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Characterization of new putative adhesion factors in Shiga toxin-producing Escherichia coli

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Characterization of new adhesion factors in Shiga-toxin producing *Escherichia coli*

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Abstract

Worldwide nearly 2.8 million people are infected by STEC with acute illness, among them approximately 0.14% lead to HUS and 0.008% lead to death. Shiga toxins are essential virulence factors of STEC, but also other factors have been reported to be involved in virulence of such bacteria. Adherence to the target intestinal epithelial cell is an important initial step to establish STEC infection, with intimin is the major adhesin. Besides intimin, STEC express fimbrial proteins such as long polar fimbriae (LPF) which also have a role as adhesion factors. For some fimbrial and putative fimbrial proteins the function in virulence is still not clear. The aim of this study was to clone the putative fimbrial operon *lpf*$_{B1}$ into a wild type STEC strain and analyse expression of fimbrial operon genes in this strain under different growth conditions.

First PCR primers for the *lpf*$_{B1}$ operon and one of the genes within the operon were designed, based on the nucleotide sequence of the operon from a genome sequenced strain. After optimization of the PCR, the operon with up- and downstream adjacent sequence was amplified. The identity of the PCR product was confirmed by gel electrophoresis and Sanger sequencing. The PCR product was then inserted in a cloning vector pCR-4 TOPO, which was first transformed in TOP10 competent cells. This resulted in TOP10 cells with *lpf*$_{B1}$ insert-vector. Plasmid was extracted from the transformed cells and plasmid with the l*pf*$_{B1}$ insert (named pDA00) and plasmid without insert (named pDA00) were amplified with PCR and performed electrophoresis to confirm the presence of *lpf*$_{B1}$ operon. These plasmids (pDA00 and pDA09) were further cloned into a clinical wild type strain STEC O103:H2, which had lost its *stx* genes. Transformed cells were again tested with PCR and electrophoresis to confirm the *lpf*$_{B1}$ operon. Transformed bacterial cells were then cultured in LB broth and SILACE broth. From these cultures RNA was extracted, and reverse transcribed to cDNA using random primers. The amplification efficiency of the real-time PCR for the *lpf*$_{B1}$*C* gene, and the housekeeping gene *rpoA* was tested, and found to be 99% and 105% respectively. Then cDNA was amplified by real time PCR of the *lpf*$_{B1}$*C* gene, using *rpoA* as reference, to study the expression of the operon under different growth conditions. The reference gene *rpoA* was expressed when grown both in LB and SILACE broth, while the *lpf*$_{B1}$*C* gene was not found to be expressed in any of the two growth conditions tested. There could be several reasons why the *lpf*$_{B1}$*C* gene was not expressed in this study, but due to time constrains there was not sufficient time to investigate why the *lpf*$_{B1}$*C* gene was not expressed.

In conclusion, PCR amplification and cloning of the *lpf*$_{B1}$ operon were successful, but the operon was not expressed under the two growth conditions tested. Nevertheless, this study has

expanded further possibilities to analyze the expression of long polar fimbriae in non-O157 STEC.

Preface

This thesis is a part of my masters' degree at the department of Biology, NTNU. This study was carried out at Department of Clinical and Molecular medicine (IKOM) at Faculty of Medicine and Health Sciences, and the Department of Medical Microbiology, St. Olavs hospital, Norway.

I would first like to thank my supervisor Professor Jan Egil Afset for his help and support throughout the work with this thesis. Thank you for keeping the door of your office always open for me. Your warm behavior, scientific experience and knowledge, and your inspiration have made possible to complete this thesis.

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I express my profound gratitude to my parents who allowed me to carry out my study in NTNU, Norway. The journey from Nepal to Norway would not have been possible without their support. Thank you and love you! And finally, thanks to the invisible power 'god' for giving me strength.

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1 Introduction

1.1 Escherichia coli

Genus *Escherichia*, a member of Enterobacteriaceae family, comprises gram-negative, non-spore forming, facultatively anaerobic rod-shaped bacterial species. In general, based on the biochemical reactions, Escherichia is differentiated into six species: *E. coli, E. hermannii, E. fergusonii, E. vulneris, E. blattae,* and *E. albertii* [1].

Escherichia coli was named as Bacterium coli commune when Theodore Escherich first isolated this bacterium from colon of an infant in 1885. The size of rod-shaped *E. coli* ranges from 2.0 to 6.0 micrometer (µm) in length and 1.1 to 1.5 µm in width. Presence of peritrichous flagella (Fig. 1), protruding 15 to 20 µm from the cell surface, makes the bacterium motile. Most strains have polysaccharide capsules. The optimum temperature for growth is 37 ˚C with respiratory as well as fermentative type of metabolism [1].

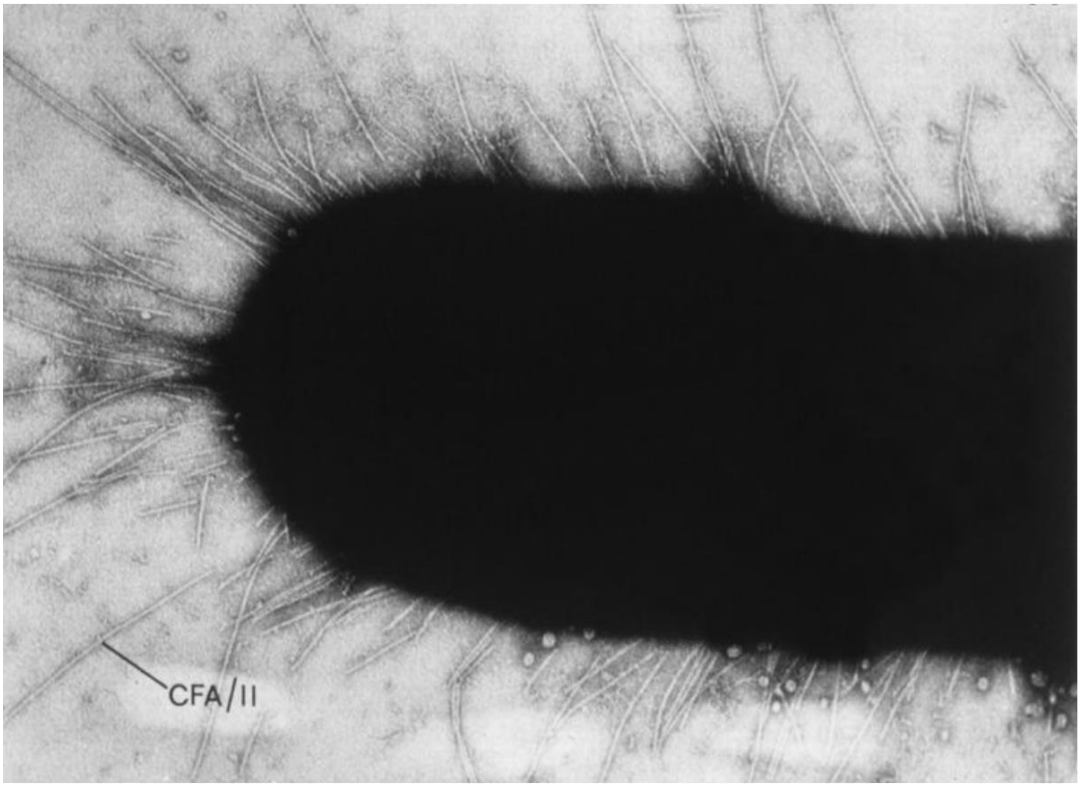

Figure 1: Morphology of ETEC fimbriae (labelled as CFA/II in the figure) and peritrichous flagella observed under transmission electron microscopy [2]

Serotyping of *E. coli* is based on the polysaccharide O antigen, flagellar H antigen, and capsular K antigen. O antigen, one of the components of lipopolysaccharides (LPS) of the cell wall, has a significant role in establishing infection. Currently, in *E. coli*, there are approximately 186

distinct types of O antigen and 53 different types of H groups, and several combinations of O and H antigens give the *E. coli* specific serogroups (e.g. O157:H7) [2, 3].

E. coli, one of the microbes found as a commensal of the intestine, is isolated from fecal contaminated water and food. *E. coli* can be described as an indicator organism for faecal contamination. It is transmitted to the host via contaminated food and water and may cause different diseases. Microbes that cause disease usually contain virulence factors. The intestine is the natural reservoir of *E. coli*. Because of gain or loss of functions in genes, commensal *E. coli* may receive different types of virulence factors and thereby become pathogenic. Route of infection might be endogenous or exogenous. Infections by *E. coli* are differentiated in two main types: intestinal disease (e.g. diarrhea) and extra-intestinal disease (e.g. urinary tract infections, meningitis) [1, 4].

Diarrhea is a major intestinal disease worldwide causing death of children, particularly under the age of 5, usually in developing countries. About 1.7 billion children suffer from diarrhea every year and nearly 0.5 million deaths occurs each year [5]. Pathogenic *E. coli* is one of the etiologic agents of diarrhea and is known as diarrheagenic *E. coli* (DEC) which is of seven types: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), Shiga toxin-producing *E. coli* (STEC-e.g. enterohemorrhagic *E coli* [EHEC]), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC) and adherent invasive *E. coli* (AIEC) [6].

EPEC, first identified pathotype of *E. coli*, has the ability to form attaching and effacing (A/E) lesions on the surface of intestinal epithelial cells (Fig. 2). Between 1940 to 1960, EPEC was first identified as an important causative agent of infantile diarrhea in developed countries. However, nowadays it is more prevalent in less developed as well as developing countries [2].

EIEC is a causative agent of bacillary dysentery and has common biochemical, pathogenic and genetic features with *Shigella* species. Nevertheless, *Shigella* species have higher degree of virulence and expression of virulence genes in comparison to EIEC. The fecal-oral route and direct contact from person to person are the major ways of transmission [6]. General path of pathogenesis of EIEC follows invasion of epithelial cells then endocytosis leading to breaking down of endocytic vacuole. Organisms or toxins then enter the cytoplasm and eventually migrate into adjacent cells (Fig. 2). In case of severe infection, a strong inflammatory reaction is produced that can cause ulceration in the intestinal wall. Periodic watery diarrhea followed

by dysenteric stools with blood and mucus are typical symptoms experienced by patients with EIEC infection [2].

EAEC, one of the most common etiologic agents of traveler's diarrhea, is also causing persistent diarrhea in AIDS patients as well as in children in EAEC endemic areas [6]. EAEC has a distinctive type of adherence termed aggregative adherence (Fig. 2), which is facilitated by aggregative adherence fimbriae, encoded by at least three different *aaf* genes. Fimbriae promote formation of biofilm in the intestinal mucosa. Enterotoxins and cytotoxins produced by EAEC can induce mucosal secretion and promote inflammation that leads to diarrhea [6, 7].

