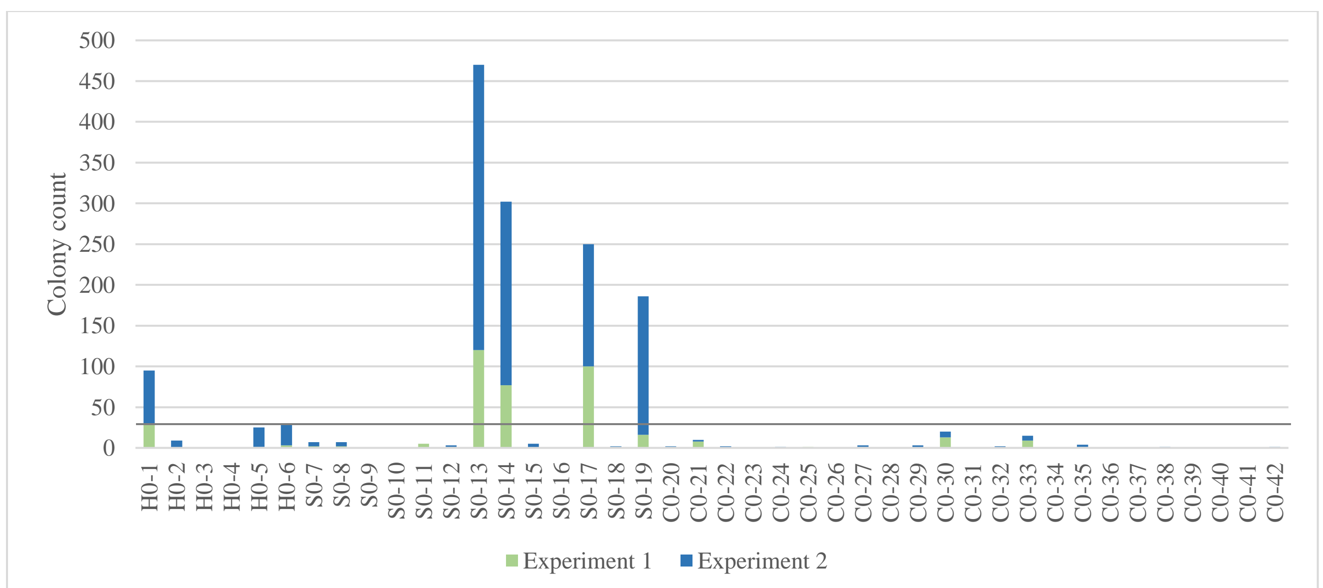


Figure 3.2: Lysogenic infection of aEPEC O26 isolates with phage ϕ731. The approximate colony count of lysogenic bacteria from experiment 1 (green color) is added to the approximate colony count from experiment 2 (blue color) for each isolate. The dark grey line in the figure is placed at a total colony count of 30.

The dark grey line in figure 3.2 is placed at colony count of 30 colonies. Most isolates were beneath this value. In fact, most isolates had a low total colony count from the experiment runs, or were not susceptible to the phage at all. This is visualized in figure 3.3, where total colony counts above 30 colonies are not shown.

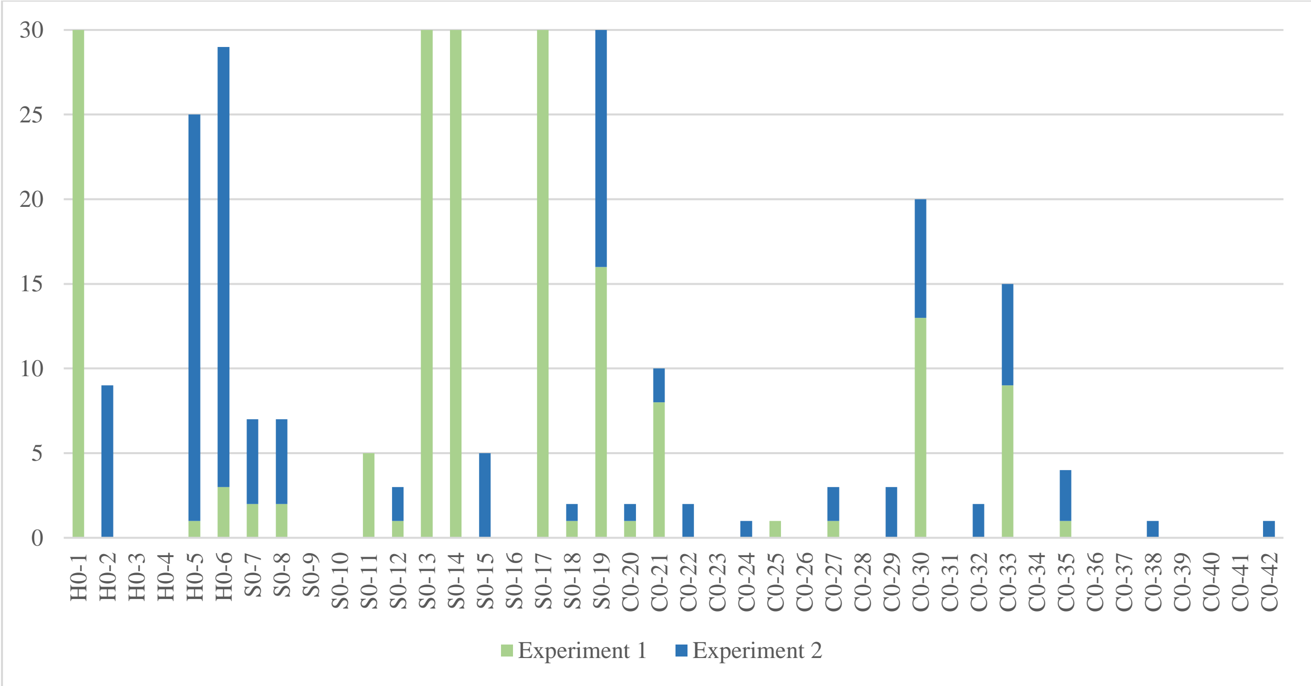


Figure 3.3: Lysogenic infection of O26 aEPEC isolates with phage ϕ731. The approximate colony count of lysogenic bacteria from experiment 1 (green color) is added to the approximate colony count from experiment 2 (blue color) for each isolate. The maximum total colony count in this figure is 30 colonies. Isolates H0-1, S0-13, S0-14, S0-17 and S0-19 had a higher total colony count, and the total amount is not shown.

Figure 3.2 and 3.3 shows that there is a difference in consistency for the lysogeny results. Regarding some recipients, lysogenic bacteria were created in the first or second experiment, but not in both. Other recipients were consistently susceptible or not susceptible to the phage. There also seemed to be a difference in how susceptible the isolates are related to source origin. These observations are listed in table 3.1.

Table 3.1: Isolates' susceptibility to phage ϕ731 by source origin and total. The table shows how many isolates that were susceptible to the phage per experiment, consistent susceptible isolates and total number of lysogenic isolates.

Source	Total number isolates	Lysogenic infection Experiment 1	Lysogenic infection Experiment 2	Consistent susceptible isolates	Total number lysogenic isolates
Human	6	3/6	4/6	3/6	4/6
Ovine	13	9/13	9/13	8/13	10/13
Bovine	23	7/23	12/23	6/23	13/23
ALL	42	19/42	25/42	17/42	27/42

As table 3.1 depicts, the isolates from bovine sources seemed to be the least susceptible. Of the 23 isolates from this group, only six isolates were consistently susceptible. Nevertheless, after two replicates of the experiment, a total of 13 isolates were infected with the phage. The isolates from ovine sources seemed to be more susceptible as a group, with ten susceptible isolates of a total of 13. This is also true for the human source isolates, where a total of four susceptible isolates was seen of six isolates in total. However, few of the lysogenic isolates had a colony count of more than five in each experiment as seen in figure 3.3.

The STEC O26 isolates were all were susceptible to the ϕ731 phage. There were some differences in number of lysogenic bacteria created. Table 3.2 shows that the isolates H1-43, H1-44, H1-44, C1-47 and C1-50 were highly susceptible to the phages, whereas H1-46 and S1-51 were susceptible, but far fewer lysogens were created.

Table 3.2: Description of growth on 25 mg/L Chloramphenicol LB agar plates from the 1. and 2. experiment of the lysogenic infection using phage ϕ731 to infect Shiga toxin-producing Escherichia coli *isolates.*

Isolate name	Experiment 1	Experiment 2
H1-43	~100 colonies	200-300 colonies
H1-44	>1000 colonies*	>1000 colonies*
H1-45	>1000 colonies*	Overgrowth
H1-46	1 colony	1 colony
C1-47	>1000 colonies*	160-260 colonies
C1-50	>1000 colonies*	>1000 colonies*
S1-51	4 colonies	11 colonies

** Too many isolates to count on the plates.*

As the experiment was set up multiple times to include all isolates twice, there were differences in the phage quantity that the isolates were subjected to. For some isolates, lower concentration yielded more lysogenic bacteria. When excluding differences of ten colonies or less, this effect was seen in various degrees for S0-13, S0-14, S0-17, S0-19, H1-43 and H1-45. The latter isolate had >1000 colonies on the 25 mg/L Cm LB agar plates when treated with phage filtrate of ~$3,1 \cdot 10^7$ PFU/ml. When the same isolate was treated with phage filtrate with ~$5,1 \cdot 10^6$ PFU/ml, the result was overgrowth. The effect of phage filtrate concentration of the other isolates is illustrated in figure 3.4. Generation of more lysogenic bacteria with higher phage filtrate concentration as opposed to lower was not seen.

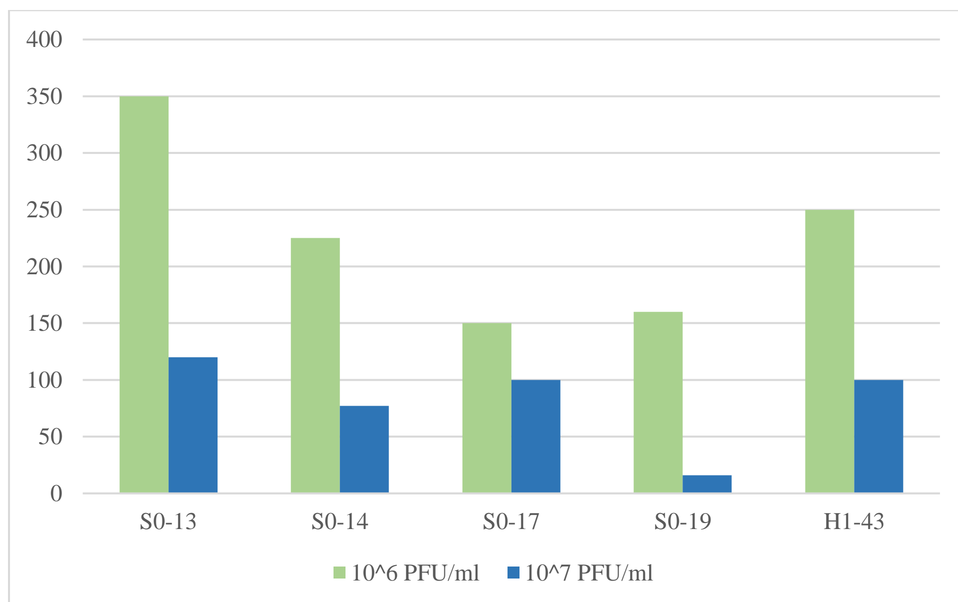


Figure 3.4: Approximate colony counts on LB agar plates with 25 mg/L Chloramphenicol after lysogenic infection with phage ϕ731 for each isolate. The colors represent different phage filtrate concentrations.

The lysogens were verified with both conventional PCR and real-time PCR. All the lysogens created with ϕ731 and the method of which they were verified is listed in table a.1 in appendix A.

3.1.2 Studies with native Stx2a phages

Native Stx2a phages were obtained from the STEC O26 isolates listed in table 2.2. The recipient strains were used as described in table 2.1. Phage ф731 was used as control with *E. coli* C600 as recipient strain in each experiment set up.

3.1.2.1 Lytic infection

The lytic infection experiments with phage filtrates from STEC O26 isolates were performed in two replicate experiments using *E. coli* C600 and *S. sonnei* 866 as recipient strains. Both spot test with dilutions of phage filtrate and whole plates with undiluted phage filtrate were carried out. The undiluted phage filtrates mainly resulted in complete lysis or almost complete lysis of the strains using whole plates. The undiluted phage filtrate from H1-43 and using *S. sonnei* 866 as the recipient was the only combination that resulted in countable PFUs. In the first experiment with this combination a total of 232 PFU was counted, in the second experiment 219 PFU was counted. Phage filtrate of C1-50 was another exception to almost complete/complete lysis of the strains. In the first experiment, too many PFU to count was created with both the recipient strains. In the second experiment, there was no lysis with *E. coli* C600, while there was almost complete lysis with *S. sonnei* 866.