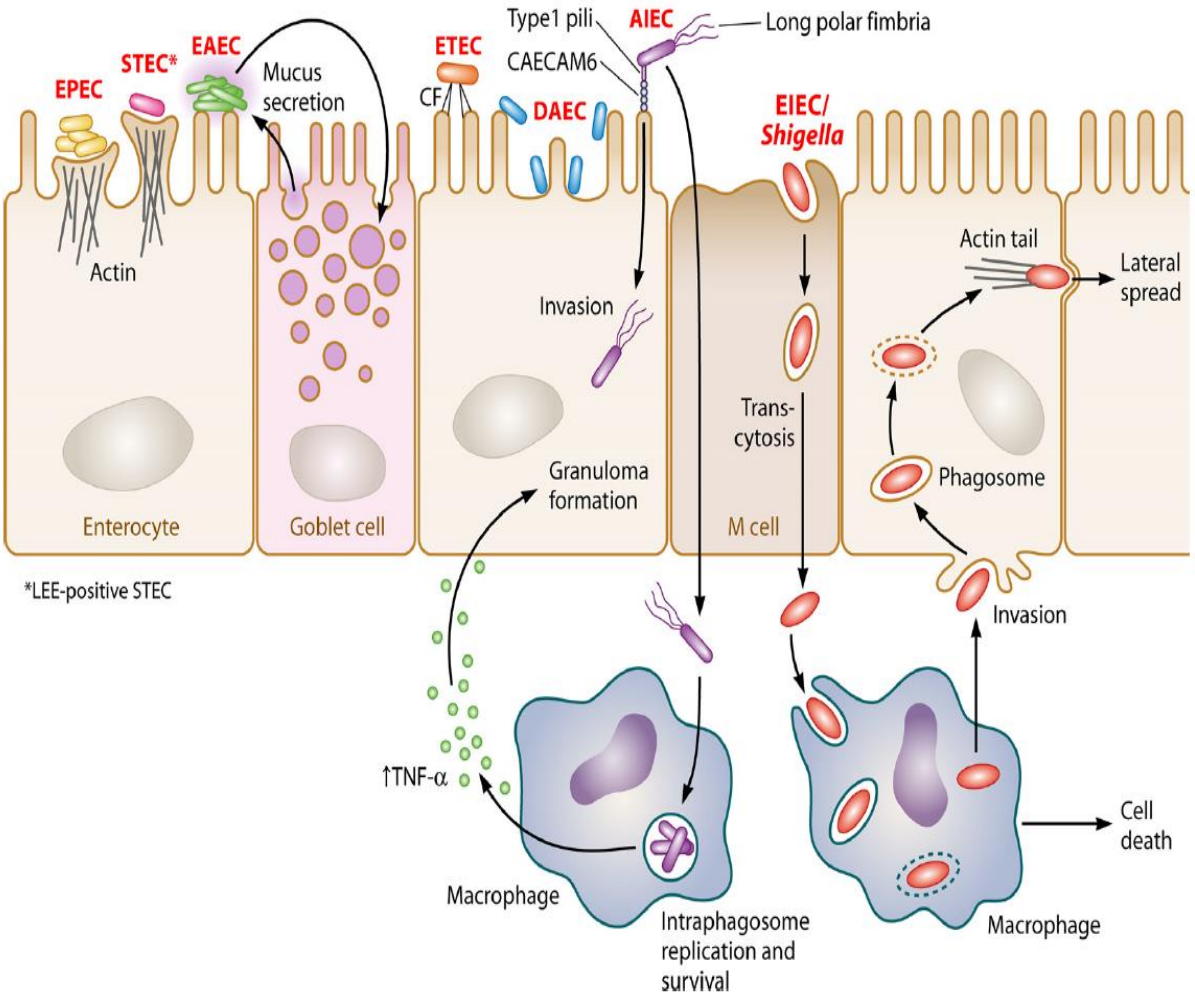

Figure 2: Pattern of adhesion of diarrheagenic *E. coli*. EPEC (yellow) and STEC (pink) are extracellular pathogens that adhere to the epithelial cells of the colon, efface microvilli, and form characteristic A/E lesions. EAEC (green) make biofilms on the mucosal surface of the intestine and adhere to the each other in addition to the cell surface to form a distinct type of aggregative adherence pattern. ETEC (orange) adhere to intestinal cells with the help of colonization factors (CFs). DAEC (blue) is scattered over the intestinal surface in the colon and forms a diffuse adherence pattern. AIEC (purple) attaches to the intestinal cells (of patients with Crohn's disease) with the help of type I pili and uses long polar fimbria to invade the cells. EIEC (red) are intracellular pathogens that invade the epithelial cells of the intestine and enter the submucosal region with the help of M-cells. EIEC escape submucosal macrophages by induction of macrophage cell death and spreads to invade other cells [6]. Image printed with permission from author.

ETEC can produce two types of enterotoxins heat-stable ST and heat-labile LT. It has a group of colonization factors that act as attachment factors to the epithelial cells of the intestine (Fig. 2). ETEC, a major cause of traveler's diarrhea, is a significant risk factor for lethal infection in children in developing countries. As with other diarrheagenic *E. coli*, ETEC is also transmitted by the fecal-oral route via contaminated food and drinking water [2, 6].

DAEC is characterized by that it adheres over the entire surface of Hep-2 epithelial cell in a scattered way i.e. known as diffuse adherence pattern, different from typical A/E adherence (Fig. 2). DAEC has been isolated from children with diarrhea as well as from healthy individuals. However, the mode of transmission and reservoir of DAEC is still not clear and specific detection methods for DAEC are yet to be developed [6].

AIEC has been reported to be associated with inflammatory bowel disease known as Crohn's disease (CD) and has been isolated from more than 30% of CD patients. AIEC has the ability to adhere to and invade epithelial cells (Fig. 2). It can replicate within epithelial cells and macrophages [6].

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

STEC is capable of producing a potent cytotoxin called Shiga toxin (Stx). Two independent studies done in 1983 gave the first reports on strain O157:H7 of *E. coli*. In a study done by Riley et al. [8], bloody diarrhea with little fever was reported from patients who had eaten undercooked hamburgers in a restaurant. Another investigation was reported by Karmali and his coworkers [9], in which cytotoxin produced by faecal *E. coli* was associated with hemolytic uremic syndrome (HUS). Furthermore, Johnson et al. also reported a case of hemorrhagic colitis (HC) from which *E. coli* O157:H7 was isolated and was found to be cytotoxic to Vero cells [10]. Eventually, Vero cytotoxin or Shiga-like toxin was considered as a virulence factor for HUS and HC [11]. After these first reported STEC cases, STEC have been associated with many diarrheal outbreaks all over the world [6].

STEC was formerly known as "verotoxigenic *E. coli*" after 1977, when Konowalchuk et. al reported STEC strains to be toxic on Vero cells [12]. The term enterohemorrhagic *E. coli* (EHEC) is used for the subpopulation of STEC that expresses Stx genes, cause A/E lesions on intestinal cells, and cause disease like HC and HUS in humans, with possessing a 60-megadalton (MDa) plasmid [13]. In this study, the term STEC will be used to denote all EHEC as well as other STEC.

1.2.1 Classification of STEC

World-wide O157:H7 is the most common serotype for STEC infections, however, non O157:H7 STEC groups are also an increasingly prevalent cause of human infections. In USA between 1983 and 2002, and in Australia in the same period, non O157:H7 human infections were mostly associated with the following serogroups: O26, O45, O103, O111, O121 and O145 which were called the "Big 6". Furthermore, O157 is differentiated into non-sorbitol-fermenting (NSF) STEC O157:H7 [14] and Sorbitol-fermenting O157:NM (SFO157:NM, NM: non motile). SFO157:NM strains were isolated from HUS patients in late 1990s to mid-2000s in some countries of Europe [6].

Another classification scheme is based on the presence of the LEE pathogenicity island. LEE positive strains include strains of serotypes O157:H7, SFO157: NM, O111:H8 and O26:H11, and LEE negative strains are found in the serotypes O113:H21, O91:H21, O45:H2 and O103:H2/H6 [6].

1.2.2 Virulence factors

Several virulence factors, both chromosomal and plasmid located factors, have been associated with STEC infections [15, 16]. Some common and potential virulence genes with their product, functions, and localization are shown in Table 1, and some of these will be described in more detail below.

Table 1: Some common virulence factors of STEC

Gene	Virulence factor	Function	Localization	Ref.
eae	Intimin	Adhesin	LEE pathogenicity island	[17]
efa-1/lifA	EHEC factor for adherence	Adhesin, lymphostatin	OI-122	[18]
ehaA, ehaB	Autotransporter EhaA, EhaB	Adhesin, autoaggregation, Biofilm formation	Chromosome, OI-15	[19]
ehxA	Enterohaemolysin	Production of proinflammatory cytokines	pO157	[20]
epeA	EpeA	Serine protease	pO113	[21]
espB	E. coli secreted protein B (EspB)	Multifunctional effector proteins	LEE pathogenicity island	[22]
espP	EspP	Serine protease: cleaves pepsin A and human coagulation factor V, cytopathic effect on vero cells, host colonization	pO113	[21]
espZ	EspZ	Inhibits cell apoptosis, regulate T3SS translocation	LEE pathogenicity island	[23]
etpD	EtpD	Type II secretion system	Plasmid	[24]
hcpA	Haemorrhagic coli pilus (Hcp)	Adhesin	Chromosome	[25]
katP	KatP	Catalase/peroxidase:hydrogen peroxide resistance	pO157	[26]
lpfA	Long polar fimbriae	Fimbrial adhesion	Chromosome	[27]
nleA	NleA	TTSS serine protease: inhibits cellular protein secretion, tight junction disruption	OI-71	[28]
nleB	NleB	Immune system modulation	OI-122	[29]
paa	Porcine attaching and effacing associated factor (Paa)	Adhesin	OI-57	[30]
pagC	PagC	Bacterial survival within macrophages	OI-122	[29]
saa	STEC autoagglutating adhesion	Adhesin	Plasmid	[31]
sfp	Sfp	Adhesin	pSFO157	[32]
stcE	StcE	Metalloprotease: remodeling the mucosal lining during infection	pO157	[33]
stx1	Shiga toxin 1	Toxin	Bacteriophage	[34]
stx2	Shiga toxin 2	Toxin	Bacteriophage	[34]
subA	Subtilase cytotoxin	Toxin	pO113	[35]
tccp/espF	Tir cytoskeleton coupling protein (Tccp)	Actin pedestal formation	Prophage	[36]
tir	Translocated intimin receptor	Intimin receptor, actin pedestal formation, inhibits NF-kB functions	LEE pathogenicity island	[37]
toxB	ToxB	adhesin	pO157	[38]

Shiga toxins (Stx)

The crucial virulence factor of STEC, the Shiga toxin (Stx), was given its name after the researcher Kiyoshi Shiga, who identified *Shigella dysenteriae* as the cause of dysentery [39]. There are two major types of Shiga toxins from *E. coli*: Stx1 and Stx2. These toxins are encoded by the genes *stx1* and *stx2*, respectively. Stx1 is almost identical to Stx from *S. dysenteriae* [40]. Stx1 and Stx2 share almost 60% identical amino acids in their structure [41].

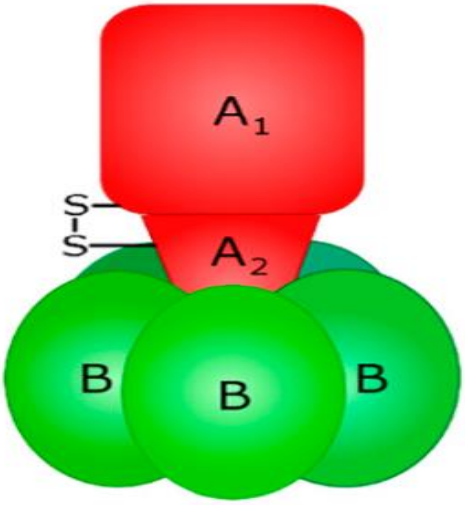

Figure 3: Structure of Shiga toxin (Stx) which is composed of two subunits, the A subunit and the pentameric B subunit. During endocytosis of toxin, the A subunit is cleaved by protease into A_1 and A_2 with disulphide linkage. The enzymatically active A_1 subunit inhibits protein synthesis of the ribosome [42]. Image printed with permission from author.

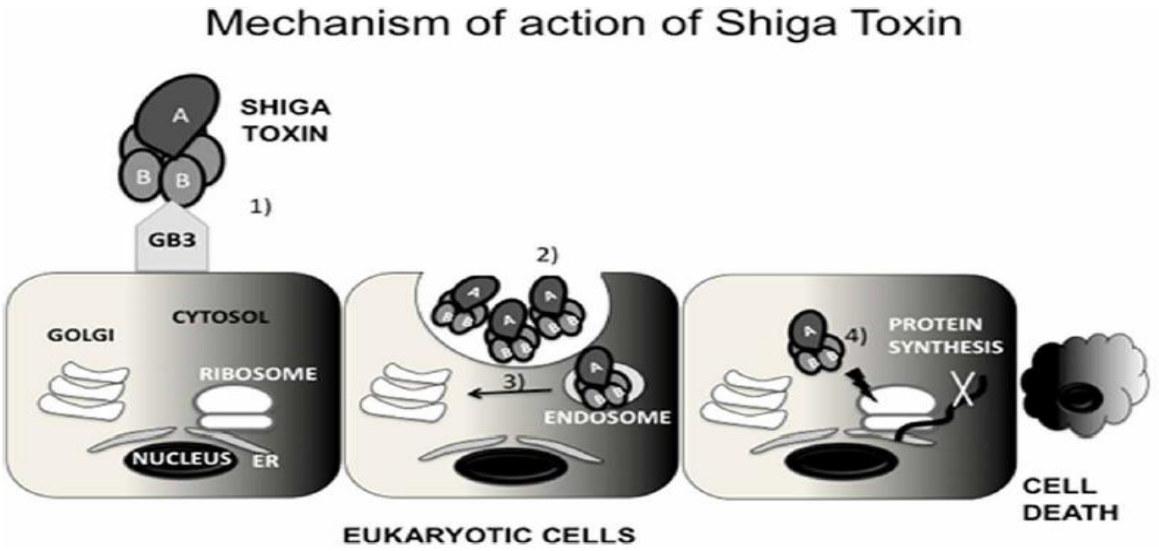

Figure 4: Mode of action of Shiga toxins (Stx): (1) The B subunits of Stx binds to the globotriaosylceramide (Gb3) receptor on the surface of the host eukaryotic cell. (2) Stx enters the cell by endocytosis. (3) Stx is then reversely translocated into Golgi bodies. From Golgi bodies Stx is then translocated into the endoplasmic reticulum (ER). (4) In the ER, Stx encounters the ribosome and inactivates it leading to inhibition of translation and causing cell death by apoptosis [43]. Image printed with permission from author.

Structure

The Shiga toxins consist of an A subunit (StxA) with a molecular weight of 32 kDa and five identical monomers of the B subunit, each of 7.7 kDa (Fig. 3). STEC reaches the gastrointestinal tract where it releases Stx, and through the Stx-B subunit the toxin binds to the glycolipid receptor globotriaosylceramide-3 (Gb3) on the surface of intestinal epithelial cells [44]. Then toxin is internalized and is reversely translocated via the Golgi apparatus into the endoplasmic reticulum (ER). During this translocation, the inactive A subunit is cleaved by protease into an active A1 subunit and a carboxyl terminal A2 subunit, but which are still linked by a disulphide bond. In ER the disulphide bond is degraded and the free enzymatically active A1 subunit is retrogradely translocated to the cytosol where it removes an adenine from 28SrRNA by its RNA-glycosidase leading to inhibition of protein synthesis, and eventually apoptosis occurs (Fig. 4) [6, 45, 46].

The actual mechanism of how shiga toxins are transported from the intestinal epithelium to the systemic circulation is not yet clarified. However, there are some hypotheses describing possible mechanisms of transportation. Etienne-Mesmin et al. suggest that STEC and Stx can cross the intestinal epithelial barrier through the microfold cells (M-cells). Then the bacterium is engulfed by and replicate inside macrophages and then release Stx. Eventually, due to excess production of Stx, host cells die, and toxin is released to and transported with the circulation to reach different organs such as the kidneys, the brain and the intestine causing injury to these organs [47].

There two Shiga-toxins types, Stx1 and Stx2, are further classified in subtypes. Currently three subtypes of Stx1 are recognized (stx1a, stx1c, and stx1d), and there are seven subtypes of Stx2 (stx2a, stx2b, stx2c, stc2d, stx2e, stx2f and stx2g) [2, 6].

Regulation

Both the Stx1 and the Stx2 genes are encoded on lamboid bacteriophages (eg. 933W and 933J) which can integrate into the bacterial chromosome, and which then is termed a prophage. The stx prophage can exist in either lytic or lysogenic form. Stx is produced and released after activation of the phage lytic cycle as well as during bacterial cell lysis. The Stx and Stx2 are phage late genes. The phage cycle and iron regulated promoter controls the Stx1 expression while Stx2 is produced after the phage goes into the lytic cycle. cI repressor protein binds to the right (O_R) and left (O_L) operator regions (Fig. 5) and makes the phage to become quiescent and eventually inhibit the activity of phage early promoters P_L and P_R. Furthermore, phage encoded

StxAB is induced by stress respons via the bacterial SOS response system (response to DNA injury in which the DNA repair is commenced with detention of the cell cycle [48]) whereby the phage enters the lytic cycle. The RecA protein is produced and activated after triggering of a SOS response eventually leading to cleavage if the cI repressor. Then the Q protein is expressed and contacts $P_{R'}$ to induce expression of late phage genes, including the StxAB genes [43].

Antibiotics have been reported to have a role in regulation of the lytic cycle of stx-phages. McGannon et al. reported that some antibiotics such as azithromycin, doxycycline and gentamycin repress the production of Stx whereas, ampicillin and ciprofloxacin were found to induce Stx production [49]. Some studies suggest that quinolones activate the SOS response in STEC, and in turn induce Stx production. Other components such as nitric oxide, hydrogen peroxide and iron have also been shown to influence on the regulation of phage replication and stx production [43].

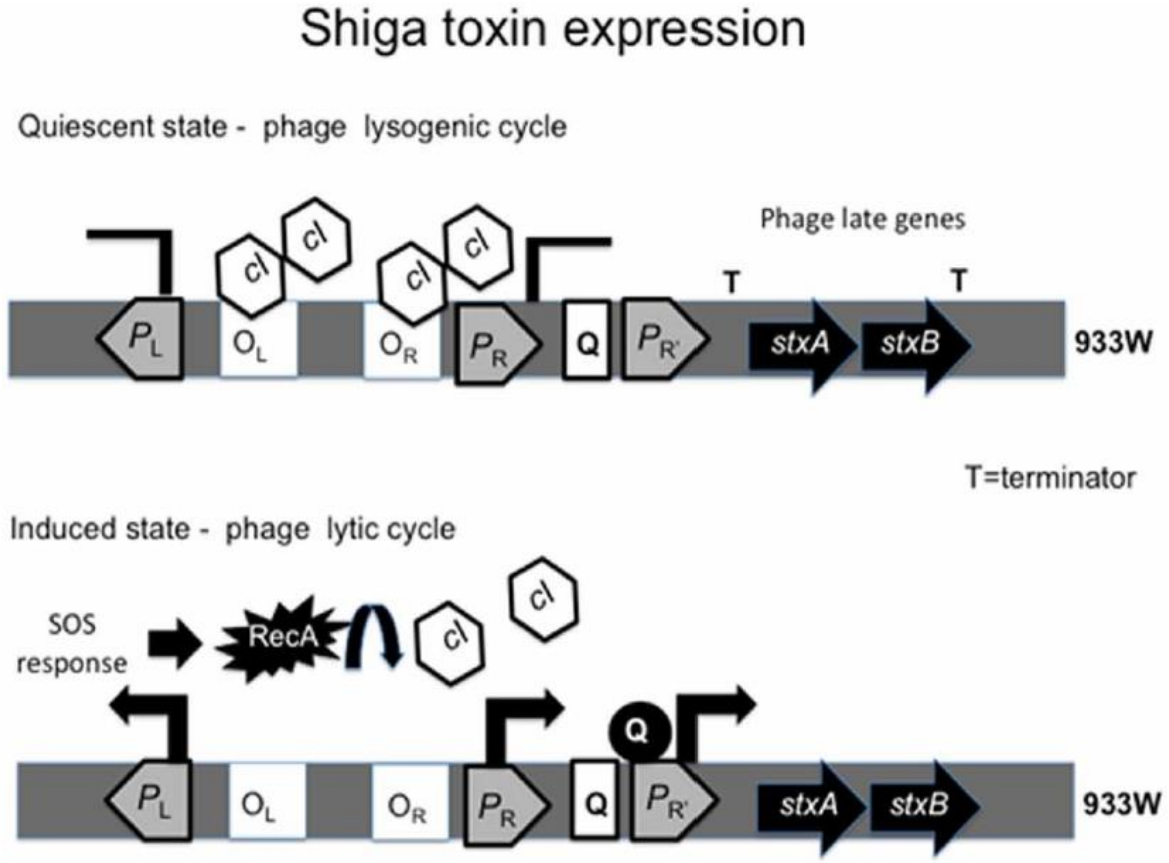

Figure 5: Regulation of lamboid phage and stx expression [43]. Image printed with permission from author.

The LEE pathogenicity islands

Some STEC strains consist of a cluster of virulence genes of a length of about 35 kb located on a chromosomal pathogenicity island termed the Locus of Enterocyte Effacement (LEE), responsible for attaching and effacing (A/E) lesions in vivo and in cell culture. The A/E lesion is characterized by effacement of microvilli at the site of bacterial attachment, accumulation of polymerized actin cytoskeleton beneath the adherent bacteria [50], and pedestal-like structures (Fig. 6) [6]. A/E lesions were described in piglets infected with STEC O157:H7 and has been reported also in ruminants [51].

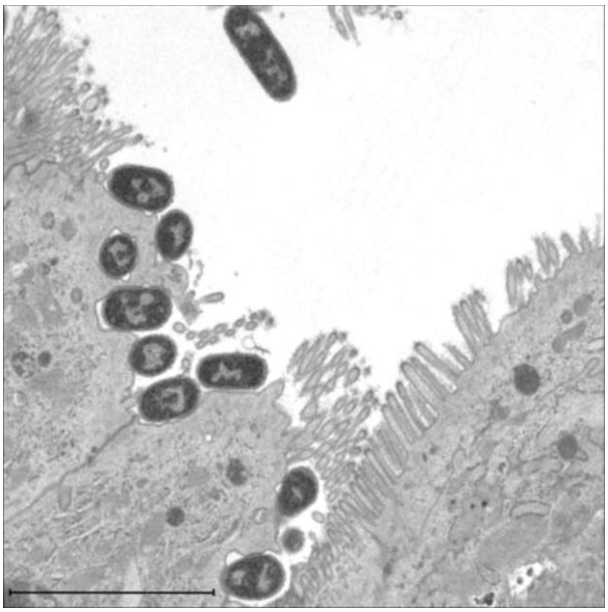

Figure 6: Transmission electron microscopy image of A/E lesions caused by O157:H7 infection on bovine intestinal cells [51]. Image printed with permission from author.

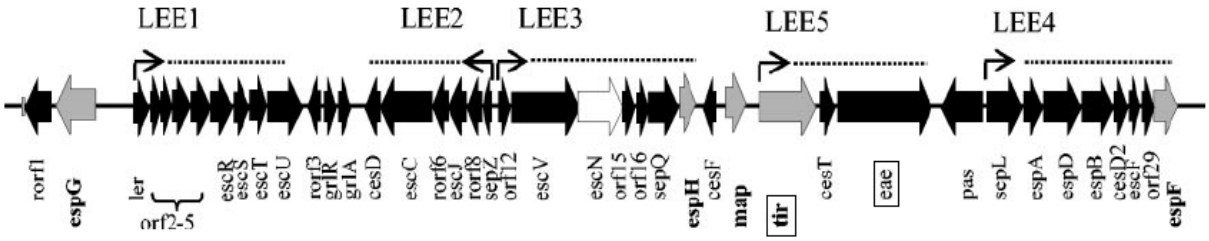

Figure 7: Organization of the LEE pathogenicity island genes of STEC O157:H7 in the five polycistronic operons LEE1 to LEE5 [51]. Image printed with permission from author.

The LEE of STEC consists of 41 genes which are localized in five polycistronic operons, termed LEE1 to LEE5. The LEE1 to LEE4 comprise genes that encode type III secretion system (T3SS) proteins, regulators, translocators, chaperons and effector proteins (Fig. 7). T3SS is a complex of proteins which interacts with host cells. Some of the proteins are injected into the host cell showing different effects on the host cells [51]. LEE5 contains genes that encode the

translocated intimin receptor (Tir) and intimin [15, 52]. The 94-kDa intimin protein is encoded by the *eae* gene, and the 80 kDa protein Tir protein is encoded by *tir*. Tir is transferred by the T3SSs into the cytoplasm of the host cell, and then is the translocated into the cell wall where it acts as receptor for intimin. Amino acids near the C- terminal part of the intimin protein take part in binding with Tir on the host cells. The interaction of Tir and intimin subsequently causes Tir clustering, actin assembly and cytoskeletal proteins are gathered at the bacterial attachment-site [2, 20, 51]. This assembly process is assisted and followed by different signaling pathways to form the A/E lesions.

Fimbrial adhesins

Besides the major adhesin intimin, fimbriae are also involved in adhesion of the bacterium to the intestinal epithelial cell. These hair-like filamentous structures are made up of protein and cover the cell surface. Fimbriae are encoded either by chromosomal or plasmid located genes. All strains of bacteria may not have fimbriae; however, some strains may have more than one kind of fimbriae [1]. For example, in *E. coli* O157:H7, about 16 fimbrial operons have been described [53].

Some well-featured fimbrial proteins found in STEC are Long polar fimbria (Lpf), the *E. coli* common pilus (ECP), the hemorrhagic *E. coli* pilus (HCP), F9 fimbriae, *E. coli* YCBQ laminin-binding fimbria and some other minor fimbrial proteins [54].

Lpf has been found to adhere to extracellular matrix protein Laminin important for establishing initial attachment before intimate attachment [54]. In STEC O157:H7, two Lpf operons- *lpf1* and *lpf2* has been found [6]. The *lpf1* operon, situated in an "O157- specific island" (O-island 141 i.e. OI141), contains 6 genes (*lpfABCC'DE*). The *lpf2* operon consists of 5 genes (*lpfABCDD'*) situated in the OI-154 region [54]. One study showed that strains with mutations in the *lpfA1* gene adhered less to intestinal epithelial cells than strains with the wild type gene [54] indicating a role of the lpf gene in attachment.

The filamentous polymeric hemorrhagic coli pilus (HCP) belongs to the type 4 pili group which helps the bacterium to adhere to extracellular matrix proteins on intestinal epithelial cells. Besides adherence, HCP also contributes in virulence, biofilm formation, cell signalling, twitching motility, and influences the host immune system [6, 54].

The *E. coli* common pilus (ECP) belongs to the type 1 fimbriae and is found in commensal as well as pathogenic *E. coli*. It is composed of fibrous protein encoded by the *yagZ* gene. It mediates microbe binding to the intestinal surface as well as to induce interactions between

microbes [6, 55]. Initially, this component was found in meningitis associated *E. coli* [56]. Temperature, oxygen level, and culture media regulate the expression of genes encoding this component [54].

F9 fimbriae, this putative type fimbrial operon, is encoded in O island 61 (OI61) in the chromosome of STEC O157:H7. This operon share homology with the operon encoding the F1 fimbrial protein of *E. coli*. Especially, it was found to be involved in adherence and colonization in the intestinal epithelium of cattle [57, 58].

E. coli YCBQ laminin-binding fimbria: This is encoded by another putative fimbrial operon containing genes with high degree of similarity to the F17 fimbrial genes. The YCBQ fimbria is encoded by the *ycbq* gene and it is significantly involved in adherence to the laminin protein [54].

One of the minor STEC fimbrial adhesins are the SFP fimbriae. Sorbitol-fermenting (SF) STEC O157:H7^{-} has a plasmid pSFO157 (different from the plasmid pO157:H7) that carries a cluster of virulence genes named *sfpAHCDJG*. These genes encode SFP fimbriae involved in attachment of bacterial cells to human intestinal cells. SF-STEC O157:H7 has been found to be one of the important causes of gastrointestinal diseas and HUS in some areas of Europe [54, 59].

pO157 plasmid

The pO157 plasmid is a virulence type of plasmid which is conserved in almost all clinical isolates of STEC O157:H7. The biological importance of this 92 to 104 kb sized plasmid is still not clear, however, some proteins encoded by pO157 is considered to have a role in virulence [2].