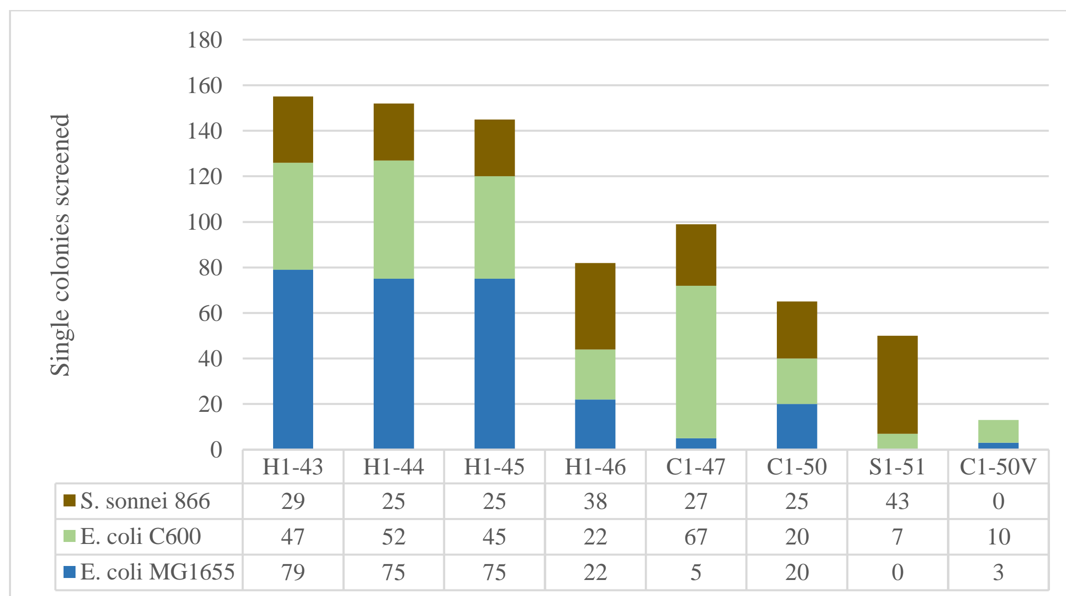
In the spot test, a dilution series of the phage filtrate from UD to 10^{-5} was used. Using UD phage filtrate, a lysis zone was apparent for all the samples. This zone became less apparent in the subsequent dilutions. The dilution at which the zone was no longer visible varied slightly between the phage filtrates. The isolates with the strongest lysis (H1-46, C1-47, S1-51) had an clearly visible zone at 10^{-3}, while isolates with the weakest lysis (H1-43, H1-44, H1-45, C1-50), lost its visible zone after 10^{-2}. Visible PFUs were not generated.

3.1.2.2 Lysogenic infection

In the initial lysogenic studies, only *E. coli* C600 and *E. coli* MG1655 were used as recipient strains. *S. sonnei* 866 were later added to the recipient strains. The experiment was dependent on generation of single colonies to screen for *stx2a*. The first experiments were carried out as described in protocol 1 in 2.4.2.1, using LB agar plates. However, too much growth and few

single colonies were generated. An amended procedure, called protocol 2 (also described in section 2.4.2.1), involved including 0,1M $CaCl_2$, less incubation time and less material deposited on the LB agar plate replaced protocol 1.

A total of 761 colonies were screened for presence of *stx2a* gene. Figure 3.5 illustrates how many single colonies were screened according to combination of recipient strain and which STEC O26 isolate the phage filtrate originated from. DNA from up to ten different single colonies was pooled for the screening. There were several positive hits in the pooled samples, but few unique combinations of recipient strain and phage. These combinations include a Stx2a phage from C1-50 and *S. sonnei* 866 as the recipient, and an Stx2a phage from C1-47 and *E. coli* MG1655 as the recipient. The Stx2a phage inserted into *S. sonnei* 866 was further transduced to *E. coli* C600. Other isolates were positive for presence of *stx2a,* but after replating two times the *stx2a* gene could no longer be confirmed within the bacteria.



	H1-43	H1-44	H1-45	H1-46	C1-47	C1-50	S1-51	C1-50V
S. sonnei 866	29	25	25	38	27	25	43	0
E. coli C600	47	52	45	22	67	20	7	10
E. coli MG1655	79	75	75	22	5	20	0	3

Figure 3.5: Number of single colonies screened divided by recipient strain and phage filtrate from the respective Shiga toxin-producing Escherichia coli (STEC) O26 isolates. C1-50V is the Stx2a phage from STEC isolate C1-50, but phage filtrate was made via Shigella sonnei *866 as host strain.*

Figure 3.5 shows there were differences in how many single colonies were screened for each combination of phage and recipient strain. High number of positive hits of *stx2a* presence did not correlate to high number of single colonies screened. For example, for the combination of *E. coli* MG1655 treated with phage filtrate from C1-47 and H1-43, five and 79 single colonies

were screened, respectively. The first combination created two stable lysogenic isolates, while *stx2a* could not be found in any of the isolates of the second combination.

The real-time PCR screening was performed on DNA from pooled samples. Usually, if the CT is above 25 cycles for any given sample, it would not be considered positive for presence of the gene (see section 2.3.2). However, as these samples are pooled, the amount of template may be less. Therefore, the cut-off for possible positives was set to 30 cycles. The samples were then extracted separately and run on real-time PCR again, this time using 25 cycles as a cut-off for positive samples.

Overview of the lysogenic isolates with native Stx2a phages created in this study is provided in table a.2 in appendix A. These isolates were further used in the plasmid experiments, which is part of the recombination experiment.

3.1.2.3 Growth curves

Growth curves were created to see effect of MitC on cultures with lysogenic bacteria. The cultures were incubated in shaking incubator and OD was periodically measured hourly over a time period of ca 7 hours, and once more after ON incubation. MitC was added when the culture had an OD of 0.3-0.5. Both culture with and without MitC were then measured.

E. coli C600::ф731 was used as control and needed 5-6.5 hours to reach the required OD. The cultures of STEC O26 isolates reached OD of 0.3-0.5 much quicker than the control, after ca. 2.5-3 hours. After addition of MitC it was possible to see a slight difference in the curve developments of culture with and without MitC after 2-3 hours. After ON incubation, the differences in OD between cultures with and without OD were substantial. Cultures without MitC had much higher OD value than those treated with MitC, which indicates cell lysis of the cultures treated with the antibiotic due to induction of phages. Growth curves of STEC isolates are added in appendix D.

3.1.3 Phage survival in filtrate

To evaluate if the same phage filtrate could be used after prolonged storage, phage filtrate was prepared using *E. coli* C600::ϕ731 and quantified with *E. coli* C600 as recipient strain the same day it was made, and quantified again after one week and after 12 days. In the meanwhile, the filtrate was kept in refrigerator. The evaluation of functional phages present in the filtrate showed a dramatic drop in phage number in just a week as illustrated in figure 3.6.

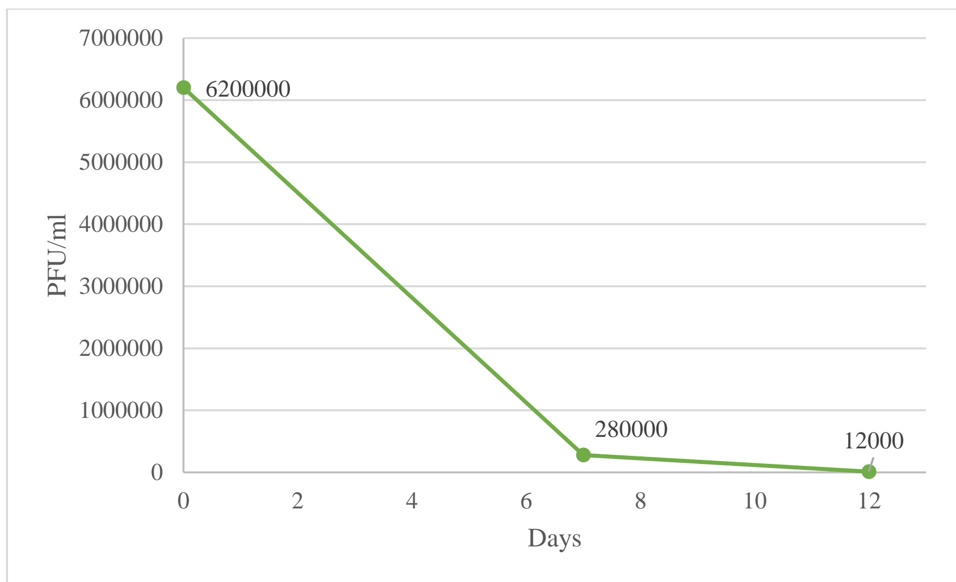


Figure 3.6: Phage stability in filtrate. PFU/ml was calculated using phage filtrate quantification.

Using chloroform in the isolation process did not improve survival of phages. This time, the original filtrate was quantified to contain $4.7 \cdot 10^7$ PFU/ml. After one week, quantification was repeated with both the original filtrate and the part that had been treated with chloroform. The original phage filtrate contained ca. 750 phages. It was not possible to quantify the filtrate that had been treated with chloroform. The undiluted phage filtrate with chloroform had created too many PFU to count and the 10^{-2} dilution did not have any visible PFU (10^{-1} dilution was not prepared). It was decided that phage filtrates were to be used and quantified the same day they were made, and not use chloroform in the isolation process.

3.1.4 Recombination experiment

The final result of the recombination experiment was recombinant phages which originate from two separate STEC isolates. Strain selection for the recombination experiment was based on the results from the plasmid experiments, which involved test for competence using pUC19 and transformation with pKD46. Confirmation of recombination was carried out using PCR and host infectivity tests.

3.1.4.1 Selection of strains

The strains used in the plasmid experiments are listed in table 2.2 and 2.4. *E. coli* DH5α was used as control. The test was run three times with the control strain, and twice for the other isolates.

Transformation efficiency reflects the cells ability to take up extracellular DNA and express genes encoded by this DNA. The plasmid experiments using pUC19 was performed to find strains with high transformation efficiency. During the experiments, issues with some of the isolates were encountered. The *E. coli* C600 cultures with the phage from C1-50 never reached target OD of 0.3-0.5 in neither of the two replicates of the experiment. The H1-46 isolate and the *S. sonnei* 866 with the phage from C1-50 strain had overgrowth on the LB agar plates with 50 mg/L Ampicillin. ON incubation of the original isolates of *S. sonnei 866* and H1-46 streaked on LB agar plates with 50 mg/L Ampicillin proved they were ampicillin resistant.

The remaining isolates had growth of single colonies in at least one of the two replicates of the experiment, and competence efficiency was estimated. The most competent strain seemed to be S1-51. The colony count for each isolate and replicate is listed in table e.1 in appendix E. The mean transformation efficiency for the isolates is illustrated in figure 3.7.

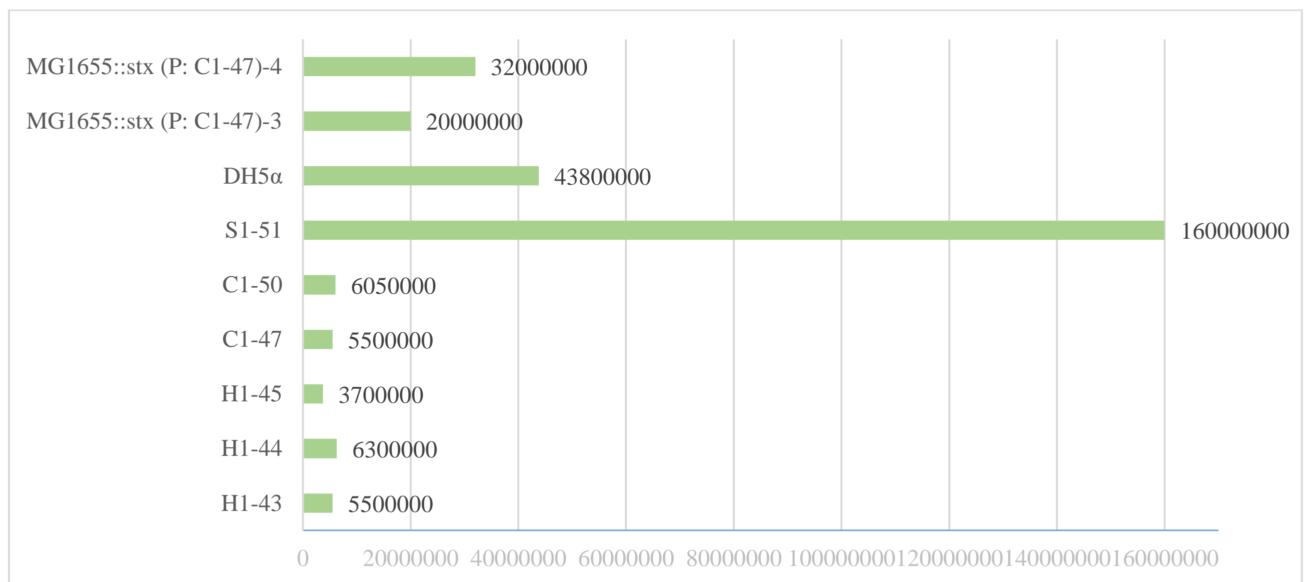


Figure 3.7: Mean transformation efficiency (transformants/µg DNA) of the isolates, calculated using experiments with pUC19.

Transformation of pKD46 was done in parallel with the pUC19 experiments. pKD46 inhabits properties important for the recombination experiment such as ampicillin resistance, temperature sensitivity and harbours the λ red gene. Successful transformation of pKD46 was therefore necessary for continuation of the recombination experiments. A total of five strains had successful transformation with pKD46. These isolates were MG1655::Stx2a-3, MG1655::Stx2a-4 H1-43, C1-50 and S1-51. Interestingly, pKD46 could not be introduced into *E. coli* DH5α.