The *katP* gene in the pO157 plasmid encodes the catalase peroxidase (KatP) protein that shows bifunctional properties [6]. It may help in reduction of oxidative burden by decomposing some toxic components into different by-products in addition of oxygen. This oxygen is utilized to reduce oxygen dependent killing of bacteria within macrophages in the host intestine [60]. The enzyme may also assist in recovery of bacteria from heat stress [reviewed by [61]].

The first described virulence protein of the pO157 plasmid is hemolysin, encoded by the 3.4 kb long operon *ehx*. This highly conserved toxin present in almost all strains of STEC can form pores and lyse the erythrocytes. In addition, hemolysin is cytotoxic to endothelial cells, a property which may be involved in development of HUS [6, 60] .

Serine protease is a type v secreted protein, encoded by the plasmid located *espP* gene, which has proteolytic activity to pepsin A and coagulation factor V [60]. One study mentioned EspP to adhere, colonize and lyse host intestinal cells [62] potentially associated with mucosal haemorrhage in patients with HC [60].

1.2.3 Epidemiology of STEC

The crucial reservoirs of STEC are ruminants which are used for meat and dairy products. STEC is transmitted by the faecal-oral route. Consumption of contaminated or undercooked meat and meat products, poor personal hygiene and use of improperly treated water may be risk factors for STEC infections. Due to high degree of toxicity of Stx, even low infectious dose of STEC is sufficient to cause illness [6].

In the beginning of the 1980s, STEC was first isolated from undercooked meat [8]. In general, STEC O157:H7 infection has been most common in humans. In the early 1990s an outbreak occurred in Western USA after eating inadequately cooked STEC O157:H7 contaminated hamburgers in a chain restaurant [63]. Similarly, in the late 1990s, in New York, USA again, another outbreak of O157:H7 happened due to consumption of water contaminated by cattle manure [6].

However, non-O157:H7 may also cause severe disease. In a study done between 1983 and 2002, the “Big 6” serogroups were found as common etiologic agents of human STEC infections [6, 64].

A report by Majowicz et al. reported that worldwide nearly 2.8 million people were infected by STEC with acute illness, among them approximately 0.14 % led to HUS and 0.008 % led to death [65].

In 2015, within 30 EU/EEA countries, 6151 cases of STEC infection were confirmed. The report showed a notification rate of 1.5 cases per 100,000 population, and this was the same rate as in the year 2014. The highest notification rate was seen in Ireland with 12.9 cases/100,000 population. In Norway the notification rate was 3.0 cases per 100,000 population. Among the total confirmed cases, 55 % were female and the age group between 0 to 4 years was found to be most susceptible to infection with almost 8.0 cases per 100,000 population. In the report it was found that there was a significant reduction in the notification rate from 2011 (2.6 cases per 100,000 population) to 2015 (1.5 cases per 100,000 population) [66].

1.2.4 Haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS)

HC is a disease caused by STEC. The illness is characterized by abdominal cramps followed by bloody diarrhea. It is normally a self-limiting disease with incubation period of 2-10 days [reviewed by [67]].

HUS is a rare type of disease first described by von Gasser and co-workers in a child of five year [68, 69]. Main etiological agents of HUS are *Shigella dysenteriae* serotype 1 especially in South Asia, and STEC (e.g. O157:H7) common in the most part of the world [68]. 90% of all HUS cases are generally found in children. HUS caused by STEC is the most common reason for acute renal failure in children [69].

Stx mediated HUS is featured by three major symptoms: hemolytic anemia, low platelet count, and acute renal failure [69]. An essential element in HUS is thrombotic microangiopathy which results from thrombosis (formation of blood clot inside the blood vessel) associated with to injury to endothelial cells [70].

2 Aim of the study

Although STEC may have the potential to cause severe disease in humans, most STEC isolated from humans do either not cause symptoms at all or only mild uncomplicated infection. Host factors or infection dose may influence on disease severity. Stx is essential for severe disease, *eae* is an important adherence factor and other factors like fimbriae may play a role adherence to the host cells which seems to be necessary for the bacterium to be able to establish infection. Among Stx, Stx2 subtypes are usually associated with severe disease [2, 71].

In 2014, Kjersti Haugum in her PhD-project compared the genomes of STEC isolated from patients with HUS to the genomes of strains from patients with non-severe disease [72]. Despite in-depth comparative genomics analyses, she was not able to identify specific genes which could be used to differentiate between the two groups of strains. In a further attempt to differentiate between HUS-associated and non-HUS STEC, Christina Gabrielsen in her postdoc project compared the level of RNA expression between 15 HUS associated and 15 non-HUS non-O157 STEC strains. These strains were similar with respect to known risk factors: all strains were from children < 5 years of age, were LEE positive and contained stx of subtype stx2a, and all belonged to STEC serogroups. Based on preliminary results of global transcriptional profiles in HUS and non-HUS STEC, she was then able to identify 13 genes upregulated in HUS strains which may be involved in cell adhesion in non-O157 STEC [73]. Among these genes there were five genes located in type 1 fimbriae operon and several not-well characterized putative fimbrial genes. Interestingly, four of the genes, located in a single putative fimbrial operon in strains of phylogroup B1, were not identical to any previously characterized fimbrial genes. The encoded proteins of this putative fimbrial operon was 42-63% similar to the long polar fimbria 2 of STEC O157:H7 Sakai strain, and was located in the same pathogenicity island I island 154. The putative fimbrial operon was therefore termed lpf_{B1}.

The aim of this master project was to clone the putative lpf_{B1} operon into a wild type STEC strain and analyse the expression of the operon genes in the wild type strain.

Specific objectives of the study were as follows:

- Develop a PCR of the lpf_{B1} operon and a quantitative real-time PCR for one of the putative fimbrial genes
- Clone the operon into a wild type STEC-strains which had lost its *stx* genes
- Analyse the expression of the $lpf_{B1}C$ gene by quantitative real-time PCR in different growth media

3 Materials and Methods

This section provides detailed information of the materials and methods that were used in this study. All equipment, media and biological reagents are listed in Appendix I.

3.1 Clinical isolates E. coli

Clinical isolates of *E. coli* were kindly provided by the Department of Microbiology at St. Olavs Hospital, Trondheim, Norway. All clinical strains and reference strains with their general features are listed in table 2.

Table 2: Characteristics of clinical and reference strains used in the study

Type	Strain ID	O type	H type	Stx1/2	LEE	eae	Source	HUS	*lpf*$_{B1}$-operon
Clinical strains	FHI11	103	25	-	+	+	Human faeces	+	+
	FHI13	103	25	-	+	+	Human faeces	+	+
	FHI15	103	25	-	+	+	Human faeces	+	+
	FHI21	103	25	-	+	+	Human faeces	+	+
	SO-ECO 13-1	103	2	-	+	+	Human faeces	+	(-)*
	SO-ECO 13-2	103	2	-	+	+	Human faeces	+	(-)*
	SO-ECO 10-1	103	2	-	+	+	Human faeces	+	(-)*
Ref.	FHI6	111	H-	Stx1a Stx2a	+	+	Human faeces	+	+
Plas.	pDA00						This study		-
	pDA09						This study		+
Clon	*E. coli*	Genotype: F- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ *lac*X74 *rec*A1 *ara*D139 Δ(*araleu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G							

*Based on the following: strains belonging to the serotype O103:H2 were shown by whole genome sequencing in a previous study [72]) to lack the *lpf*$_{B1}$ operon. Ref: Reference strain; Plas: isolated Plasmid from transformed bacterial cells; Clon: Topo 10 competent *E. coli* cell used in cloning.

3.2 Bioinformatic tools

Bioinformatics is the art of collection, classification, storage and analysis of biological data by use of computational resources. The bioinformatic tools used in this study are described in the following sections, including software which is integrated in laboratory instruments for instrument operation and data analysis.

3.2.1 Geneious software (version 11.0.2)

Geneious is a commercial software for analysis of molecular biology and genome analysis. It provides modules to visualize and manipulate DNA sequences, amino-acid sequences, alignments, 3D structures, sequence chromatograms and graphs [74].

The *lpf*$_{B1}$ operon of the non-O157 strain FHI6 used as reference strain in this study is shown in Fig. 8. The *lpf*$_{B1}$ operon has 4 structural genes. In this study, the clinical strains were found to contain a putative fimbrial operon encoding proteins 42-63% similar to LPF 2 of STEC strain O157:H7 Sakai. The *lpf*$_{B1}$ operon contained four structural genes. Based on that of the LPF 2 operon, the putative fimbrial operon was named *lpf*$_{B1}$, and genes were named as *lpf*$_{B1}$A, *lpf*$_{B1}$B, *lpf*$_{B1}$C and *lpf*$_{B1}$D, respectively.

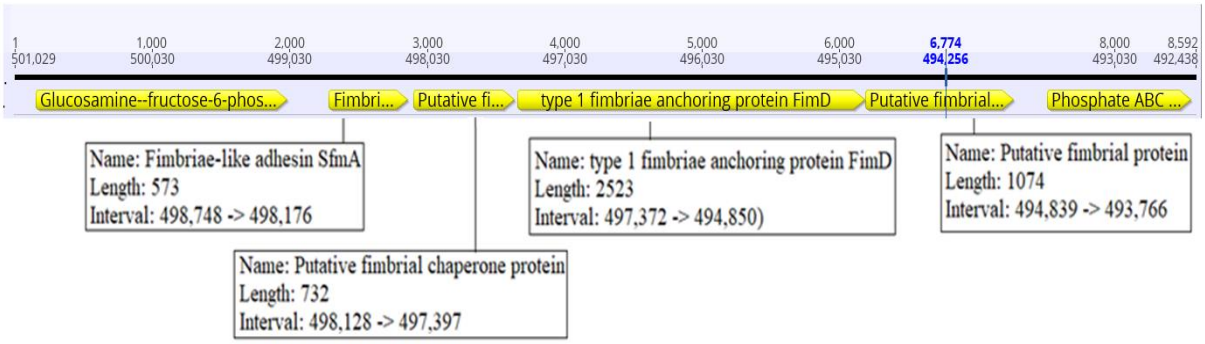

Figure 8: Structure of *lpfB1* operon of the reference non-O157 STEC strain (FHI6) extracted from the Geneious software. Gene names in figure based on automatic annotation: SfmA corresponds to *lpf*$_{B1}$A, putative fimbrial chaperone protein to *lpf*$_{B1}$B, FimD to *lpf*$_{B1}$C and putative fimbrial protein corresponds to *lpf*$_{B1}$D.

3.2.2 PromoterHunter

PromoterHunter, available at the phiSITE database (phiSITE release 2014.1), is a tool for promoter search in prokaryotic genomes [75]. The phiSITE is a free database service provided by the Institute of Molecular Biology, Slovak Academy of Sciences. The site contains a collection of phage gene regulatory elements, genes, genomes and other related information (http://www.phisite.org/main/index.php?nav=tools&nav_sel=hunter).

FASTA nucleotide sequences are uploaded and then a matrix for distribution of A, G, C, T is specified. Different input parameters such as global G+C content, space between -10 and -35 region, and Gibbs energy are specified. Then analysis is started. On the result window, possible promoters are presented with a score. The highest final score is called “best score” and promoters are chosen according to the score. Based on this principle, promoter sequences and regions were identified in this study.

3.2.3 NCBI

The National Center for Biotechnology Information (NCBI) Primer-BLAST program (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) is developed for automatic design of PCR primers. The Primer-BLAST uses Primer3 to design PCR primers and checks the target specificity of the generated primer pairs. The specificity checking function uses BLAST along with the Needleman-Wunsch global alignment algorithm to check matches between the targets and primers [76].

In this study, the nucleotide sequence of the *lpf*$_{B1}$ operon and *lpf*$_{B1}$*C* gene from the reference strain FHI6 were chosen for design of PCR primers for the whole operon and the *lpf*$_{B1}$*C* gene, respectively. Primer-BLAST was used to identify possible primer pairs, and primer pairs with high specificity based on Primer-BLAST analysis were further analysed with the OLIGO Primer Analysis Software (as discussed below) to identify the best primer pair.

3.2.4 Oligo Primer Analysis Software (version 7)

Oligo Primer Analysis Software (Molecular Biology Insights) is a commercial software for design of PCR primers, probes and synthetic genes, accepting sequence formats from GeneBank and EMBL and other text formats. The Oligo software makes it possible to visually inspect and redesign suggested primers. Required length of primers can be searched within the template sequences and then optimized by visualizing the sequence melting temperature, internal stability graph, hairpin loops and duplex formation [77]. In this study, several primer pairs were first designed in Primer BLAST, then those primers were checked with in Oligo, and the best primer pairs were chosen for further testing.

3.3 Culture of bacterial strains

Bacterial strains were available from the strain collection at Department of Medical Microbiology, St. Olavs Hospital, frozen in Greaves solution [78]. Bacterial strains were recovered from frozen state by culture and controlled for purity on blood agar. Blood agar plates were taken, well labelled and each strain of *E. coli* (as listed above) was inoculated on distinct plates with the help of sterilized plastic inoculating loops.

In the incubator the petri plates were put upside down to prevent the inoculated surface of agar from absorption of moisture resulted from condensation. The plate was incubated overnight at

37°C. After culture, an isolated colony was picked up and further sub-cultured on blood agar to get isolated pure colonies. Biosafety cabinet type 2 was used in processing of microbial materials.

Culture media used in this study were as follow:

Luria-Bertani Broth/Agar medium

LB medium is an enriched medium due to the presence of yeast extract and casein enzymic hydrolysate, which allows fast growth of many bacterial species. It contains all essential nutrients including B-vitamin to support the growth of recombinant *E. coli* strains [79, 80]. The composition and preparation of the medium is described in Appendix II.

Blood agar

Blood agar is an enriched as well as a differential medium. 5% v/v sterile defibrinated blood is added to blood agar base making the medium highly nutritious. Some bacteria able to lyse red blood cells to give hemolysis of red blood cells in the agar medium. Hemolysis may be partial (α-hemolysis), complete (β-hemolysis) or there may be no hemolysis (term γ-hemolysis) [81]. The composition of the medium is described in Appendix II.

SILACE broth

SILAC (Stable Isotope Labeling by Amino acids in Culture) broth is a method for quantitative proteomics analyses from complex protein samples like cell culture. At first media with proteins is specially formulated and later one or two essential amino acids labelled with isotopes such as lysine labelled with ^{2}H (D4), ^{13}C or $^{13}C^{15}N$ and/or arginine labelled with ^{13}C or $^{13}C^{15}N$ are added. The labelled amino acids increase the molecular weight making it easier to analyse the proteins or peptides from e.g. cell culture [82, 83].

In this study SLILACE medium developed by Ping et al. was used that was suitable for complete labelling of *E. coli* without any additional genetic modifications [84]. The formulation of SILAC broth specifically developed for auxotrophic *E. coli* (SILACE) is described in Appendix II.

3.4 DNA extraction

DNA extraction was done using the DNeasy blood and tissue kit from Qiagen. This kit is used for rapid purification of DNA with minimal handling and does not require phenol or chloroform extraction. It is provided with different buffer systems and a mini spin column with silica-based

membrane. Bacterial samples are lysed with the use of proteinase K, ALT lysis buffer and heat. Then AL buffer is added, and the mix is loaded onto the DNeasy mini spin column. AL buffer contains the chaotropic salt guanidine hydrochloride which induces binding of DNA onto the silica-based membrane inside the spin column. During centrifugation, DNA is bound to the membrane. Then the AW1 buffer is added. This buffer also contains guanidine hydrochloride that promotes binding of DNA onto the membrane. Then the mix is centrifuged. Impurities bound to the membrane are washed away with wash buffer and DNA is eluted in an elution buffer.

A colony of bacteria from a blood agar plate was mixed with 100 µl TE buffer (pH 7.4). Then 180 µl ATL buffer and 20 µl Proteinase K was added and mixed by vortexing. The mix was then incubated at 56 ℃ for 30 min. and mixed by vortexing for 15 sec. 200 µl buffer AL and 200 µl 96% ethanol was then added and mixed by vortexing, after which a white precipitation was formed. The mixture was pipetted into the spin column placed in a 2 ml collection tube, which was then centrifuged at 8000 rpm for 1 minute, and flow-through and collection tube were discarded. The DNeasy mini spin column was placed in a new 2 ml collection tube, 500 µl buffer AW1 was added, and the column was centrifuged for 1 min at 8000 rpm. Flow-through and collection tube was discarded. Then the DNeasy spin column was again placed in a new 2 ml collection tube and 500 µl AW2 buffer was added and centrifuged for 3 min. at high speed to dry the DNeasy membrane. The flow-through and collection tube was discarded. The DNeasy column was then placed in a clean 1.5 ml microcentrifuge tube and 200 µl buffer AE was pipetted directly onto the DNeasy membrane. The column was thereafter incubated at room temperature and centrifuged at 800 rpm for 1 minute. The eluate with extracted DNA was stored at 4℃ for further use. The concentration of DNA was measured by nanodrop method (is described in appendix V).

3.5 Polymerase chain reaction (PCR)

PCR allows the exponential amplification of specific regions of a DNA template with the help of a pair of small fragments of single stranded DNA known as primers. In a PCR buffer with magnesium chloride ($MgCl_2$), deoxynucleotide triphosphates (dNTPs), primers, water and *Taq* polymerase, amplification of PCR products happens during repeated temperature cycles of 3 steps: denaturation of template, annealing of primer with target and extension of primer. *Taq* polymerase is a heat stable DNA polymerase enzyme which is isolated from the bacterium

Thermus aquaticus. $MgCl_2$ acts as a cofactor and catalyzes the PCR reaction. Commercial PCR buffers usually contain 1.5mM $MgCl_2$ in 1x PCR buffer. In conventional PCR, amplified product is generally run on gel electrophoresis after end of thermocycling to detect the amplification product [85, 86].

The AmpliTaq Gold DNA polymerase is derived from a thermostable DNA polymerase encoded by a recombinant *Thermus aquaticus* DNA polymerase gene in *E. coli*. AmpliTaq Gold is provided by the manufacturer in an inactive state and due to this characteristic provides flexibility to assemble the mix of reagents of the PCR reaction at room temperature. The AmpliTaq Gold DNA polymerase is activated at high temperature (94°C) before thermocycling is started (Qiagen).

3.5.1 Protocol to confirm the presence or absence of *lpf$_{B1}$C* gene in *E. coli*

Primers were provided by the manufacturer as dry substance, which was then dissolved in Molecular Grade Water (MGW: i.e. high-quality water free from DNase, RNase and protease contamination) to make stock solutions according to the OLIGONUCLEOTIDE Technical Data Sheet (TDS) for each primer. Then working solutions of 10mM of primer was made by dilution from the stock solution. PCR reagents (buffer, dNTPs, primer) were thawed, vortexed and spun down except for the enzyme (Ampli Taq Gold). PCR mix was made for the number of samples plus one, according to the protocol shown in table 3. All reagents except enzyme were added to a 1.5 ml Eppendorf tube and mixed by vortexing and spun down. Polymerase was then added and pipetted gently to mix well. 48 µl of the mix was transferred to the required number of PCR tubes. Then 2 µl of DNA template was added in respective tubes and mixed by gentle pipetting. A negative control with molecular grade water (MGW) used as replacement for template was also included. All tubes were placed in a conventional T100 thermocycler (Bio-Rad) and run according to a specific protocol as given in table 3 below.

Table 3: Protocol for *lpf$_{B1}$C* gene PCR (product length 141 bp)

Components	**Volume per reaction (total 50 µl)**	**Protocol**
10x PCR buffer with $MgCl_2$	5.0 µl	Activation of Ampli Taq Gold enzyme: 94^0C for 10 min. Denaturation: 94^0C for 30 sec. Annealing temp. 59^0C for 30 sec. Extension 72^0C for 30 sec. Extra elongation step 72^0C for 7 min. Cooling 10^0C Amplification cycles: 25x
1mM dNTPs	2.5 µl	
10 µM forward primer	2.5 µl	
10 µM reverse primer	2.5 µl	
MGW	35.0 µl	
Ampli Taq Gold	0.5 µl	
DNA template	2.0 µl	

3.5.2 Protocol to confirm the presence or absence of lpfB1 operon in *E. coli*

LongAmp *Taq* 2X Master Mix was used to amplify the larger sized lpf_{B1} operon. The LongAmp Taq 2X Master Mix provides two-fold higher fidelity than *Taq* DNA polymerase and it has an optimized blend of enzymes to generate long PCR products up to 30 kb (BioLabs). The PCR product from the lpf_{B1} operon PCR was calculated to be 6,303 bp.

LongAmp *Taq* 2X master mix was pipetted into a 1.5 ml Eppendorf tube, primers and MGW were added and mixed by gentle pipetting. The mix was then transferred to PCR tubes and DNA template (MGW for negative control) was added in respective tubes. All reaction components were assembled on ice and quickly transferred to the thermocycler. PCR was run according to the protocol in table 4. First a standard protocol, as shown in table 4, was used to amplify the operon. Due to non-specific products with this protocol, the PCR was optimized with touch down PCR.

3.5.3 Touch-down PCR

Spurious smaller bands of nonspecific bands in the PCR-product spectrum may be due to mis-priming of one or both primers to the target template. With increasing numbers of amplification cycles, these non-specific products are accumulated and may overshadow the correct product. One alternative approach to overcome this problem is to adjust the annealing temperature of the PCR [87]. In touch down PCR, annealing temperature is set 3-10°C higher than the primer T_m during the first 5 to 10 cycles of PCR, whereafter it is reduced to the actual primer T_m for the rest of the PCR cycles (Advantage-HF 2 PCR User Manual). High annealing temperatures during the first cycles ensure high amplification specificity, although at the expense of low amplification efficiency. During later amplification cycles this is compensated for with more efficient amplification efficiency after that the annealing temperature is lowered.

Touch down PCR was performed with reagents and amplification conditions as described in table 4.

Table 4: Protocol for *lpf*$_{B1}$ operon PCR (product length: 6,303 bp)

Components LongAmp Taq2X Master Mix	**Volume per reaction (25 µl)**	**Final Conc.**	**Protocol**

10 µM Forward primer	1 µl	0.4 µM (0.05-1 µM)	**Standard PCR:**
			• Initial denaturation- 94°C for 30 sec.
			• 30 cycles- 94°C for 30 sec.
			  ○ 59°C for 30 sec.
			  ○ 65°C for 10 min (50 sec per kb)
			• Final extension- 65°C for 10 min
			• Hold- 10°C
			
			Touch-down PCR:
			• Initial denaturation: 94°C- 30 sec
			• Denaturation: 94°C- 30 sec
			• (high) Annealing temperature: 62°C- 30 sec
			• 65°C- 6 min (Go to step 2, 9 cycles)
			• Denaturation: 94°C- 30 sec
			• (actual/low) Annealing temperature: 55°C- 30 sec
			• 65°C- 6 min (Go to step 6, 19 cycles) (Total 30 cycles)
			• 65°C -10 min
			• Hold -10°C

10 µM Reverse primer	1 µl	0.4 µM (0.05-1 µM)	
LongAmp Taq 2X Master Mix	12.5 µl	1x	
Nuclease-free water	9.5 µl		
Template DNA	1 µl	< 1,000 ng	

3.6 Agarose Gel Electrophoresis

Agarose gel electrophoresis is used to separate, purify and identify fragments of nucleic acids (DNA or RNA). DNA is a negatively charged molecule and will migrate toward the positively charged cathode. Small fragment moves faster through the agarose gel toward whereas longer fragment moves more slowly. The location of nucleic acid fragments within the gel can be determined by staining with fluorescent dyes such as ethidium bromide, SYBR Safe or Gel red and examination of the gel in UV light. Concentration of the gel also determines the rate of migration of nucleic acid. Low concentration of agarose has large pore size that makes suitable for large DNA fragment to migrate. To resolve short fragments (100 to 3000 bp) of DNA, gel with 2% agarose is usually used whereas to resolve longer fragment of DNA, 0.5 to 1% (700 to 12,000 bp) agarose gel is more suitable [88]. The Gel Doc XR$^+$ System (Biorad) system is an equipment developed for imaging and documentation of nucleic acids and proteins suspended within polyacrylamide or agarose gels. When a gel is processed with the Gel-doc system, fluorescent stain bound to nucleic acid fragments are excited by ultraviolet radiation generating images of bands formed in gel which may be captured and stored.

In this study, E-Gel™ Agarose Gels (2%) with SYBR Safe™ (Invitrogen, Thermo Fisher Scientific) was used to resolve 141 bp-PCR products, and 1% agarose gels were used to resolve long PCR products. E-Gel with SYBR Safe gels contain buffer-less E-Gel pre-cast agarose gel with SYBR Safe DNA gel stain. Like ethidium bromide, SYBR Safe is a fluorescent DNA gel stain which is safer and environmental friendly, than formerly used stains like ethidium bromide. E-Gel agarose gel eliminates the need to prepare the agarose gel and as well as the need to buffers, and it makes possible fast, safe and high-resolution electrophoresis.

The E-Gel agarose gel was removed from the package and the comb was removed from the cassette before it was inserted into the base with care. A working solution of 1 kb-DNA ladder (1:10 in TE buffer) was for use as size standard: 20 µl of DNA ladder solution was loaded into the first well from the right side and the last well at the left side. Then 20 µl of each sample was loaded into separate remaining wells. Then 30-minute button on the E-Gel™ PowerBase™ device was pressed and released to begin electrophoresis. After 30 minutes, it automatically shut off. The gel cassette was removed from the base and processed with the Gel Doc XR^+ System and fluorescent bands were analysed using the Quantity one 1-D analysis software, and results were recorded. Readymade 2% agarose gel cassette was used to analyse the size of *lpf*$_{B1}$*C*-PCR products.