After transformation, the presence of the *Stx2a* gene in the isolates containing pKD46 was analyzed by real-time PCR. It was found that the isolates MG1655::Stx2a(P:C1.47)-3 and MG1655::Stx2a(P:C1-47)-4 had lost the *stx2a* gene. The isolates H1-43, C1-50 and S1-51 with pKD46 still carried the *stx2a* gene, and were used in the recombination experiment.

3.1.4.2 Generation of the long fragment

The long fragment used in the recombination experiment was generated through several conventional PCRs. The long fragment consists of the amplimers called product 1, product 2 and product 3 (see figure 2.6). When first generating product 1, the annealing temperature was set to 54°C and elongation time was set to 15 seconds. This annealing temperature and

elongation time had successfully been used by Serra-Moreno *et al.* (2006). Gel picture with all the amplimers is shown in figure 3.8.

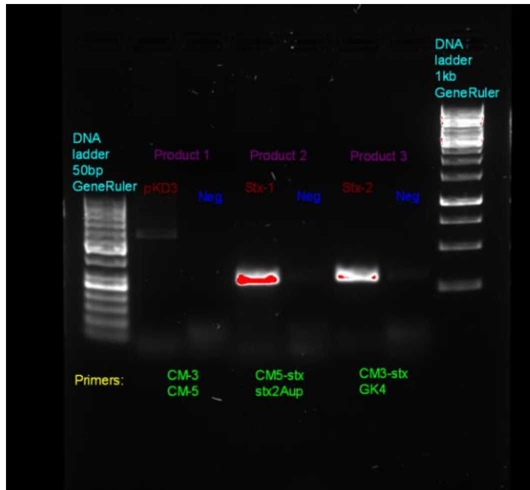


Figure 3.8: Gel picture of PCR products 1-3.

In this study, it was found that the PCR program as described by Serra-Moreno *et al*. (2006) to generate product 1 was not optimal, as figure 3.8 depicts. To optimize the PCR, the elongation time was first changed to 45 seconds. Another attempt used elongation time of 30 seconds and different temperatures. Using the temperature gradient option on the PCR machine, optimum temperature was identified using only a single run. The final optimal PCR program for product 1 is listed in table 2.7. Figure 3.9 shows the gel picture with the PCR products from the temperature gradient run, and the PCR product from using elongation time of 45 seconds.

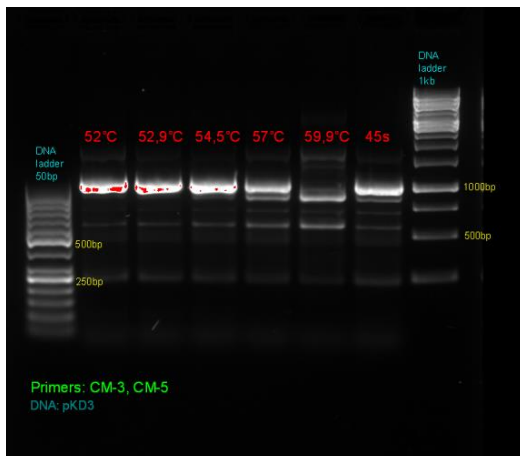


Figure 3.9: Gel picture of PCR products 1 using temperature gradient and 30 second elongation time, and 45 seconds elongation time with original program.

Product 1 was purified from the gel shown in figure 3.9 by cutting out the bands at ca 1000 bp from both 52°C and 52.9°C. Product 2 and 3 were purified from PCR mixes. After purification, the products were run on a gel. Purity and concentration were also evaluated using NanoDrop2000 (*results not shown*). Figure 3.10 shows a gel picture of the purified products.

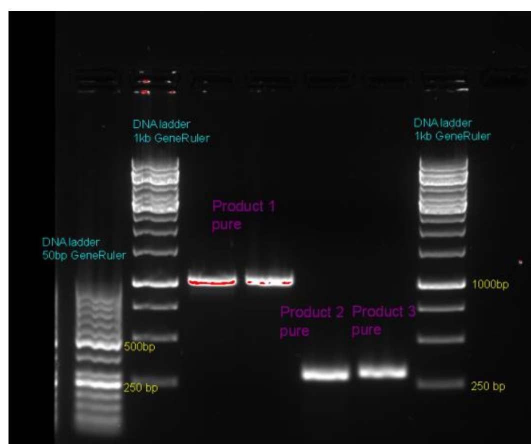


Figure 3.10: Gel picture of purified PCR products.

To generate the final product (the long fragment) four identical reactions were run. The PCR products were run on a gel. Bands of approximately 1500 bp were excised from the gel and the four samples were pooled in pairs and purified. Figure 3.11 shows the gel picture of the purified products.

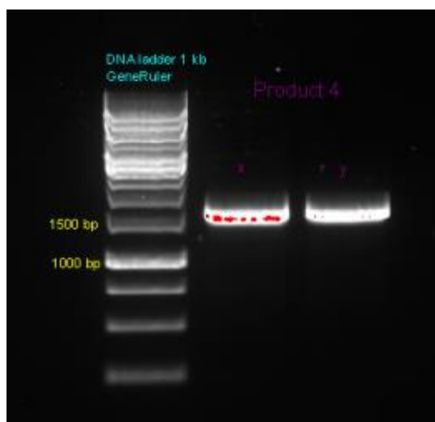


Figure 3.11: Gel picture of purified long fragment.

After evaluating the purity of the PCR products, both visually on gel and using NanoDrop2000 (*result not shown*), purification was performed on the remaining PCR products. To increase concentration, less elution buffer was used (*20 µl instead of 50 µl*). The purified PCR products were pooled and the final product was then measured on NanoDrop2000, results are shown in table 3.3.

Table 3.3: NanoDrop measurement of PCR product concentration and purity.

Sample ID	Nucleic Acid Concentration	260/280 ratio	260/230 ratio
Long fragment (final product)	142,4 ng/µl	1,82	1,5

3.1.4.3 Recombination

The isolates H1-43, C1-50 and S1-51 from the transformation experiments were used in the recombination experiment. When growing the isolates in LB broth in shaking incubator to prepare electrocompetent cells, the culture of STEC isolate S1-51 with pKD46 did not reach the target OD and was not included further. The long fragment was electroporated into the pKD46 carrying STEC isolates H1-43 and C1-50. After plating, the isolates had grown in single colonies on both the LB agar plates supplemented with 5 mg/L and with 20 mg/L Cm.

3.1.4.4 Confirmation of recombinant phages

Colonies from the LB agar plates supplemented with 20 mg/L Cm were replated twice. After replating, some colonies were selected for verification of recombinant phages using PCR. Both conventional PCRs and real-time PCR were used. All the selected colonies were confirmed carriers of the *stx2a::cat* gene.

Two isolates containing a recombinant phage confirmed with PCR were further used. The recombinant phages were named ɸH1-43(*stx2a::cat*) and ɸC1-50(*stx2a::cat)*. The host isolates with these phages were streaked onto LB agar plates supplemented with 25 mg/L Cm and incubated ON at 37°C to verify that this concentration of Cm could be used in lysogenic infection experiments.

Host infectivity experiments were carried out to confirm that the phages ɸH1-43(*stx2a::cat*) and ɸC1-50(*stx2a::cat*) were viable, and both lysogenic and lytic infective abilities were tested. These tests were used in addition to PCR to evaluate if the functional phage has been correctly inserted, as PCR can give positive result for the *stx2a::cat* gene independent of phage location and even if only parts of the phage have been inserted. The lysogenic infection experiments were performed using all three protocols described in 2.4.2.1 and 25 mg/L Cm LB agar plates with recipient strains as described in table 2.1. For the selected aEPEC O26 isolates as listed in table 2.3, lysogenic infection protocol 1 and 2 using 25 mg/L Cm LB agar plates were used.

Lytic infection with ɸH1-43(*stx2a::cat*) *and* ɸC1-50(*stx2a::cat*)

Lytic infection was performed as spot test and on whole plates. Both *E.coli* C600 and *S. sonnei* 866 were used as recipient strains.

Spot test with phage ɸC1-50(*stx2a::cat*) had similar results with both recipient strains. The undiluted phage filtrate created a clearly visible zone where the phage filtrate had been deposited, but did not result in complete lysis or any visible PFU. The zone became less visible with each dilution, and there was no lysis from dilution 10^{-3} to 10^{-5}. The lytic infection of *S. sonnei* 866 with undiluted phage filtrate resulted in complete lysis, while the 10^{-1} dilution almost complete lysis in form of thin growth covering the plate and no visible PFU. The latter result also applies for undiluted phage filtrate and *E. coli* C600.

Spot test with phage ϕH1-43(*stx2a::cat*) also had similar results with both recipient strains. The undiluted phage filtrate resulted in complete lysis. The 10^{-1} dilution created a clear zone, but did not result in complete lysis or any visible PFU. With *E. coli* C600 as recipient strain there was a barely visible zone at the 10^{-2} dilution and no lysis from dilution 10^{-3} to 10^{-5}, while with *S. sonnei* 866 as recipient there was no lysis from dilution 10^{-2} to 10^{-5}. The lytic infection of both *S. sonnei* 866 and *E. coli* C600 with undiluted phage filtrate resulted in complete lysis. The 10^{-1} dilution and *S. sonnei* 866 did not show any lysis.

Lysogenic infection with ϕH1-43(*stx2a::cat*) *and* ϕC1-50(*stx2a::cat*)

Lysogenic infection of recipient strains using both phage ϕH1-43(*stx2a::cat*) and ϕC1-50(*stx2a::cat*) generated several lysogenic bacteria. There where however differences in success associated with phage, lysogenic infection protocol and recipient strains.

The lysogenic infection of *E. coli* C600, *E. coli* MG1655, *S. sonnei* 866 and *E. coli* DH5α as recipient strains all generated lysogenic bacteria using protocol 1 and 3 (protocol 3 was not performed with lab strain C600 and phage ϕH1-43(*stx2a::cat*)). Far more lysogenic *E. coli* DH5*α* was generated using protocol 3, while this effect was not obvious for the other isolates. Protocol 2 was not successful for lysogenic infection using phage ϕH1-43(*stx2a::cat*). For phage ϕC1-50(*stx2a::cat*), protocol 2 generated fewer lysogenic bacteria than the other protocols. Table 3.4 shows the number of lysogens generated using phage ϕH1-43(*stx2a::cat*).

*Table 3.4: The number of lysogens infected with ϕH1-43(*stx2a::cat*) and ϕC1-50(*stx2a::cat*) divided by lysogenic infectivity experiment protocol.*

Recipient strain	**Lysogenic infection of lab strains with phage ϕH1-43(*stx2a::cat*)**			**Lysogenic infection of lab strains with phage ϕC1-50(*stx2a::cat*)**		
	Lysogenic infection protocol			*Lysogenic infection protocol*		
	1	**2**	**3**	**1**	**2**	**3**
***Escherichia coli* C600**	~100 colonies	No growth	Not done	>1000 micro and small colonies	~200 colonies	>1000 micro and small colonies
***E. coli* MG1655**	2 colonies	No growth	40 colonies	Overgrowth	22 colonies	Overgrowth
***E. coli* DH5α**	1 micro colony	No growth	~500 faint, small colonies	~300 small colonies	No growth	>1000 micro colonies
***Shigella sonnei* 866**	>1000 colonies	No growth	>1000 small colonies	Overgrowth	37 colonies	>1000 micro colonies

The recombinant Stx2a phages also infected 13 aEPEC O26 isolates as described in table 2.3. Infection with ϕH1-43(*stx2a::cat*) produced only two lysogenic aEPEC isolates (H0-1 and S0-13) using protocol 1, while protocol 2 did not provide any lysogenic bacteria. The phage ϕC1-50(*stx2a::cat*) created more lysogenic aEPEC isolates than phage ϕH1-43(*stx2a::cat*). In total nine isolates using protocol 1 (H0-1, H0-4, H0-5, H0-6, S0-13, S0-14, S0-17, S0-19 and C0-41), but only two isolates using protocol 2 (H0-1 and H0-4). This result is illustrated in figure 3.12.

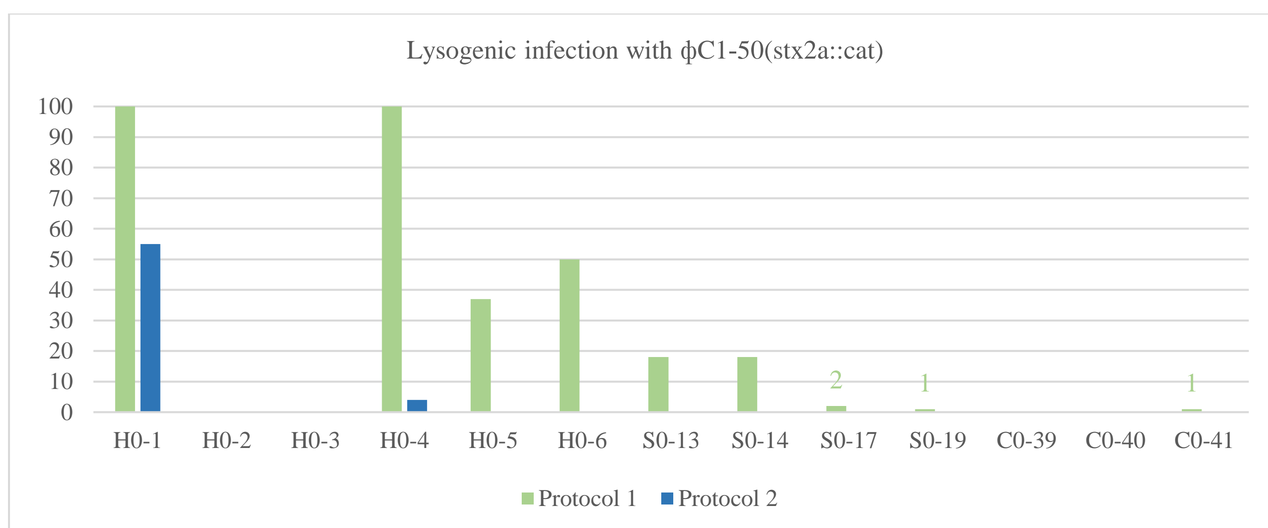


*Figure 3.12: Lysogenic infection with phage ϕC1-50(*stx2a::cat*). Approximate number of the lysogenic isolates (y-axis) created in the lysogenic infectivity experiment using different protocols; protocol 1 (green) and protocol 2 (blue).*

The lysogenic bacteria generated were not confirmed using PCR. Growth on LB agar plates with 25 mg/L Cm was used as indicator for presence of phage. The lysogenic isolates created in the recombination experiment are shown in table a.3 in appendix A.

3.2 Characterization of *E. coli* O26

Results from the characterization of the *E.coli* O26 isolates include RDF, presence of *ehxA* and data analysis of WGS.

3.2.1 Rhamnose and dulcitol fermentation (RDF)

The RDF results for isolates listed in table 2.2 and 2.3 are shown in table 3.5. Which isolates were tested in this thesis is described in table 2.2 and 2.3.

3.2.2 Characterization of virulence-associated gene ehxA

The results for the isolates listed in table 2.2 and 2.3 regarding presence of the virulence-associated gene *ehxA* using real-time PCR is shown in table 3.5. Which isolates were tested in this thesis is described in table 2.2 and 2.3. In the real-time PCR run, only NTC and C0-27 had no CT. The other isolates were considered positive as each sample in the run crossed CT before 25 cycles.

Comparison of the O26 isolates susceptibility to phage (described in section 3.1.1.2) and characterization by RDF and presence of *ehxA* are listed in table 3.5.

Table 3.5: Escherichia coli O26 isolates and their rhamnose and dultictol fermentation (RDF), presence of the ehxA *gene and susceptibility to phage ϕ731.*

Isolate	RDF	*ehxA*	Susceptible to phage ϕ731
H0-1	-/-	+	+
H0-2	-/-	+	+
H0-3	-/-	+	-
H0-4	-/-	+	-
H0-5	-/-	+	+
H0-6	+/+	-	+
S0-7	+/-	+	+
S0-8	+/-	+	+
S0-9	-/-	+	-
S0-10	-/-	+	-
S0-11	-/-	+	+
S0-12	-/-	+	+
S0-13	+/+	-	+
S0-14	+/+	-	+
S0-15	-/-	+	+
S0-16	-/-	+	-
S0-17	+/+	-	+
S0-18	-/-	+	+
S0-19	+/+	-	+

C0-20	+/+	-	+
C0-21	-/-	+	+
C0-22	-/-	+	+
C0-23	-/-	+	-
C0-24	-/-	+	+
C0-25	+/+	-	+
C0-26	-/-	+	-
C0-27	+/+	-	+
C0-28	-/-	+	-
C0-29	-/-	+	+
C0-30	-/-	+	+
C0-31	-/-	+	-
C0-32	-/-	+	+
C0-33	-/-	+	+
C0-34	-/-	+	-
C0-35	-/-	+	+
C0-36	-/-	+	-
C0-37	-/-	+	-
C0-38	-/-	+	+
C0-39	-/-	+	-
C0-40	+/-	+	-
C0-41	-/-	+	-
C0-42	**-/-**	+	+
H1-43	-/-	+	+
H1-44	-/-	+	+
H1-45	-/-	+	+
H1-46	-/-	+	+
C1-47	-/-	+	+
C1-50	-/-	+	+
S1-51	-/-	+	+

3.2.3 Whole genome sequencing of **E. coli** *O26*

Whole genome sequences of the *E. coli* O26 isolates were used in data analysis to evaluate insertion sites and virulence genes. Some isolates (see table 2.3, and table a.1 in appendix A) were sequenced during this study, other isolates had already been sequenced in previous studies. The isolates H0-3, H0-5, H0-5::ϕ731, S0-9, S0-14, S0-14::ϕ731, H1-46 and H1-46::ϕ731 were used in the insertion site screening. Apart from H1-46, the isolates sequence data were *de novo* assembled in this study. For the virulence gene study using CGE

VirulenceFinder 1.5, only isolate C1-47 were *de novo* assembled in this study. The WGS data for the remaining STEC isolates had already been assembled.

3.2.3.1 Screening for possible Stx phage insertion sites

Screening for possible Stx phage insertion sites was done in CLC Genomics Workbench 9. Bonanno *et al.* (2015) had used conventional PCR to determine if an insertion site was intact (*attB*) or occupied (*attL*), the same primer sequences was used in this study for *in silico* searches in the genome of selected isolates. In the Bonanno *et al.* (2015) study, if no *attB* DNA amplification occurred, amplification of the *attL* junction site was performed to demonstrate presence of inserted Stx phage. In this study, all the primer sequences were evaluated.

Data analysis of the genome sequences involved *in silico* BLAST searches for the primer binding sites and evaluation of possible amplicon size from the hit was used to investigate the insertion sites. The insertion sites investigated include *wrbA, yehV, yecE, sbcB, Z2577, argW, prfC* and *torST* . If none or only one of the primers in a primer pair (for *attB* or *attL*) were found, the insertion site was determined not found. The result is shown in table 3.6.

*Table 3.6: Stx phage insertion sites and status of insertion sites as intact (*attB*) or occupied (*attL*). + = *attB*: intact/available insertion site, - = *attL*: occupied, N.F.= Not found (neither *attB *or* attL *complete).*

Strain	***Insertion site***							
	wrbA	*yehV*	*yecE*	*sbcB*	*Z2577*	*argW*	*prfC*	*torST*
H0-3	+	-	+	+	+	+	+	+
H0-5	+	+	+	+	+	+	+	+
H0-5::ϕ731	N.F.	+	+	+	+	+	+	+
S0-9	+	-	+	+	+	+	+	+
S0-14	+	+	+	+	+	+	+	+
S0-14::ϕ731	N.F.	+	+	+	+	+	+	+
H1-46	+	-	N.F.	N.F.	+	+	+	+
H1-46::ϕ731	N.F.	-	-	+	+	+	+	+

3.2.3.2 CGE VirulenceFinder 1.5

Assembled genomes of the STEC isolates listed in table 2.2 were uploaded to the CGE VirulenceFinder 1.5 database. The results confirmed that all the STEC isolates carried *stx2a* and *ehxA* genes. Selected virulence genes and their presence in the isolates are shown in table 3.7.

Table 3.7: Presence of a selection of virulence genes found by the CGE VirulenceFinder 1.5 database in the Shiga toxin-producing Escherichia coli O26 isolates.

	Toxins		**Colicins**			**Non-LEE encoded effectors**			**Intimin and adherence**		
	toxB	*cdtB*	*celB*	*cba*	*cma*	*nleA*	*nleB*	*nleC*	*eae*	*iha*	*efa1*
H1-43				x	x	x	x		x	x	x
H1-44				x	x	x	x		x	x	x
H1-45				x	x	x	x		x	x	x
H1-46	x					x	x	x	x	x	x
C1-47				x	x	x	x		x		x
C1-50	x					x	x	x	x	x	x
S1-51	x		x			x	x	x	x	x	x

Other results from CGE VirulenceFinder 1.5 showed that all the STEC isolates carried several genes for Type III secretion system including *espA*, *espB*, *espF*, *espJ* and *cif*, and *lpfA* gene associated with fimbria. Several other genes were also assessed, but will not be presented in this study.

3.3 Validation of new primers

To validate the presence of *stx2a::cat* in lysogenic isolates, conventional PCR with the primers that had been used in Serra-Moreno *et al.* (2006) were carried out on the first lysogenic isolates generated with phage ϕ731. The recipient strains are as described in table 2.1, and all were susceptible to the phage. Figure 3.13 shows the gel picture from the conventional PCR run using the primers from Serra-Moreno *et al.* (2006) on the lysogenic strains created (positive control *E. coli* C600::ϕ731 and negative control samples *E. coli* C600 not shown).

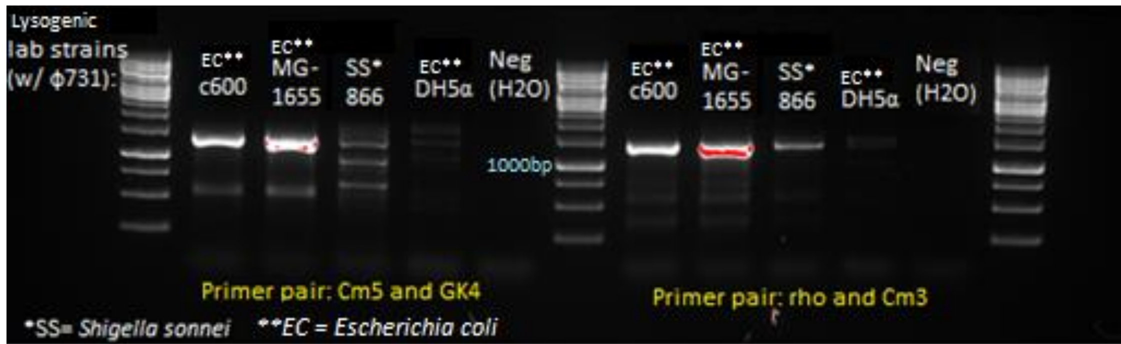


Figure 3.13: PCR to confirm stx2a::cat *in lysogenic strains. The ladder used was the GeneRuler 1kb DNA ladder.*

As figure 3.13 shows, the PCR created several unspecific products and the specific amplicon was weak for some of the samples. New primers were therefore designed using CLC Genomics Workbench. The original primers used by Serra-Moreno *et al.* (2006) created PCR products of more than 1000 bp. The new primers designed in CLC Genomics Workbench generated PCR products of 300-400 bp in length. This is illustrated in figure 2.2. The gel picture of the PCR products generated using the new primers is shown in figure 3.14.

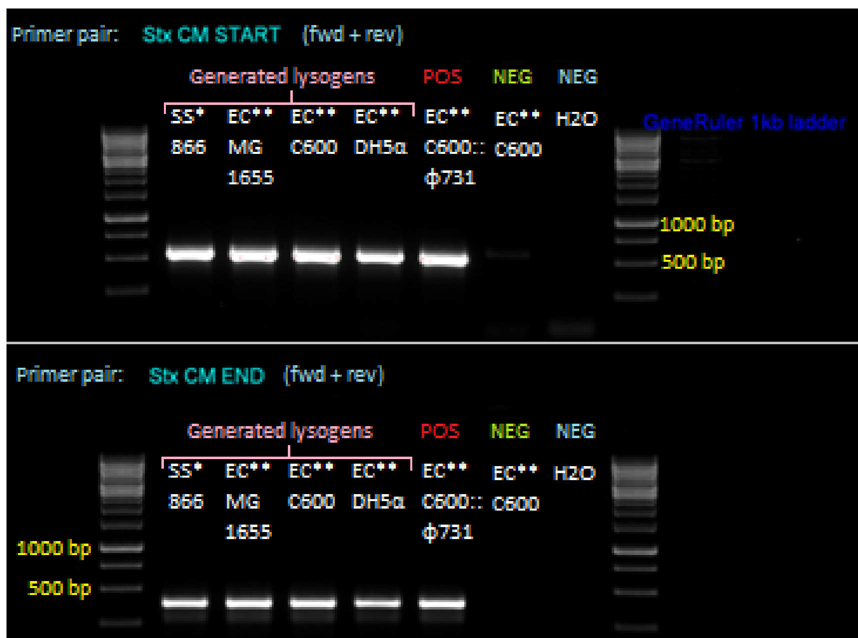


Figure 3.14: Gel picture of stx2a::cat presence confirmation PCR using primers designed in this study.

Figure 3.14 shows that the primers designed for confirmation of *stx2a::cat* presence using conventional PCR created specific products at expected size. Negative and positive control generated satisfactory results. The primers were validated for use.

Primers and probe for real-time PCR were also tested to see if this method could replace conventional PCR. The same lysogenic strains tested with conventional PCR were also used in this test. In addition, the possible lysogens of H0-1, H0-5, H0-6, S0-7, and S0-8 were also tested with the real-time PCR method. The amplification plot from the run is shown in figure 3.15.