For analysis of PCR product of the putative fimbrial operon, which was longer than 6000 bp, gels with a lower concentration of agarose were used. Gel with 1% agarose was prepared by mixing 0.4-gram agarose with 40 ml 0.5x TBE buffer (volume can be adjusted according to the size of electrophoresis cell available in the laboratory) in a glass bottle. The agarose was melted, and the solution was equilibrated to 60°C for at least 30 minutes. The solution was then mixed carefully and poured into an electrophoresis cell with casting gates and a comb and left for 30 minutes to settle. Samples were prepared by mixing with BlueJuice gel loading buffer (Invitrogen). This buffer contains the colour bromophenol blue which makes loading and tracking of DNA samples in agarose gel surface easier. 10 µl of DNA sample and DNA ladder solution were mixed with 1 µl loading buffer in a microtitre plate. After half an hour the casting gates and comb were removed carefully from the electrophoresis cell and 0.5x TBE buffer was added so that it covered the gel. Then DNA ladder and PCR products were applied into wells in the gel, the gel was covered by a lid. Electricity cables were connected, and the power supply was turned on and run at 75V constant voltage for 60 minutes. Then the power supply was turned off, cables were disconnected, lid was removed, and the gel was transferred to a GelRed staining bath for 30 minutes, before destaining in water (Protocol for preparation of GelRed

solution has been described in Appendix III). The gel was then placed under UV light in the Gel Doc system, an image was taken, and the results were stored electronically.

3.7 Sequencing of the putative fimbrial operon *lpf*$_{B1}$

DNA sequencing is a method of deciphering the exact order of nucleotides within a DNA molecule or fragment. It is useful to identify of the product of PCR, clone and mutation. The Sanger method (also termed the Dideoxy method) is one of the most widely used methods of DNA sequencing**.** In this method dideoxynucleoside triphosphates (ddNTPs) are used along with dNTPs. ddNTPs contain an oxygen (O) instead of a hydroxy group (OH) at the 3'-carbon position of pentose sugar. Main role of ddNTPs in Sanger sequencing is to terminate DNA synthesis in a base-specific manner. Four ddNTPs corresponding to dNTPs are mixed in a ratio of e.g. 1:10. During DNA synthesis, whenever ddNTNP are inserted at random frequency, DNA synthesis is terminated at a specific position, and different oligonucleotide fragments are produced which may be further analysed by capillary agarose gel electrophoresis [88].

In this study, sequencing was done in fully automated capillary electrophoretic genetic analysis system (CEQ). CEQ is used to determine the nucleotide sequence and the size of DNA fragments. "Four-color, dye-labelled terminator chemistry kits are used to process samples for nucleotide sequence analysis. Generation of samples for fragment length analysis is performed using dye-labelled primers. The CEQ incorporates two plate holders and accepts two 96-well plates at one time. Each row of eight samples (sample set), containing labelled DNA fragments, is automatically denatured and then separated by capillary electrophoresis. The replaceable medium (separation gel) is automatically replaced in the eight capillaries after each separation. The separation gel supply is an easily replaced cartridge with a capacity sufficient for two full microplates. Detection is by laser-induced fluorescence in four spectral channels. The four-channel raw data sets generated by each of the eight capillaries are automatically processed to produce high quality base sequences or fragment lists after separation. Raw and analysed data are stored in a database and may also be exported in file formats compatible with common analysis applications" (CEQ™ 8800 Genetic Analysis System – User's Guide, Beckman Coulter).

In this study, PCR products of the putative fimbrial operon were sequenced to confirm the identity of the PCR products as that of the *lpf*$_{B1}$ operon. Before starting sequencing, it is essential to remove non-specific PCR products, residues of enzymes, dNTPs, primers and buffers. For

this purpose, a PCR cleanup enzyme set (BioLabs) was used: Exonuclease (Exo I) and Shrimp Alkaline Phosphatase (rSAP). 5 µl of the PCR product was transferred to a 1.5 ml microcentrifuge tube. Then 1 µl of each enzyme was added and the mix was incubated at 37℃ for 5 min. The mix was then transferred to heating at 80℃ on a heat block for 10 min. to inactivate both enzyme. This mix was then spun down and used for the sequencing reaction. Sequencing was done by Dye Terminator Cycle Sequencing (DTCS) with Quick Start Kit (GenomeLab^{TM}) by following the manufacturer´s guidelines. The method was completed in four major steps described as below:

Preparation of the DNA sequencing reaction

All reagents were kept on ice while preparing the sequencing reactions. The PCR product, measured to 450 ng/µl by the Nanodrop method (is described in Appendix V), was diluted 1:2, and 2 µl of diluted template was mixed with 8 µl DTCS, 2 µl (1.6 mM) forward primer for the lpf_{B1} operon, 8 µl DNase/RNase free water to a total volume to 20 µl. Similarly, in another tube, mix was prepared and 2 µl (1.6mM) reverse primer was used instead of the forward primer.

Thermal cycling

Two mixes with forward and reverse primer, respectively, were amplified in a T100 thermal cycler (Bio-Rad) with the following protocol: 96℃ (denaturation) for 20 sec., 59℃ (annealing), 60℃ (extension) for 5 min. for 30 cycles.

Ethanol precipitation

Fresh Stop solution/Glycogen mixture was prepared as follows: 2 µl of 100 mM Na_2EDTA (pH 8.0) and 1 µl of 20 mg/ml of glycogen. Then 5 µl Stop solution/Glycogen mixture was added in well labelled microcentrifuge tube (separate tubes for forward and reverse primer-sequencing reaction). Then the sequencing reaction was added to the Stop solution/Glycogen mixture. 60 µl cold 95% (v/v) ethanol/dH_2O from -20℃ freezer was then added and mixed thoroughly. Immediately the mix was centrifuged at 14,000 rpm at 4℃ for 15 min., before the supernatant was carefully removed with the micropipette. The visible pellet was then rinsed 2 times with 200 µl 70% (v/v) ethanol/dH_2O at -20℃ from freezer. For each rinse, the mix was centrifuged immediately at 14,000 rpm at 4℃ for 2 min., before the supernatant was carefully removed with a micropipette, and kept for drying for 30 min. Finally, the sample was resuspended in 40 µl of the Sample Loading Solution.

Sample preparation for loading into the capillary electrophoretic genetic analysis system (CEQ)

The resuspended samples were transferred to the appropriate wells of the sample plate. Each of the resuspended samples was overlaid with one drop of light mineral oil. The sample plate was then loaded into the instrument and capillary electrophoresis was initiated.

3.8 Molecular Cloning of the *lpf*$_{B1}$ operon

The TOPO TA cloning kit (Invitrogen, California, United States) for sequencing is used as a one-step cloning technique where *Ta*q-polymerase amplified PCR products are directly inserted into the pCR^{TM}4-TOPO linearized plasmid vector (Fig. 9) available in the kit. During PCR, *Taq* polymerase adds a single deoxyadenosine (A) to the 3'end of the PCR product. The plasmid vector supplied with the kit has a single 3' deoxythymidine (T) overhang-residue. The A-residue of the PCR-product and the T-residue of the vector allows ligation between the two nucleic acid fragments. The plasmid vector is double stranded, and one end of each strand has 5'-CCCTT. Topoisomerase (isolated from *Vaccinia* virus) binds covalently to the vector at specific sites and cleaves the phosphodiester backbone after 5'-CCCTT in one strand. Energy produced from this cleavage reaction is used to form a covalent bond between the 3'-phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of the topoisomerase I. This phospho-tyrosyl bond can then be broken by the 5'hydroxyl of the cleaved strand to release the topoisomerase enzyme. This mechanism is used to efficiently clone PCR products [89]. Salt is used to increase the efficiency of DNA uptake in the cloning reaction.

Transformation is the process of taking up free DNA directly from the environment. Bacteria that can take up DNA are termed competent, and bacterial cells that have taken up DNA are called transformants [90]. The TOPO TA cloning kit is supplied with TA cloning reagents as well as One Shot TOP10 competent *E. coli* cells. To distinguish between transformants and non-transformants, after transformation *E. coli* cells are cultured on agar media with antibiotics. Usually, the plasmid vector has been designed with an antibiotic resistance marker gene. If bacterial cells grow on the medium with antibiotic, this indicates that the transformation was successful and that the bacterial cells contain the plasmid vector. Non-transformants should in principle not grow on agar medium with antibiotic because they lack the resistance mechanisms to this antibiotic.

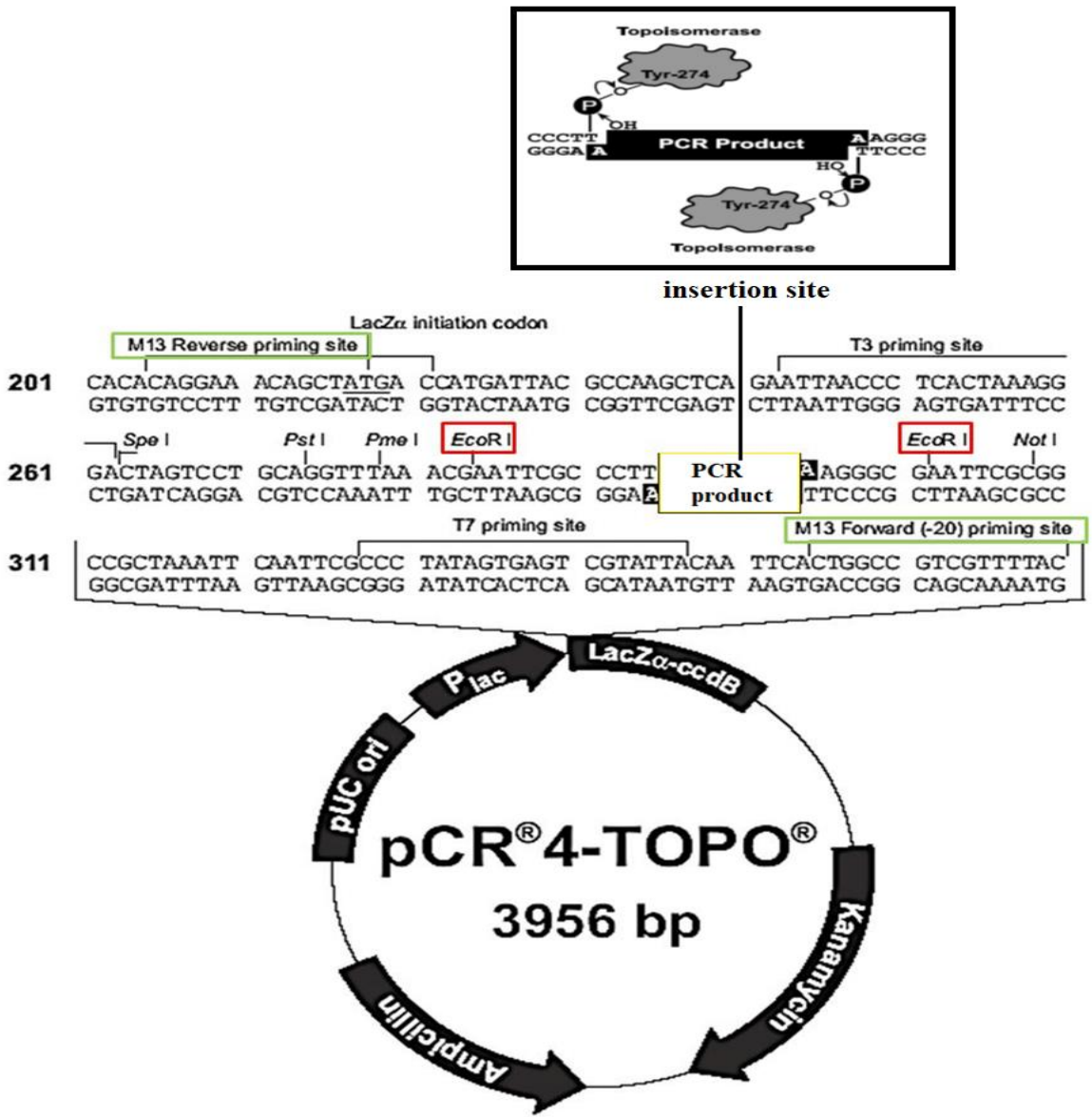

Figure 9: Ligation of PCR product with pCR™4-TOPO plasmid vector (source: User guide of TOPO TA Cloning Kit for sequencing, Invitrogen).

Resistance to the antibiotic is due not only to the presence of plasmid in the transformed cells, it also need to be expressed. The resistance gene can start its expression immediately after transformation, but it takes some time for transformed cells to produce enough of the resistance mechanism (e.g. an enzyme) to tolerate the toxic effect of the antibiotic. For this reason, the transformed cells should not be cultured onto the selective medium immediately after the heat shock treatment, but should first be incubated a short period of time (e.g. 1 hour) in a small volume of liquid medium not containing antibiotic [e.g. Super Optimal broth with Catabolite repression (S.O.C) media]. In this period, plasmid replication occurs, and a sufficient amount of the antibiotic resistance mechanism is synthesized. Now some volume of this inoculum can

be transferred to antibiotic containing medium [91]. The pCR^{TM}4-TOPO plasmid vector has two marker genes: the ampicillin resistance gene (*amp^r*) and the kanamycin resistance gene (*kan^r*).

3.8.1 Ligation of lpfB1 operon with the pCRTM4-TOPO plasmid vector

In this procedure, 4 µl fresh PCR product was mixed with 1 µl Topo vector and 1 µl salt solution. Then reaction was mixed gently and incubated for 30 min. (because of large product) at room temperature. For use as negative control, 4 µl water was added instead of the PCR product. After 30 min., the tube with mix was transferred to ice, and transformation reaction was done, as described in the following text.