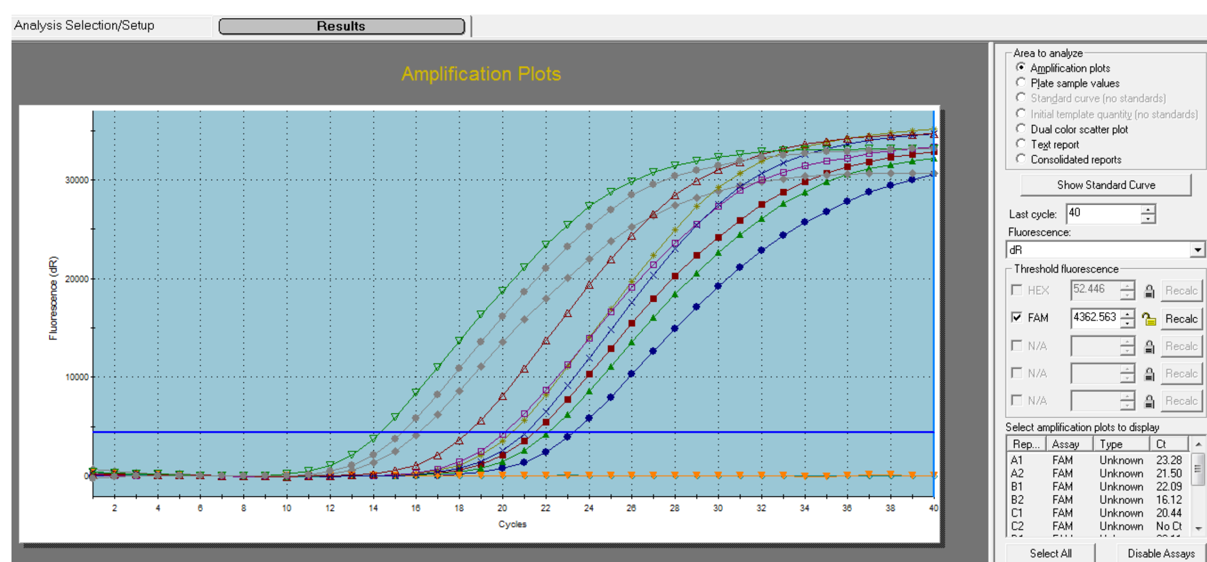


Figure 3.15: Amplification plot from real-time PCR for verification of stx2a::cat gene.

The real-time PCR for verification of *stx2a::cat* could also be approved. Both negative controls (*E. coli* 600 without ϕ731 and no template) had no CT, all the other samples crossed the threshold before 25 cycles as expected.

Other primers that were designed include the Stx-cm3 and alternative GK4 primers used in the recombination experiment. When the primers used by Serra-Moreno *et al.* (2006) for recombination of the phages were aligned with the sequence from the isolates used in this study, one of the primers did not match perfectly. There was one nucleotide difference in the Stx-cm3 primer, and the primer was altered accordingly (illustrated in figure 2.3). There was no specific validation of the primer. It was used as described in the recombination experiment, and amplicons of expected size were created.

Alternative GK4 was run in primer pair with the rho primer. Using CLC Genomics Workbench and available WGS data, the length of PCR product of the *stx2a* gene with and without *cat* incorporated was calculated. Samples with *stx2a::cat* and samples with *stx2a* were both run and the size was evaluated.

4 Discussion

4.1 Comparison of phage susceptibility by different *E. coli* O26

Previous studies have described environments that harbours both *E. coli* O26 *stx* positive isolates and O26 *stx* negative isolates where they may convert to one form from the other by loss or uptake of a Stx phage (Bonanno *et al.*, 2016, Bielaszewska *et al.*, 2007). Susceptibility towards phages has been described as dependent on phage/host compatibility, including receptors and insertion sites (Muniesa and Schmidt, 2014, Allison, 2007, Rakhuba *et al.*, 2010). In this study, 42 aEPEC O26, seven STEC O26 and four well-characterized lab strains were used as recipients in phage susceptibility experiments. In total, three labelled Stx2a phages and seven native Stx2a phages were evaluated for their infective abilities, both lytic and lysogenic, towards the different bacteria. Two of the labelled phages (ϕC1-50(*stx2a::cat*) and ϕH1-43(*stx2a::cat*)) were generated in this study. To investigate potential markers of Stx2a phage susceptibility, *E. coli* O26 isolates in this study were compared by Stx2a phage susceptibility, RDF, *ehxA* presence and availability of insertion sites.

Lysogenic isolates were generated via infection by all the labelled phages. The O26 isolates were considered more susceptible to a given phage if a high number of colonies were achieved on LB agar plates supplemented with 25 mg/L Cm. A high number of colonies imply that many of the bacteria in the LB solution were infected by the phage. Some isolates were susceptible to certain phage/phages. In this study, 27 of 42 aEPEC isolates and all seven STEC isolates were susceptible to phage ϕ731. In addition, nine of 13 aEPEC isolates tested were susceptible to phage ϕC1-50(*stx2a::cat*) and two of 13 aEPEC isolates were susceptible to phage ϕH1-43(*stx2a::cat*). In total, 29 of 42 aEPEC isolates (69%) and all seven STEC isolates (100%) were susceptible in various degrees to a labelled Stx2a phage. Other studies have also found that aEPEC and other *E. coli* isolates are susceptible in different degrees to various Stx phages (Bonanno *et al.*, 2016, Muniesa *et al.*, 2003, Serra-Moreno *et al.*, 2007).

Lysogenic infection of the *E. coli* O26 isolates by phage ϕ731 showed that some isolates were more susceptible to the phage than others. Especially some of the aEPEC isolates from ovine sources and most STEC isolates (H1-46 and S1-51 being the exceptions) were highly susceptible. The fact that all seven STEC O26 were susceptible to phage ϕ731 indicates that several insertion sites may be occupied simultaneously, as seen in previous studies (Hayashi *et al.*, 2001, Scheutz, 2014, Allison, 2007). Carrying two Stx prophages within the *E. coli*

genome has been shown to decrease the lytic cycle activation and subsequent Stx production (Serra-Moreno *et al*., 2008). This may give lysogenic bacteria with double Stx phage infection some survival advantage as opposed to those infected with one Stx phage. This may be a reason for the phage susceptibility by all the STEC isolates in this study.

Phage ϕ731 is a recombinant Stx2a phage originated from a STEC O103:H25 strain isolated from a HUS patient, transduced into *E. coli* C600 (Solheim *et al*., 2013). In this study, we examined the uptake of both phage ϕ731 and the recombinant phages created from STEC O26:H11 isolates. Comparison of the lysogenic infective abilities of the phages has several limitations. First, a smaller number of O26 isolates were tested with the recombinant phages created in this study. Secondly, the phage filtrate was prepared from STEC isolates with the recombinant phage directly, and not using an intermediate host such as *E. coli* C600. Virulence profile and factors such as colicin production may therefore interfere with host susceptibility and survival. The quantity of phages from the STEC isolates were not possible to calculate as no PFU were visible at any dilution. Finally, no replicates were performed. Furthermore, the new lysogens were not confirmed with PCR methods due to limited time, only using growth on LB agar plates supplemented with 25 mg/L Cm as indication of successful lysogenic infection.

When comparing host susceptibility of phage ϕC1-50(*stx2a::cat*) and phage ϕ731, it was observed that aEPEC isolates from human sources were more susceptible to the former phage. Interestingly, the phage ϕ731 did not generate any lysogenic bacteria with H0-4 as recipient strain, but this isolate was highly susceptible to phage ϕC1-50(*stx2a::cat*), as can be seen in figure 3.13. Differences between the phages may explain this observation. This is interesting as phage ϕC1-50(*stx2a::cat*) originates from a STEC 026 in a bovine source, while phage ϕ731 originates from STEC O103 in a human source. This could be an indication that human-adapted *E. coli* O26 are more susceptible to phages from other O26 isolates than to phages from other serogroups of human-adapted *E. coli*. The four aEPEC isolates from ovine sources did not share this result. All were susceptible to both phages, but more susceptible to phage ϕ731. C0-41 was another isolate susceptible to phage ϕC1-50(*stx2a::cat*), and not phage ϕ731. However, if the ϕC1-50(*stx2a::cat*) phage was integrated into a well characterized strain such as *E. coli* C600 and further induced from that isolate, different results may arise.

Phage ϕH1-43(*stx2a::cat*) was far less successful in creating lysogens of the aEPEC O26 isolates than both phage ϕ731 and phage ϕC1-50(*stx2a::cat*). The phage filtrate concentration was not possible to calculate, but presence of phages was however obvious as some lysogenic

bacteria were generated. Using the CGE VirulenceFinder 1.5, it was found that STEC H1-43 carried genes for colicins, while STEC C1-50 did not. This may affect the host cell survival as colicin present in phage filtrate could lyse the potential host, and could explain why phage ϕH1-43(*stx2a::cat*) had less successful lysogenic infection (Cascales *et al.*, 2007).

Several factors influence the lysogenic infection, including phage amount. In this study, concentration of phage ϕ731 in filtrate varied between ca. $1.0 \cdot 10^5$ PFU/ml and $5.0 \cdot 10^7$ PFU/ml. For some isolates in this study, more lysogenic isolates were created when the phage concentration was lower, as shown in figure 3.4. Other factors include host susceptibility such as host cell receptors and available insertion sites.

To investigate insertion sites, WGS data from eight selected isolates were screened for primer sites *in silico* to examine phage insertion sites and their vacancy or occupancy status. The sites evaluated in this study are the same as described by Bonanno and coworkers (2015), using the same primer sequences. H0-3 and S0-9 were not susceptible to infection by the ϕ731 phage and both isolates had an occupied insertion site at *yehV*. The S0-14 and H0-5 isolates which were susceptible to phage ϕ731 in both replicates of the lysogenic infection experiment had all insertion sites vacant. From these isolates, the results suggest that not having any insertion sites occupied increase the chance for incorporating bacteriophage DNA. Previous studies have observed that several Stx phages may be incorporated to the same genome, and that if the preferential site in the host strain is unavailable, a secondary insertion site is chosen (Kruger and Lucchesi, 2015). This was not seen with the aEPEC O26 isolates analysed in this study, however only few isolates were investigated.

Further data analysis evaluated the insertion site of phage ϕ731 in selected isolates. For S0-14::ϕ731 and H0-5::ϕ731 isolates, the result was similar as for the respective isolates without phage ϕ731, but neither *attB* or *attL* of *wrbA* could be found. This may suggest changes in that area of the genome after phage DNA integration. For H1-46 *yecE* and *sbcB* were not found, and only *yehV* of the remaining sites was occupied. The result for H1-46::ϕ731 was similar to H1-46 apart from the *wrbA* (which could not be found), *yecE* (which was occupied) and *sbcB* (which was vacant). That some bacteria carry several phages has been shown before, e.g. STEC carrying both Stx1 and Stx2 phages (Scheutz, 2014). It was not possible to confirm the insertion site of phage ϕ731 in either of the isolates S0-14::ϕ731, H0-5::ϕ731 or H1-46::ϕ731. In other studies, *wrbA* and *yehV* have been recognised as the main insertion site of Stx1a phages in STEC O26:H11, and *wrbA, argW* and *yecE* for Stx2a phages (Bonanno *et*

al., 2015, Scheutz, 2014, Serra-Moreno *et al*., 2007). Our data could neither confirm or refute these findings.

The O26 isolates were characterized by presence of *ehxA* and RDF ability to evaluate if there could be a connection between these characteristics and uptake of phage. A study by Brandal *et al.* (2012) indicated that aEPEC with certain characteristics could be grouped as EHEC-like isolates, which had the potential of converting to EHEC if a Stx phage was acquired. That study suggested a link between no RDF and presence of *ehxA* to EHEC or EHEC-like isolates. To investigate further, we compared O26 isolates by these characteristics and susceptibility to Stx2a phages. In our study, it was found that all *ehxA* negative isolates were RDF positive. The STEC isolates in this study were RDF negative and *ehxA* positive, similar to the EHEC isolates in the Brandal *et al.* (2012) study. Several of the aEPEC isolates had the same pattern of these characteristics, but not the aEPEC isolates most susceptible to phage ϕ731 (S0-13, S0-14, S0-17, and S0-19). In fact, aEPEC isolates of both patterns of characteristics varied in susceptibility to the phage. No clear connection between RDF ability, occurrence of *ehxA,* and uptake of phage could be established.

In addition to host and phage variabilities, the method of which to promote lysogenic infection can influence success or failure of the lysogenic infection experiment. Different lysogenic infection protocols were compared using the results from phage ϕC1-50(*stx2a::cat*) and phage ϕH1-43(*stx2a::cat*). Lysogenic infection protocol 1, protocol 2 (less incubation time, $CaCl_2$, less material spread on the plates than protocol 1) and protocol 3 (similar to protocol 1, but with addition of $CaCl_2$) was set up using *E. coli* C600, *E. coli* MG1655, *S. sonnei* 866 and *E.coli* DH5α as recipients. The result showed that long incubation time (ON) had positive effect on generating lysogens. Possible explanations include that the phage need more time to infect bacteria, the infected bacteria need more time to replicate, or both. Serra-Moreno *et al.* (2006) have suggested that lysogenic bacteria need more time to stabilize after infection. This is in accordance with our findings. Protocol 2 with short incubation time generated relatively few or no lysogenic bacteria. It was also noted that lysogenic infection of *E. coli* DH5α was more successful when 0.1M $CaCl_2$ was added (protocol 3).

Experiments with native Stx2a phages were more challenging than experiments with labelled phages. The lysogenic abilities of the Stx2a phages could not be thoroughly evaluated, but in this study it seemed to be a rare event. This may perhaps reflect the true nature of phage/bacteria interaction. Using recombinant phages are likely to overestimate the amount of lysogeny because they are easier to separate using selective medium. It may also be a

selective pressure from the antibiotic for the bacteria to keep the phage. In addition, high numbers of bacteria can be added to one plate because of the selective media, which means the lysogenic bacteria can have more time to grow. This is not possible for lysogenic bacteria with native phages which needs to be plated earlier to avoid overgrowth.

To investigate lysogenic infective abilities of the native Stx2a phages, lysogenic infection protocol 2 was mainly used. Initially, lysogenic infection protocol 1 was used, but too much growth and few single colonies were generated and no positive hits were achieved. Protocol 2 generated several single colonies, but far fewer lysogenic bacteria as previously described. Fewer bacteria are spread onto the plates as selecting single colonies is important for subsequent screening. This, in addition to non-selective media permitting growth of all bacteria, consequently lead to finding fewer lysogens.

Some positive results in the PCR screening were achieved, but after replating multiple times several of the lysogenic strains lost the Stx2a phage. Loss of Stx phage has been seen in several studies (Bonanno *et al.*, 2015, Scheutz, 2014, L'Abee-Lund *et al.*, 2012, Bielaszewska *et al.*, 2007). Two combinations of recipient strain and phage directly isolated from a STEC strain persisted; *S. sonnei* 866 with phage from C1-50 (*S. sonnei* 866::ϕC1-50(Stx2a)) and *E. coli* MG1655 with phage from C1-47 (MG1655::ϕC1-47(Stx2a)-3 and MG1655::ϕC1-47(Stx2a)-4). *S. sonnei* 866::ϕC1-50(*stx2a*) was used to further infect the other recipient strains. In this case, very few colonies grew on the LB agar plates, but there were positive hits of Stx2a presence within two *E. coli* C600 isolates which remained stable after several replating events (C600L::ϕC1-50(Stx2a)V and C600H::ϕC1-50(Stx2a)V).

Other studies have struggled to create lysogens of e.g. *E. coli* O26 *stx* negative isolates (Bonanno *et al.*, 2016). This may be due to using different phages, or having other conditions that are less suited for lysogeny. As seen in this study as well, the different phages and protocols for lysogenic infection created varying results.

Lytic infection of the isolates was used for quantification, spot test and evaluation of the lytic abilities of the phage. The quantification of phage filtrate with phage ϕ731 was performed using lytic infection of *E. coli* C600 using different dilutions of the phage filtrate, while other studies have used qPCR (Bonanno *et al.*, 2016). Using qPCR has the potential of over representing the number of phages as only a gene is to be verified. This means free DNA and non-infective phages may also give a positive result, without being part of a phage. Using DNAse treatment would reduce this challenge. Using lytic infection may underrepresent the

real number of phages as the method is dependent on host susceptibility. In this study, it was usually possible to count PFU on the plates with 10^{-4}, the 10^{-5} and/or the 10^{-6} dilution. When assessing phage filtrate stability in storage, it was found that the number of infective phages present in the filtrate dropped drastically in only a few days. Chloroform has been suggested for increased survival of phages in phage stocks for storing (Sambrook and Russell, 2001). However, in this study adding of chloroform did not contribute to increased survival, on the contrary, the phages died even faster.

The quantification set up was not successful for the Stx2a phages, as they did not generate PFUs in any dilution (*except for phage filtrate from H1-43 using* S. sonnei *866 as host strain and UD phage filtrate*), and the concentration could not be estimated. The plates had complete lysis, almost complete lysis or no lysis. Spot test replaced quantification for these phage filtrates, which was less laborious and could just as well evaluate the lytic abilities of the phage filtrate. However, the phage filtrate can contain more than just isolated phages, e.g. colicins, which could contribute to the lysis seen on the plates (Cascales *et al*., 2007). By entering the sequences of all the STEC isolates in the CGE VirulenceFinder 1.5 server/database, it was discovered that all the isolates apart from C1-50 had one or more gene for colicin production present. Presence of colicins could be a reason why no isolated plaques were generated. Lytic infection of aEPEC isolates using the ɸ731 phage was not successful. This was verified with plaque hybridization. Gamage and coworkers also found lytic infection to be a rare event, more so than lysogenic infection (Gamage *et al*., 2004).

In the lytic experiments with phage filtrates from STEC, phage filtrate from some isolates were generated more lysis than others. Phage filtrate from S1-51 created strong lysis, and when the culture of the isolate was grown in shaking incubator in the recombination experiment, it lysed. Other isolates also lysed during incubation. This include C600L::ɸC1-50(*stx2a*)V and C600H::ɸC1-50(*stx2a*)V. The lysis was probably due to phage activation and lysis since the OD of the culture initially increased, but started to decline again in early to mid-log phase. Spontaneous induction of the lytic cycle has previously been observed in other studies, reporting that the level of spontaneous induction is higher for Stx phages in comparison with non-Stx phages (Kruger and Lucchesi, 2015, Serra-Moreno *et al*., 2006).

In the recombination experiment, two native Stx2a phages were successfully recombined. In this experiment, lysogenic infection of *E. coli* C600, *E. coli* MG1655 and *S. sonnei* 866 by native Stx2a was first performed. The aim was to create lysogens of these strains for use in recombination experiments in this study and in future experiments. These strains are well

characterized. Higher transformation efficiency is expected as opposed to wild type strains and lysogenic variants of these strains are more likely to create the recombinant phages. Quantification of phages using plaque assays would also be less troublesome. No other phages are inserted in the genome, and the bacterial genome and background is well-studied. With wild type strains, quantification can be disrupted by presence of other phages and gene products, such as colicins, as seen suggested in this study. Lysogenic infection was also performed to evaluate the Stx2a phages ability to create lysogens.

In total, three different combinations of native Stx2a phage and recipient strain was generated: *S. sonnei* 866::ϕC1-50(*stx2a*), *E. coli* C600::ϕC1-50(*stx2a*)V and MG1655::ϕC1-47(*stx2a*) . During the experiments, *S. sonnei* 866 proved resistant towards ampicillin. Since the pKD46 plasmid encoding the λ-Red recombinase necessary to introduce the Cm cassette into the *stx2a* gene of the phages is ampicillin resistant, ampicillin resistant isolates made counter selection of transformants impossible. The *E. coli* C600::ϕC1-50(*stx2a*)V lysogens did not reach target OD when grown in LB agar in shaking incubator at 37°C, probably due to lysis as earlier described. Finally, the *E. coli* MG1655::ϕC1-47(*stx2a*) lysogens did no longer possess the *Stx2a* gene after the plasmid experiment. Loss of phage can occur as previously described in this study. Therefore, neither of the lysogenic lab strains created could be used further.

As neither of the above-mentioned strains could be used, using the STEC isolates and directly recombine the native phages was considered. The H1-46 isolate was excluded from the recombination experiment, as this isolate was ampicillin resistant. The transformation efficiency for the remaining STEC isolates was calculated using pUC19. The results show similar competence, ranging from 10^6-10^7 CFU/µg DNA, which was like the control strain *E. coli* DH5α. One isolate, S1-51, had the highest transformation efficiency of approximately10^8 transformants per µg DNA. This isolate, along with C1-50 and H1-43 were also able to incorporate pKD46 into their genomes. The transformation efficiency of the two latter isolates ranged from 0 CFU/µg DNA to 10^7 CFU/µg DNA. No clear connection between the competence efficiency and uptake of pKD46 could be established. The very low transformation efficiency was surprising. However, the STEC strains are poorly characterized and may inhabit other plasmids leading to plasmid incompatibility, in addition to other unknown factors. Since the plasmids used in the study are different, one may be more compatible than the other towards the certain STEC strain.

The recombination experiment in this study was a modified version of the method described by Serra-Moreno *et al.* (2006), which in turn used a modified version of the original protocol

using the lambda Red recombinase system for genetic manipulation of chromosomal DNA in *E. coli* described by Datsenko and Wanner (2000). Increased length of the homologous region to reduce the number of false recombinants and higher concentration of bacteria for electroporation were among the changes made by Serra-Moreno and coworkers (2006). Of the three isolates that had transduced the pKD46 plasmid in our study, only H1-43 and C1-50 reached target OD when the recombination experiment was performed. The growth of S1-51 probably failed due to lysis that is possibly a result of spontaneous induction of bacteriophages, as seen earlier in the experiments.

Serra-Moreno *et al.* (2006) did not produce any colonies of recombinant strains that grew directly on 20 µg/ml Cm LB agar plates, and suggested that the bacteria needed more time or lower concentrations of the antibiotic to recover. Because recombinant phages can be problematic to make, all precautions possible was made in our study, choosing the optimizations found by Serra-Moreno *et al*. (2006). In our study, several recombinant strains grew directly on the 20 µg/ml Cm LB agar plates. A selected few were replated and validated using both conventional and real-time PCR.

For the conventional PCRs, several primer pairs were run to validate the presence of *stx2a::cat* gene fragment as illustrated in figure 2.6. An alternative primer to the primer GK4 further downstream was designed, as it is technically more accurate to use different primers for the verification of successful recombination and the fragment construction. When using the same primers as used in the construct, one might potentially just amplify the PCR product at any location in the genome of the bacteria. Using primers further downstream and upstream of the construct provides more support that the whole phage is incorporated, and therefore more accurately verify the successful integration of the phage in the bacterial chromosome. The loss of the vector using high temperature ON incubation was not performed in our study. This was not necessary as after the phage was created it was purified and transferred to new strains.

4.2 Limitations

Limitations in this study includes that a moderate number of isolates were used and few replicates of the experiments were performed. There was also little time to test the recombinant phages from the STEC O26 isolates, which limited comparison with phage ϕ731. For data analysis of insertion sites a small dataset was used, and the raw data was not

evaluated, due to time-shortage. In this study, it was decided that if the genomes had hits during BLAST search, this would be sufficient evidence that the sequence was "usable". But poor sequence quality could be the reason that not all insertion sites could be found for all isolates in the *in silico* searches. More time would also allow better processing of the genomic data.

4.3 Conclusion

In our study, 29 of 42 aEPEC O26 isolates were susceptible to Stx2a phages, which indicate that aEPEC can convert to STEC if Stx phages are present in the environment. The STEC isolates were more likely to incorporate a second Stx2a phage (100% susceptible), than the aEPEC isolates were to incorporate one Stx2a phage (69% susceptible). Some isolates were more susceptible to a Stx2a phage and susceptibility could depend on the phage introduced. No clear connection could be found between Stx2a phage susceptibility, RDF and *ehxA*.

The lytic abilities of the phages varied. Phage ϕ731 was unsuccessful in lytic infection of any of the aEPEC and STEC isolates. The phage was however easy to quantify using lytic infection of *E. coli* C600. The reason for this is difficult to conclude. Native Stx2a phages and recombinant Stx2a phages isolated from STEC isolates were difficult to assess due to other factors that influenced the lytic infection of the recipient strains, such as possible presence of colicins.

Preliminary results regarding insertion sites suggest that aEPEC O26 isolates are more likely to incorporate a Stx2a phage if neither of the insertion sites *wrbA, yehV, yecE, sbcB, Z2577, argW, prfC* or *torST* are occupied. However, sequence data from very few isolates were analysed.

In conclusion, this study found no connection between host susceptibility, presence of *ehxA* and rhamnose and dulcitol fermentation profile. Some isolates were more susceptible to lysogenic infection by Stx2a phages than others, and some isolates were more susceptible to a certain phage. However, we found no distinct differences between susceptible and non-susceptible isolates. Investigation of occupancy and availability of the insertion sites on these isolates indicated that aEPEC O26 isolates were more likely to incorporate a Stx2a phage if all insertion sites were available. No aEPEC or STEC isolate in this study was susceptible to lytic infection by phage ϕ731.

5 Further work

In this study, two recombinant phages in two different wild type STEC strains were created. The aim of the recombination experiment was to evaluate if the method could be used to create recombinant Stx2a phages in our lab, which could be established. We could not successfully use the recombination method on native Stx2a phages transferred to of *E. coli* C600, *E. coli* MG1655 or *S. sonnei* 866. Instead, native Stx2a phages within STEC O26 isolates were used, and after the phage had been recombined, several strains were be infected with the phage through lysogeny. Another important aim of this experiment was the generation of recombinant phages from O26 isolates to prepare for future studies which compare host infectivity to these Stx2a phages (O26:H11 phages) and phage ϕ731 (originated from O103:H25) to find if there is any difference between the isolates' susceptibility in regards to phage origin. To generate comparable results in other phage susceptibility tests the same host should be used, e.g. *E. coli* C600. This isolate was susceptible to the recombinant phages created in this study and freeze stocks of the lysogenic strains were made. Future experiments include verifying the presence of the *stx2a::cat* fragment within these lysogenic bacteria, before use in further studies.

Screening for native Stx2a phages proved difficult in this study. Longer incubation time could provide more lysogens, but this may also increase the risk of uninfected bacteria out-growing the lysogenic. The challenge was to create single colonies, and avoid overgrowth at the same time create lysogenic bacteria. Future studies should evaluate using ON incubation, but deposit less material on the LB agar plate.

In this study, WGS data was obtained for all the *E. coli* O26 isolates, aEPEC and STEC, and some lysogenic isolates generated in this study. This data could be used for further in *in silico* searches of both insertion sites, virulence genes and other relevant genomic constituents. Whole genome comparison could also be conducted, with phylogenetic studies to evaluate similarity and differences between aEPEC vs STEC and bovine vs ovine vs human isolates. And evaluate how the aEPEC isolates will relate to the STEC isolates, and is it possible to identify factors essential for Stx2a phage susceptibility. The Stx2a phages within the STEC isolates could also be identified and compared to each other.