In this work, the TOPO TA cloning kit was used for cloning of the *lpf$_{B1}$* operon PCR product into Topo-10 competent *E. coli* cells. After successful transformation, the plasmid was extracted from the transformed cells. After purification, the plasmid was inserted into wild type *E. coli* SO-ECO 13-1without the putative fimbrial operon. These bacterial cells were made competent in the laboratory and the plasmid was cloned into the competent cells. This is described in detail in the following text.

3.8.2 Transformation of PCR-product into One Shot TOP10 Competent E. coli cells

To perform the transformation reaction, 4 µl from the TOPO cloning (or ligation) reaction above was added into a vial of One Shot Chemically Competent *E. coli* cells and mixed gently. Separate vials were used for sample and negative control. Then the mix was incubated on ice for 30 min. Then heat shock was given for 30 sec. at 42ºC without shaking, before the tubes were immediately put ice. 250 µl S.O.C medium at room temperature (will be discussed in following text) was added. The lid was put tightly on the tube, and the tube was shaken horizontally at 300 rpm and 37°C for one hour.

Then 20 µl and 200 µl volume of mix from each vial (sample and negative control were inoculated on LB agar with ampicillin 50 µg/µl. Two different volumes (20 µl and 200 µl) were inoculated on separate agar plates both from sample and negative control, to ensure growth of

separate colonies. The plates were incubated overnight at 37°C, and next day the plates were examined for bacterial growth.

3.9 Plasmid extraction from Plasmid Miniprep Kit (Quantum Prep)

DNA extraction with the Plasmid Miniprep kit involves use of the alkaline lysis method where non-supercoiled genomic DNA is denatured, while plasmids, which are supercoiled, are not. The kit contains a cell lysis solution containing the strong base NaOH and sodium dodecyl sulphate (SDS) which lyse cells and denature proteins. SDS solubilize lipids in the cell wall where it also reversibly denatures fragile protein structures and breaks the hydrogen bond between the two helices of DNA strand. A neutralization solution containing high salt (potassium acetate) allows renaturation of plasmids because they have supercoiled structures which holds the two strands of helix tightly together. Chromosomal DNA is not able to renature because of its long length and that it is mixed with proteins during precipitation [90, 91]. The Plasmid Miniprep kit also contains a silicon dioxide matrix as DNA binding membrane where plasmids are bound. During binding of the plasmids, impurities are washed away by washing buffer and centrifugation, leaving high-quality plasmid to be eluted (Quantum Prep Plasmid Miniprep Kit Instruction Manual, Bio-Rad).

A single transformed colony (from sample plate as well as negative control plate) was inoculated into 2 ml LB broth with ampicillin (50 µg/µl) which was then incubated at 37°C overnight. Then the culture was centrifuged (maximum speed, 16000 rpm) for 30 sec. The supernatant was discarded, and the pellet was mixed with cell resuspension solution (200 µl), and vortexed to resuspend the pellet completely. Then 250 µl of the cell lysis solution was added and mixed by inverting the tight-lid tube, and 250 µl of the neutralization solution was added and mixed by inverting the tube. The tube was observed for precipitate. This step was critical because the precipitate contained chromosomal DNA with protein which should be discarded. Then the mix was centrifuged for 5 min., the pellet was discarded, and the plasmid-containing supernatant was transferred to a spin column with collection tube. 200 µl quantum prep matrix was added, mixed, and the tube was centrifuged for 30 sec. The filtrate was removed, 500 µl wash buffer was added, before the mix was centrifuged for 30 sec. Then again, the filtrate was discarded and wash buffer (500 µl) was added and the mix was centrifuged for 2 min., to remove residual traces of ethanol. The collection tube was discarded, and plasmid was eluted with 100 µl TE buffer. The plasmid solution was stored at -20°C.

Plasmid extraction was done separately of a colony from sample plate and from the negative control plate. The plasmid from the sample plate contained a *lpf$_{B1}$* operon-insert which was named pDA09. The plasmid from the negative control plate did not have an operon insert and this plasmid was named pDA00.

3.9.1 Conformation of presence of the *lpf$_{B1}$C* gene and the *lpf$_{B1}$* operon in the plasmid

PCR was used to confirm the presence and absence of the *lpf$_{B1}$C* gene and the *lpf$_{B1}$* operon in the plasmids, as described above, both in pDA09 and pDA00. After confirming the presence of the fimbrial gene and operon, the plasmid pDA09 was then transformed into a competent wild type *E. coli* strain SO-ECO 13-1previously confirmed by PCR not to lack the *lpf$_{B1}$* operon. This strain will hereafter be termed "wild type *E. coli*". Wild type *E. coli* cells were made competent by the TSS (Transformation and Storage Solution) method.

Most bacterial cell types do not to take up naked DNA efficiently from the surroundings and are therefore not naturally transformable. Because of the highly hydrophilic nature of DNA, it cannot easily pass through the bacterial cell membrane. To make DNA permeable so that the cell can take up DNA, chemical treatment or electrical treatment can be done [90].

The Transformation Storage Solution (TSS) buffer is made up of polyethylene glycol (PEG), $MgCl_2$, dimethyl sulfoxide (DMSO) and LB medium. PEG shields the negative charges on the DNA molecule and the host cell membrane to reduce repulsion between them. $MgCl_2$ is a divalent cation which alters the permeability of the bacterial cell to increase the ability of taking up DNA. All reagents in the reaction are brought together by DMSO. It also acts as a preserving agent. The mix is then exposed to cold and heat shock counteracting the repulsive forces between the negatively charged DNA backbone and LPS on the surface of the bacterial cell. A sudden increase in temperature creates pores in the plasma membrane allowing plasmid DNA to enter into the bacterial cell [92].

3.9.2 Protocol for making competent cells for transformation

The wild type *E. coli* (strain SO-ECO 13-1) was inoculated from freezing stock and cultured on blood agar. Then a distinct isolated colony from agar plate was inoculated into 5 ml of LB broth and incubated overnight at 37°C, before it was diluted into 50 ml of fresh LB medium in a 200-

ml conical flask. The diluted culture was incubated at 37 ºC and was grown to an OD_{600} (optical density at 600 nm wavelength) of 0.4. To determine the exact OD, the culture was measured with spectrophotometer at 30 min. intervals until OD reached 0.4. The culture was then split equally into two 50 ml tubes which were incubated on ice for 10 min. The tubes with culture were then centrifuged for 10 min. at 4000 rpm at 4°C. The supernatant was discarded, and the pellet was collected. Pellet was resuspended in 5 ml. chilled TSS buffer and was mixed gently. 100 µl of this solution was pipetted out and put into the chilled microcentrifuge tube. The tube was stored at -80^0C till further use.

The purified plasmids pDA09 (with lpf_{B1} operon) and pDA00 (negative control) were inserted into the newly prepared wild type *E. coli* competent strain (SO-ECO 13-1) by transformation as described above. This was done by adding 6 µl of plasmid into a vial of competent wild type bacterial cells. All volumes of transformed cells were cultured on LB agar with 50µg/ml ampicillin and incubated overnight before they were inspected for bacterial growth.

A distinct colony from both sample plate and negative control plate were inoculated in separate tubes with 5 ml LB broth with 50 µg/ml ampicillin. 2 ml of the culture was used for extraction of plasmids by the Plasmid Miniprep Kit as described above. Then PCR was done to confirm the presence of the *lpfb1C* gene as well as the lpf_{B1} operon.

Bacterial LB culture with plasmid was then stored at -80^0C for further use, by mixing with 750 µl of bacterial LB broth culture with 250 µl glycerol in 2 ml cryo-tubes.

3.10 Gene expression analysis

Housekeeping genes are genes that are vital for the survival of the bacterial cell. Such genes are expressed at more or less constant rates under all or most growth conditions. The genes encoding the RNA and DNA polymerase enzymes are examples of house-keeping genes. Many genes are expressed in certain environments but not in other because the cell does not want to waste energy to make enzymes, proteins and RNAs which are not required at that time [90]. Gene expression can be analysed by quantitative real-time reverse transcription PCR, where bacterial mRNA is first reversely transcribed to cDNA before quantitative PCR is done.

Quantitative Real time PCR (qPCR) is the technique where fluorescent signals from one or more polymerase chain reactions is continuously collected over a range of cycles and fluorescent signals from each cycle is converted into a numerical value. qPCR can be used for reliable detection and measurement of amplification products which is directly proportionate to

the amount of template prior to the start of the PCR process. To detect and measure the amount of template DNA, fluorescent dyes such as SYBR Green and EvaGreen dyes are used. The quantity of the PCR products amplified during each cycle in real time is detected by plotting the fluorescent signal as a function of cycle number [18]. At the initial stage of the PCR amplification process the fluorescent signal that is generated is mainly due to background noise which is more prominent than that created from the target amplification and cannot normally be distinguished within the first 10-15 cycles. As the thermocycling proceeds, more product is aggregated and the fluorescent signal increases exponentially. When the fluorescence signal is significantly greater (usually 10 times) than the background noise then a cycle-threshold (Ct) is reached. Ct values are directly proportional to the number of initial concentration of target template [93].

Quantitative PCR can be done by absolute or relative quantification. In absolute quantification, at first a standard curve is generated from serially dilutions of a control template of known concentrations. The standard curve is calculated by plotting the log of the initial amount of target sample against the observed Ct value for every dilution. This standard curve can then be used for comparison of the Ct values of templates with an unknown concentration of template, and the initial concentration of template can be quantified [94, 95]. In relative quantification, variation in the quantity of a target gene in unknown samples is compared to a reference sample or control that is also called as calibrator. Ct values of both control and unknown samples is noted and Ct value of control is subtracted from the Ct value of unknown target samples. The resulted value is expressed as "fold-changes" relative to the control sample. This method is often used in analysis of gene expression [96].

In this study, gene expression of the *lpf$_{B1}$C* gene in the transformed wild type *E. coli* strain SO-ECO 13-1 was analysed in two different culture media, LB broth and SILACE broth, media distinct with respect to nutritional ingredients. Expression of *rpoA* encoding the RNA polymerase α-subunit was used as reference for normalization of expression of the *lpfb1C* gene.

Two types of inoculum were used: one inoculum containing transformed wild type *E. coli* SO-ECO 13-1with plasmid pDA00 that contained no fimbrial operon insert, and SO-ECO 13-1with plasmid pDA09 with the *lpf$_{B1}$* operon. Both samples were cultured separately on LB agar with 50µg/ml ampicillin and incubated at 37ºC for 18 hours. The agar plates were then inspected for growth, and a distinct colony from each plate was picked and cultured in LB broth. One colony from the plate with growth of SO-ECO 13-1with pDA00 were inoculated into LB broth, and one colony was inoculated in SILACE broth, both broths with 50µg/ml ampicillin. Similarly,

one colony from a plate with SO-ECO 13-1 with pDA09 was inoculated into each of LB broth and SILACE broth, both broths with 50µg/ml ampicillin. Then next day, 2 ml of inoculum was taken from each tube and diluted with 20 ml. of respective antibiotic-containing-broth. Inoculum from LB was diluted with LB broth and inoculum from SILACE broth was diluted with SILACE broth. Then cultures were incubated, and ODs were observed at every 30-minute interval until the OD at 600nm wave length reached 0.4. Reaching an OD_{600} of 0.4 took 2 hrs longer for culture in the SILACE broth. At this point, there were:

- LB broth cultured *E. coli* cell with pDA00
- LB broth cultured *E. coli* cell with pDA09
- SILACE broth cultured *E. coli* cell with pDA00
- SILACE broth cultured *E. coli* cell with pDA09

When the culture reached the expected OD, 2 ml. culture was taken in separate 2 ml microcentrifuge tubes which were centrifuged for 5 minutes at 5000x rpm at 4ºC. The supernatant was discarded, and the pellet was collected. The collected pellet was used in RNA isolation.

3.10.1 RNA Isolation from transformed bacteria with the use of RNeasy Mini kit

Bacterial samples are pre-treated with lysozyme enzymes to break down the cell wall [97]. RNeasy Mini kit is a rapid, easy and well-established method for RNA extraction. This kit contains RLT buffer, RW1 buffer, RPE buffer, and spin columns with collection tubes. RLT buffer is a highly denaturing guanidine-thiocyanate- containing buffer which supports binding of RNA to a silica membrane, enhances the lysis procedure and inactivate RNases to induce the rate of purification of intact RNA. The RW1 buffer does also contain guanidine salt and ethanol, and is used as washing buffer to remove carbohydrates, proteins and other impurities that are bound to silica membrane. In this method, RNA molecules larger than 200 bases are attached to the column. RPE is also washing buffer that removes traces of salt bound to the membrane (User manual of RNeasy Mini kit). Mercapto-ethanol is also added in RPE buffer to help break the disulphide bonds, to increase denaturation of proteins and to aid in RNase deactivation [98, 99].