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Appendix A

Overview of the lysogenic isolates with phage ϕ731 is listed in table a.1.

Table a.1: Overview of ϕ731 lysogenic bacteria created in this study. Confirmation of stx2a::cat was either done using both conventional PCR (=C PCR) and real-time PCR (qPCR) or just real-time PCR (qPCR).

Isolate name	Isolate name (this thesis) before lysogeny	Stx profile	*stx2a::cat* confirmation
C600::ϕ731-2	C600	*stx2a::cat*	C PCR, qPCR
DH5α::ϕ731	DH5α	*stx2a::cat*	C PCR, qPCR
***Shigella sonnei* 866::ϕ731**	*Shigella sonnei* 866	*stx2a::cat*	C PCR, qPCR
MG1655::ϕ731	MG1655	*stx2a::cat*	C PCR, qPCR
NVI-164::ϕ731	H0-1	*stx2a::cat*	C PCR, qPCR
NVI-134::ϕ731	H0-2	*stx2a::cat*	qPCR
NVI-116::ϕ731 [A, B]	H0-5	*stx2a::cat*	C PCR, qPCR
NVI-102::ϕ731	H0-6	*stx2a::cat*	C PCR, qPCR
S-24::ϕ731	S0-7	*stx2a::cat*	C PCR, qPCR
S-26::ϕ731	S0-8	*stx2a::cat*	C PCR, qPCR
S-46::ϕ731 [A]	S0-11	*stx2a::cat*	C PCR, qPCR
S-60::ϕ731	S0-12	*stx2a::cat*	C PCR, qPCR
S-62::ϕ731	S0-13	*stx2a::cat*	C PCR, qPCR
S-85::ϕ731 [A, B]	S0-14	*stx2a::cat*	C PCR, qPCR
S-86::ϕ731	S0-15	*stx2a::cat*	qPCR
S-102::ϕ731	S0-17	*stx2a::cat*	C PCR, qPCR
S-103::ϕ731	S0-18	*stx2a::cat*	C PCR, qPCR
S-118::ϕ731	S0-19	*stx2a::cat*	C PCR, qPCR
S2014-138::ϕ731	C0-20	*stx2a::cat*	C PCR, qPCR
S2014-144::ϕ731	C0-21	*stx2a::cat*	C PCR, qPCR
S2014-154::ϕ731	C0-22	*stx2a::cat*	qPCR
S2014-197::ϕ731	C0-24	*stx2a::cat*	qPCR
S2014-206::ϕ731	C0-25	*stx2a::cat*	C PCR, qPCR
S2014-228::ϕ731	C0-27	*stx2a::cat*	C PCR, qPCR
NVI-468::ϕ731	C0-29	*stx2a::cat*	qPCR
NVI-469::ϕ731	C0-30	*stx2a::cat*	C PCR, qPCR
NVI-471::ϕ731	C0-32	*stx2a::cat*	QPCR
NVI-472::ϕ731 [A]	C0-33	*stx2a::cat*	C PCR, qPCR
NVI-474::ϕ731	C0-35	*stx2a::cat*	C PCR, qPCR
NVI-477::ϕ731	C0-38	*stx2a::cat*	qPCR
NVI-405::ϕ731	C0-42	*stx2a::cat*	qPCR
NVI-160::ϕ731	H1-43	*stx2a::cat, stx2a*	C PCR, qPCR
NVI-130::ϕ731	H1-44	*stx2a::cat, stx2a*	C PCR, qPCR
NVI-126::ϕ731	H1-45	*stx2a::cat, stx2a*	C PCR, qPCR
NVI-98::ϕ731 [A, B]	H1-46	*stx2a::cat, stx2a*	C PCR, qPCR

S2014-124::ϕ731	C1-47	*stx2a::cat, stx2a*	qPCR
S2014-148::ϕ731	C1-50	*stx2a::cat, stx2a*	C PCR, qPCR
S-17::ϕ731	S1-51	*stx2a::cat, stx2a*	qPCR

[A] = Whole genome sequenced, [B] = Data analysis of insertion sites

Overview of the lysogenic isolates with Stx2a phage created in this study is provided in table a.2.

Table a.2: Overview of Stx2a lysogenic bacteria created in this study.

Isolate name	Isolate name before lysogeny	Stx2a phage origin (STEC isolate name – this study)
S.s.866::ϕC1-50(*stx2a*)	*Shigella sonnei* 866	C1-50
C600L:: ϕC1-50(*stx2a*)V	C600	C1-50
C600H:: ϕC1-50(*stx2a*)V	C600	C1-50
MG1655::ϕC1-47(*stx2a*)-3	MG1655	C1-47
MG1655::ϕC1-47(*stx2a*)-4	MG1655	C1-47

The lysogenic isolates created in the recombination experiment are shown in table a.3.

Table a.3: Overview of lysogenic bacteria created in the recombination experiment of this study.

Isolate name	Isolate name (this thesis) before lysogeny	Phage	Stx profile
H1-43::ϕH1-43(*stx2a::cat*)	H1-43	ϕH1-43(*stx2a::cat*)	*stx2a::cat, stx2a*
C600::ϕH1-43(*stx2a::cat*)	C600		*stx2a::cat*
DH5α::ϕH1-43(*stx2a::cat*)	DH5α		*stx2a::cat*
***Shigella sonnei* 866::ϕH1-43(*stx2a::cat*)**	*Shigella sonnei* 866		*stx2a::cat*
MG1655::ϕH1-43(*stx2a::cat*)	MG1655		*stx2a::cat*
H0-1::ϕH1-43(*stx2a::cat*)	H0-1		*stx2a::cat*
S0-13::ϕH1-43(*stx2a::cat*)	S0-13		*stx2a::cat*

C1-50::ϕC1-50(*stx2a::cat*)	C1-50	ϕC1-50(*stx2a::cat*)	*stx2a::cat*, *stx2a*
C600::ϕC1-50(*stx2a::cat*)	C600		*stx2a::cat*
DH5α::ϕC1-50(*stx2a::cat*)	DH5α		*stx2a::cat*
***Shigella sonnei* 866::ϕC1-50(*stx2a::cat*)**	*Shigella sonnei* 866		*stx2a::cat*
MG1655::ϕC1-50(*stx2a::cat*)	MG1655		*stx2a::cat*
H0-1::ϕC1-50(*stx2a::cat*)	H0-1		*stx2a::cat*
H0-4::ϕC1-50(*stx2a::cat*)	H0-4		*stx2a::cat*
H0-5::ϕC1-50(*stx2a::cat*)	H0-5		*stx2a::cat*
H0-6::ϕC1-50(*stx2a::cat*)	H0-6		*stx2a::cat*
S0-13::ϕC1-50(*stx2a::cat*)	S0-13		*stx2a::cat*
S0-14::ϕC1-50(*stx2a::cat*)	S0-14		*stx2a::cat*
S0-17::ϕC1-50(*stx2a::cat*)	S0-17		*stx2a::cat*
S0-19::ϕC1-50(*stx2a::cat*)	S0-19		*stx2a::cat*
C0-41::ϕC1-50(*stx2a::cat*)	C0-41		*stx2a::cat*

Appendix B

The PCR master mixes used in this study varied depending on the primers.

Conventional PCR

PCR master mix for verification of *stx2a::cat* is shown in table b.1.

Table b.1: Reagents (amount per sample and end concentration) in the PCR master mix associated with primers for verification of stx2a::cat.

PCR Master Mix		
Reagent	*µl per sample*	*End concentration*
PCR buffer, 10x (contains 15 mM $MgCl_2$) (Qiagen, Germany)	2.5	1x
Primer 1 (10 µM)	0.5	0,2 µM
Primer 2 (10 µM)	0.5	0,2 µM
dNTP Mix (Qiagen, Germany) (10 mM each)	0.5	0,2 µM
Taq DNA polymerase (Qiagen, Germany) (5 units/µl)	0.1	0,5 U
H_2O (MilliQ)	18.9	#
Template	2	#
TOTAL	**25**	

The PCR master mix used in the recombination experiment to generate amplimers (short fragments) is shown in table b.2.

Table b.2: Reagents (amount per sample and end concentration) in the PCR master mix associated with primers for generation of short fragments used in the recombination experiment.

PCR Master Mix		
Reagent	*µl per sample*	*End concentration*
5X Phusion HF buffer* (Thermo Fisher Scientific, USA)	10	1x
Primer 1 (5 µM)	5	0,5 µM
Primer 2 (5 µM)	5	0,5 µM
dNTP mix,10 mM	1	200 µM
Phusion DNA Polymerase (2 U/µL) (Thermo Fisher Scientific, USA)	0.5	1 U
MilliQ water	27.5	#
Template	1	#
TOTAL	**50**	

*1.5 mM $MgCl_2$ in the 1X final concentration.

The PCR master mix used in the recombination experiment to generate the long fragment is shown in table b.3.

Table b.3: Reagents (amount per sample and end concentration) in the PCR master mix associated with primers for generation of the long fragment used in the recombination experiment.

PCR Master Mix		
Reagent	*µl per sample*	*End concentration*
5X Phusion HF buffer* (Thermo Fisher Scientific, USA)	10	1x
Primer GK4 (5 µM)	5	0,5 µM
Primer Stx2Aup (5 µM)	5	0,5 µM
dNTP mix,10 mM	1	200 µM
Phusion DNA Polymerase (2 U/µL) (Thermo Fisher Scientific, USA)	0.5	1 U
Product 1 (10 ng/µl)	1	0,2 ng/µl
Product 2 (2.5 ng/µl)	1	0,05 ng/µl
Product 3 (2.5 ng/µl)	1	0,05 ng/µl
MilliQ water	27.5	#
TOTAL	**50**	

*1.5 mM $MgCl_2$ in the 1X final concentration.

PCR master mix for the PCR run to compare *stx2a* and *stx2a::cat* presence is shown in table b.4.

Table b.4: Reagents (amount per sample and end concentration) in the PCR master mix associated with primers for comparison of stx2a::cat *and* stx2a.

PCR Master Mix		
Reagent	*µl per 4 samples*	*End concentration*
10X DreamTaq Buffer (with 20 mM $MgCl_2$) (Thermo Fisher Scientific, USA)	10	2x
Primer Rho (5 µM)	5	0,5 µM
Primer Alternative GK4 (5 µM)	5	0,5 µM
dNTP mix,10 mM	1	200 µM
DreamTaq DNA Polymerase (5 U/µl) (Thermo Fisher Scientific, USA)	0,5	2,5U
MilliQ water	24.5	#
Template	(4x) 1	#
TOTAL	50	

Real-time PCR

PCR master mix for verification of *stx2a::cat* is shown in table b.5.

Table b.5: Reagents (amount per sample and end concentration) in the PCR master mix associated with primers for verification of stx2a::cat.

Real-time PCR Master Mix		
Reagent	*µl per sample*	*End concentration*
2xBrilliant III Ultra fast QPCR Mastermix (Agilent technologies, USA)	12.5	1x
StxCAT_F1 (10 µM)	2.25	0,9 µM
StxCAT_R2 (10 µM)	2.25	0,9 µM
StxCAT_probe 1 (10 µM)	0.5	0,2 µM
dH2O	5.5	#
Template	2	#
TOTAL	**25**	

PCR master mix for verification of *stx2a* is shown in table b.6.

Table b.6: Reagents (amount per sample and end concentration) in the PCR master mix associated with primers for verification of stx2a.

Real-time PCR Master Mix		
Reagent	*µl per sample*	*End concentration*
2xBrilliant III Ultra fast QPCR Mastermix (Agilent technologies, USA)	10	1x
Primer VT2a-QfLNA1, (10 µM)	0.66	0,33 µM
Primer VT2a-QrLNA2, (10 µM)	0.66	0,33 µM
Probe VT2a-Qp (HEX) (10 µM)	0.2	0,1 µM
MilliQ water	3.48	#
Template	**5**	#
TOTAL	20	

PCR master mix for verification of *ehxA* is shown in table b.7.

Table b.7: Reagents (amount per sample and end concentration) in the PCR master mix associated with primers for verification of ehxA.

Real-time PCR Master Mix	
Reagent	*µl per sample*
2xBrilliant III Ultra fast QPCR Mastermix (Agilent technologies, USA)	10
20x qPCR assay*	1
MilliQ water	8
Template	1
TOTAL	**20**

Appendix C

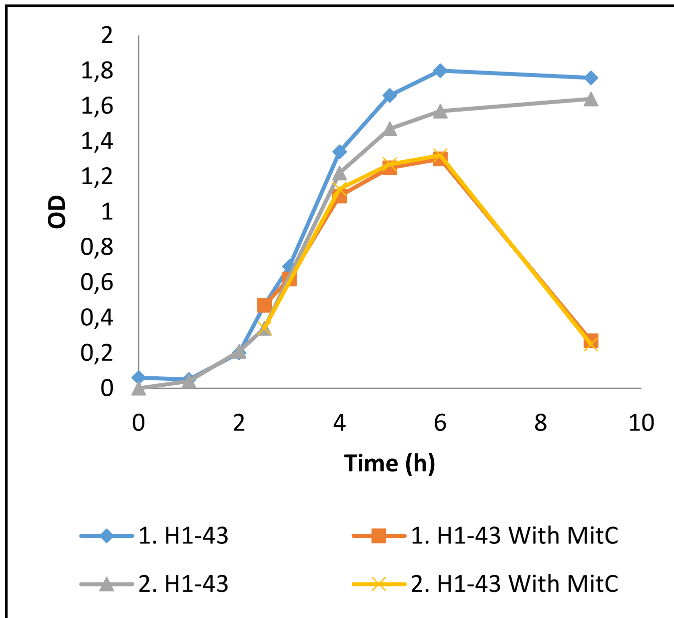
Buffer name and composition of the buffers used in plaque hybridization is listed in table c.1.

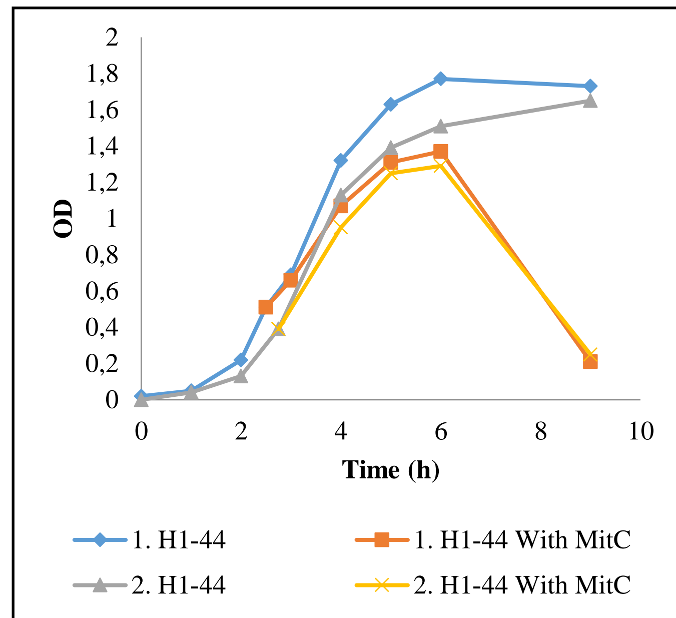
Table c.1: Overview of buffer solutions and their composition.

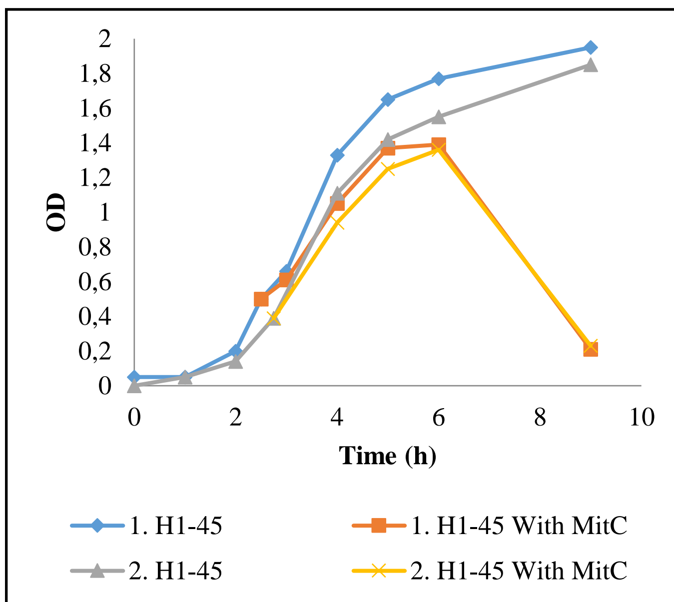
Buffer name	Volume	Composition	Other info
Denaturalization buffer	1L	20g NaOH 0.5N 87.66g NaCl 1.5M Up to 1 L H_2O	
Neutralization buffer	1L	87.66 g NaCl 1.5M 500 ml TrisHCl 1M pH 7.4 Up to 1L H_2O	Autoclave
20xSSC	1L	175.32g NaCl 88.23g Sodium citrate Up to 1L H_2O	Autoclave
2xSSC, 1% SDS	500 ml	50 ml 20 x SSC 50 ml 10% SDS up to 500 ml H_2O	
2xSSC, 0,1% SDS	500 ml	50 ml 20 x SSC 5 ml 10% SDS up to 500 ml H_2O	
0,04% SSC, 0,1% SDS	500 ml	1 ml 20 x SSC 5 ml 10% SDS up to 500 ml H_2O	
Hybridization buffer	200 ml	50 ml 20 x SSC 2 ml 10% N-Laurylsacrosin 0.4 ml 10% SDS 2 g 1 % Blocking reagent up to 200 ml H_2O	Keep at -20ºC
Hyb.buffer with probe		Add 10 µl of the purified probe to 50 ml hybridization buffer	
Buffer 1	1 l	100 ml TrisHCl pH 7.5 1M 100 ml NaCl 1.5M up to 1 l H_2O	Autoclave
Buffer 2	1 l	5 g Blocking reagent up to 1 l Buffer 1	
Antibody solution		Add 10 µl of antibodies to 50 ml Buffer 2.	
Buffer 3	200 ml	20 ml TrisHCl pH 9.5 1M 20 ml NaCl 1M 10 ml $MgCl_2$ 1M up to 200 ml H_2O	
Washing buffer	1 l	1 l Buffer 1 3 g Tween 20	Autoclave
Colouring solution		40 µl colouring solution (NBT/BCIP) pr 2 ml Buffer 3.	

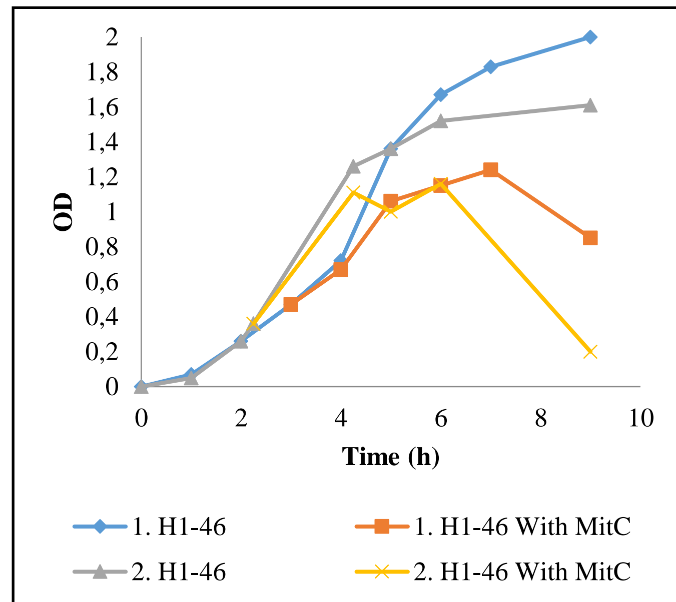
Appendix D

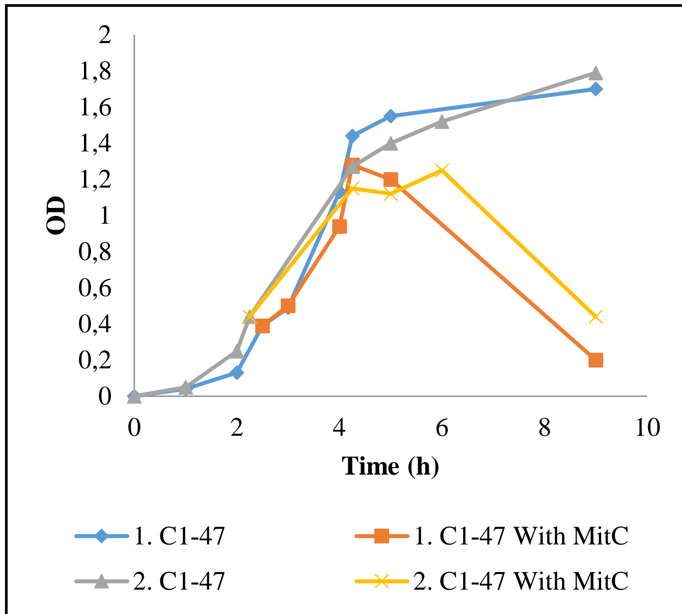
Growth curves for the STEC isolates in two replicates, with and without MitC is illustrated in the following figures, description is within each figure (Time (h) = 9 shows OD after ON incubation):










OD
2
1,8
1,6
1,4
1,2
1
0,8
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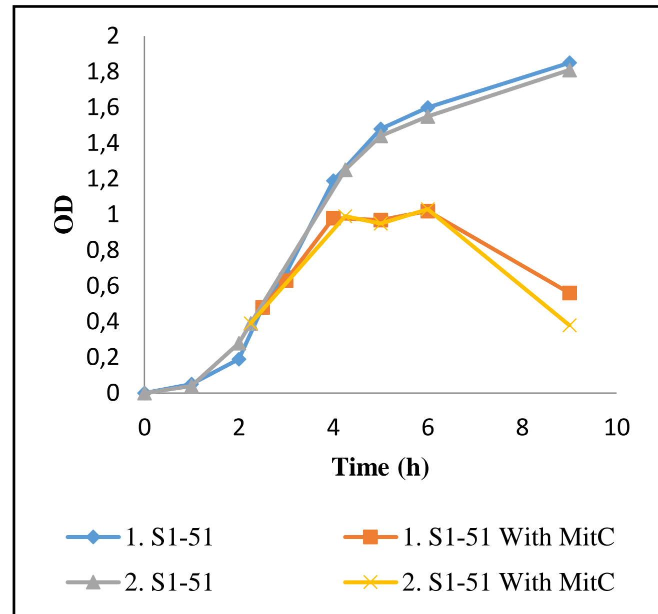
0,4
0,2
0
0
2
4
6
8
10
Time (h)
1. C1-47
1. C1-47 With MitC
2. C1-47
2. C1-47 With MitC


OD
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2
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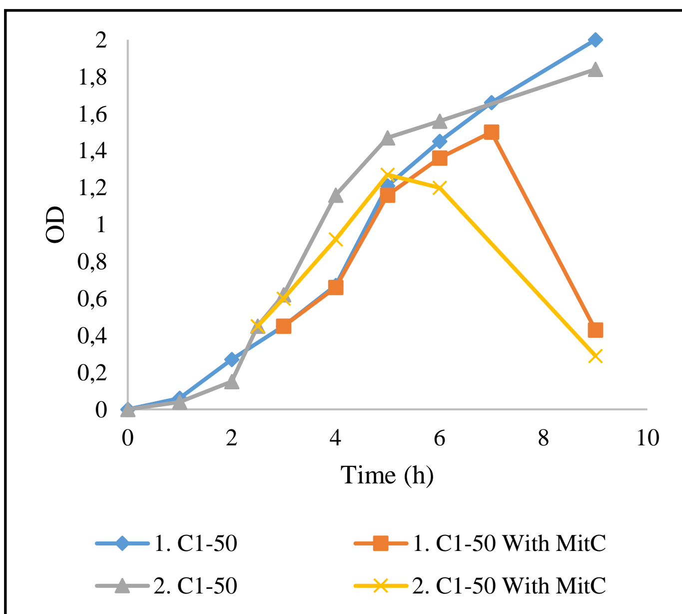
6
8
10
Time (h)
1. S1-51
1. S1-51 With MitC
2. S1-51
2. S1-51 With MitC


OD
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0
2
4
6
8
10
Time (h)
1. C1-50
1. C1-50 With MitC
2. C1-50
2. C1-50 With MitC

Appendix E

Number of colonies and calculated competence for each isolate is listed in table e.1.

Table e.1: Number of colonies and calculated competence for each isolate in the transformation efficiency experiment using pUC19.

Isolate name	pUC19		
	Date	Number of colonies	Calculated competence
H1-43	*17.11.2016*	10	$1,1 \cdot 10^7$
	13.12.2016	0	-
H1-44	*17.11.2016*	9	$9,5 \cdot 10^6$
	28.11.2016	3	$3,1 \cdot 10^6$
H1-45	*17.11.2016*	2	$2,1 \cdot 10^6$
	28.11.2016	5	$5,3 \cdot 10^6$
H1-46	*17.11.2016*	Overgrowth*	-
C1-47	*17.11.2016*	0	-
	28.11.2016	10	$1,1 \cdot 10^7$
	13.12.2016	0	-
C1-50	*17.11.2016*	1	$1,1 \cdot 10^6$
	28.11.2016	10	$1,1 \cdot 10^7$
	13.12.2016	1	$1,1 \cdot 10^6$
S1-51	*17.11.2016*	209	$2,2 \cdot 10^8$
	13.12.2016	98	$1,0 \cdot 10^8$
176 DH5α	*17.11.2016*	8	$8,4 \cdot 10^6$
	13.12.2016	38	$4,0 \cdot 10^7$
Roger DH5α	*13.12.2016*	79	$8,3 \cdot 10^7$
S.s 866::stx (P: C1-50)	*15.12.2016*	Overgrowth*	-
MG1655::stx (P: C1-47)-3	*15.12.2016*	OD did not reach target	-
	19.01.2017	38	$4,0 \cdot 10^7$
MG1655::stx (P: C1-47)-4	*13.01.2017*	23	$2,4 \cdot 10^7$
	19.01.2017	38	$4,0 \cdot 10^7$
c600H::stxV(P: C1-50)	*13.01.2017*	OD did not reach target	-
	19.01.2017	OD did not reach target	-
c600L::stxV(P: C1-50)	*13.01.2017*	OD did not reach target	-
	19.01.2017	OD did not reach target	-

*Ampicillin resistant

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