Lysozyme enzyme in 200 µl TE (in a proportion of 1mg/ml) buffer was added to the pellet and incubated at room temperature for 5 min. Then 700 µl buffer RLT (before use buffer RLT was

mixed with β- mercapto-ethanol in the proportion of 10 µl m-ethanol/1 ml buffer) was added and mixed well with pipetting, before the mix was centrifuged for 10 seconds at maximum speed (16000xrpm). The supernatant was then transferred into a new tube (2 ml) and 500 µl volume of ethanol (70%) was added and mixed well by pipetting. Precipitation was formed. 700 µl lysate (with precipitate) was then transferred into a spin column with 2 ml. collection tube. The lid was closed and centrifuged for 15 sec. at maximum speed and the flow through was discarded. 700 µl buffer RW1 was added to the spin column, centrifuged for 15 sec. at maximum speed and the flow through was discarded. 500 µl RPE buffer (with 96% ethanol) was added and centrifuged for 2 minutes and the flow through was discarded. The spin column was placed in a new 2 ml collection tube and was centrifuged for 1 minute at maximum speed. Then the spin column was placed into a new 1.5 ml collection tube, and 50 µl RNase free water was added to the column and the tube was centrifuged for 1 minute to elute RNA. RNA was put on ice at -20^{0}C. In this study, there were 4 samples, and all were processed individually. At this point there were:

- RNA sample isolated from LB broth cultured *E. coli* strain SO-ECO 13-1 with pDA00
- RNA sample isolated from LB broth cultured *E. coli* strain SO-ECO 13-1 with pDA09
- RNA sample isolated from SILACE broth cultured *E. coli* strain SO-ECO 13-1with pDA00
- RNA sample isolated from SILACE broth cultured *E. coli* strain SO-ECO 13-1with pDA09

3.10.2 Preparation of cDNA by reverse transcriptase real time PCR (RT-qPCR)

For reverse transcription reverse transcriptase enzyme is used to synthesize single stranded DNA complementary to the RNA template, known as complementary DNA (cDNA). The first step in RT-qPCR is to produce the cDNA. In addition to reverse transcriptase enzyme, oligonucleotide primers are also essential to begin cDNA synthesis. Primer used in RT-PCR may be of two-types: gene specific or non-specific random primers. Random primers contain 6-base oligonucleotide that may anneal randomly to RNA or bind to all RNAs present and make single stranded cDNA. Single stranded cDNA is then converted into double stranded DNA with the help of DNA polymerase and another complementary primer **[100]**

SYBR Green or Eva green fluorescent dyes that can be used in qPCR. Eva green dye is provided by BioRad as SsoFast EvaGreen Supermix. EvaGreen Supermix is a 2x concentrated, ready to use component which contains: 2x reaction buffer with dNTPs, Sso7d-fusion polymerase,

$MgCl_2$, EvaGreen dye, and stabilizers. The mixture has been prepared to get higher PCR efficiency, sensitivity and strong fluorescent signal. EvaGreen dye is a fluorescent dye like SYBR Green I dye. It binds to and detects any dsDNA synthesized during amplification. Unlike SYBR Green I, EvaGreen dye has low PCR inhibition activity that makes it an ideal dye for qPCR. Sso7d-fusion polymerase consists of antibody and less-active DNA polymerase. During the initial heating at 95^0C in the first PCR step, antibody denatures irreversibly, and highly-active DNA polymerase is released so that primer extension and DNA synthesis can start. Furthermore, Sso7d stabilizes the polymerase-template hybrid, increases processivity and makes the reaction faster than conventional PCR (User manual, Bio-Rad).

3.10.3 cDNA synthesis and real time PCR

cDNA synthesis was done by using the cDNA synthesis-mix which was available from Department of medical microbiology, St. Olavs hospital. The cDNA synthesis-mix contained 4 µl RT- buffer, 1 µl 0.1 M DTT, 2.4 µl random primer (100 ng/ µl), 2 µl 2.5 mM dNTP, 0.3 µl M-MLV reverse transcriptase, and 0.3 µl RNasin. 10 µl RNA solution was mixed with 10 µl cDNA synthesis- mix and then run on the CFX-96 thermocycler (Bio-Rad) according to the following protocol:

- 37°C- for 60 min- cDNA synthesis
- 94°C – for 10 min - to inactivate reverse transcriptase enzyme
- 10°C - for cooling

Prepared cDNA was stored at 4^0C. There were 4 samples of RNA and all were processed individually. At this point there were samples as below:

- cDNA sample prepared from RNA isolated from *E. coli* strain SO-ECO 13-1 with pDA00 (LB broth)
- cDNA sample prepared from RNA isolated from *E. coli* strain SO-ECO 13-1 with pDA09 (LB broth)
- cDNA sample prepared from RNA isolated from *E. coli* strain SO-ECO 13-1with pDA00 (SILACE broth)
- cDNA sample prepared from RNA isolated from *E. coli* strain SO-ECO 13-1 with pDA09) SILACE broth)

3.10.4 Amplification efficiency test of qPCR

For use of a real-time PCR method in quantitative analysis, it is important that the amplification efficiency of the method is optimal, and as similar as possible for each target gene. It was therefore necessary to check the efficiency of primers used in the qPCR experiments. The *lpf$_{B1}$C* gene was selected as target for analysis of expression of the *lpf$_{B1}$* operon during different growth conditions, and the housekeeping gene *rpoA* was selected as reference gene. Before the method could be used for gene expression analysis, amplification efficiency of both primers was checked.

Plasmid sample pDA09 used in six dilutions: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. The diluted samples were divided into two sets: in one set the selected *lpf$_{B1}$C* gene-primer pair (see Results section) was used to amplify *lpf$_{B1}$C* -PCR products whereas in second set "in house" primer pair was used to amplify the *rpoA* gene. SsoFast EvaGreen Supermix was used to amplify the PCR product. Reagents and protocol used for qPCR was as shown in table 5. In both sets, 3 parallels of each dilution (triplicate) were amplified in qPCR.

Table 5: Protocol for amplification of *lpf$_{B1}$C* and *rpoA* to evaluate efficiency of real time PCR

Component	Volume per reaction	Protocol of qPCR
SsoFast EvaGreen supermix (2x)	10 µl	Cycling step Enzyme activation at 95^{0}C for 30 sec. Denaturation at 95^{0}C for 10 second Annealing/extension at 58^{0}C for 10 sec. Melt curve: at 65-95^{0}C (in 0.5^{0}C increment) at 5 sec/step
Forward primer (5mM)	2 µl	
Reverse primer (5mM)	2 µl	
RNase/DNase-free water	4 µl	
Plasmid-DNA template	2 µl	
Total volume	20 µl	

The samples were prepared in a 96 well-micro titre plate and analysed on a CFX-96 real time PCR (Bio-Rad). Settings were adjusted according to the used dyes and placement of samples. Negative template control was also included. The instrument was then actuated. After completing the PCR reaction, results were analysed with the software incorporated in the instrument. To analyse efficiency of primers, Ct values were plotted against dilutions in a Excel sheet, and the efficiency of each PCR was calculated using the formula:

$$\% \text{ Efficiency} = (10^{-1/\text{slope}} - 1) \times 100\%$$

After it was established that both PCRs were of sufficient efficiency, the 4 samples of cDNA were analyzed. Each cDNA sample was divided into two sets, one for PCR for *lpf$_{B1}$C* and one for *rpoA*, with 3 parallels of each set. The PCR protocol used was the same as described above (Table 5). Negative template controls were not used in this analysis. On the PCR instrument, baseline and threshold values for the PCR amplification were generated automatically.

4 Results

The main objective of the study was to confirm the absence of the putative fimbrial operon and it´s genes in a set of clinical STEC strains of serotype O103:H2 which had lost the Shiga toxin (wild type strains), then clone the *lpf*$_{B1}$ operon into one of the wild type strains and analyze the expression of one of the genes within the putative fimbrial operon in the wild type strain under different growth conditions.

4.1 Establishing a PCR for the *lpf*$_{B1}$ operon and the *lpf*$_{B1}$*C* gene

4.1.1 Identification of promoters and design of primers

STEC strains of phylogroup B1 were in a study by Gabrielsen Ås et al, 2018 (submitted) found to contain a putative fimbrial operon with 42-63% similarity to long polar fimbria 2 (LPF 2) proteins of *E. coli* O157:H7 Sakai strain. This operon of phylogroup B1 strains was therefore named *lpf*$_{B1}$ by Gabrielsen et al. The *lpf*$_{B1}$ operon of the non-O157 STEC reference strain FHI6 (accession no. GCA_000941895) was used as reference strain in this study. The *lpf*$_{B1}$ operon has 4 structural genes, named by Gabrielsen Ås et al. as *lpf*$_{B1}$*A*, *lpf*$_{B1}$*B*, *lpf*$_{B1}$*C* and *lpf*$_{B1}$*D*, respectively (Figure 10a).

Search for the native promoter of the *lpf*$_{B1}$ operon was done using the nucleotide sequence of reference strain FHI6 (Table 2) [72]. Using PromoterHunter, several possible promoters were first identified immediately upstream of *lpfB1A*, as shown in figure 10 (a) and (b). The best probable promoter was then identified based on the final score. The possible promoter sequence having the highest final score was assigned as best possible promoter.

Primer pairs targeting the fimbrial operon were then identified in the region upstream of the promoter (forward primer) and downstream of the *lpf*$_{B1}$*D* gene (reverse primer), using the NCBI-Primer BLAST tool and Oligo software, ensuring that the native promoter should be included in the amplification product.

In addition, PCR-primers for an amplification product within the *lpf*$_{B1}$*C* gene were identified for use in expression analysis, using the same softwares Primer BLAST and Oligo (Figure 10 a). This gene (2523 bp) named FimD in the automatic annotation of the reference strain FHI6 was homologous to genes encoding type 1 fimbriae anchoring protein, which may have a role in adhesion, and has been used in typing of STEC.

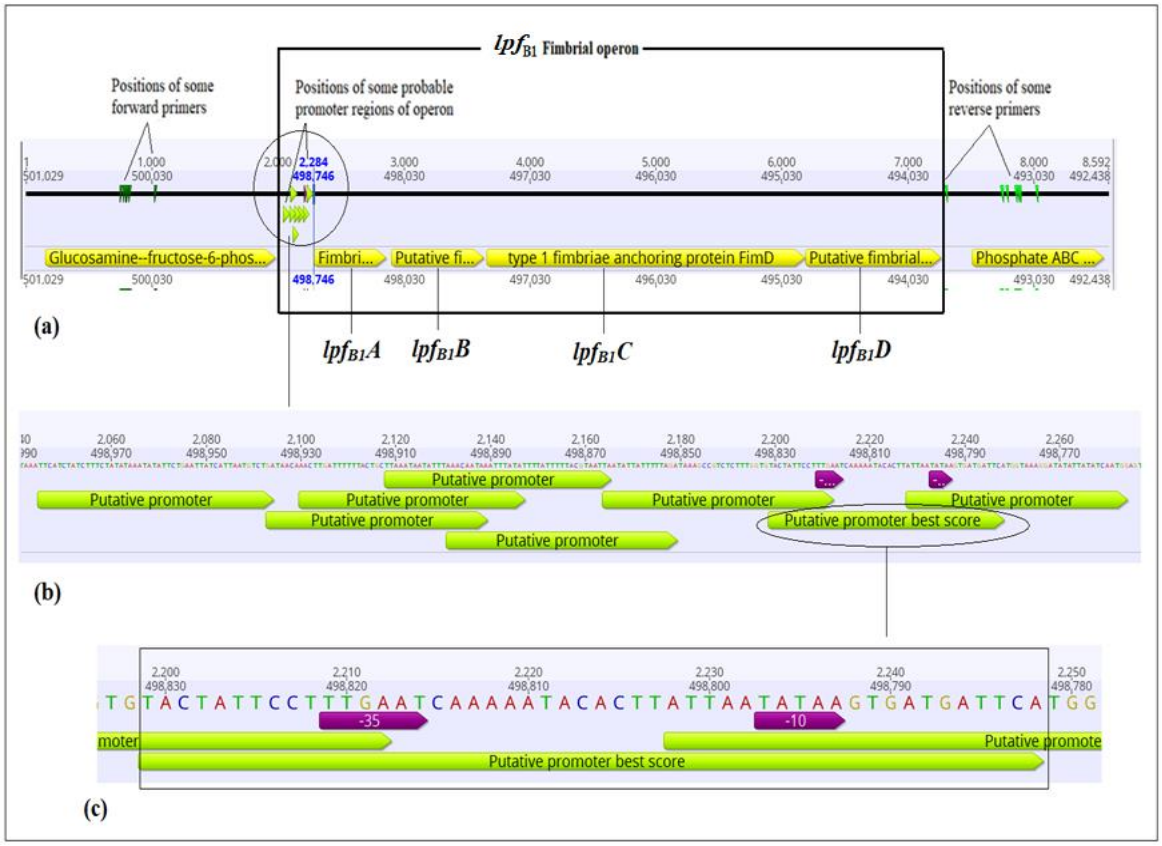

Figure 10: (a) Structure of *lpfB1* operon of the reference non-O157 STEC strain (FHI6) shown in Geneious software. Gene names in figure was based on automatic annotation: SfmA corresponds to lpf_{B1}A, putative fimbrial chaperone protein to $lpf_{B1}B$, FimD to $lpf_{B1}C$ and putative fimbrial protein corresponds to lpf_{B1}D (genes named according to Gabrielsen Ås et al.); overview of positions of possible promoters, forward and reverse primers for the lpf_{B1} operon are shown. (b) Zoomed in image of possible promoters. (c) The sequence of best score promoter is shown with -10 and -35 regions. Positions of forward primers for the lpf_{B1} operon were located upstream of the probable best promoter.

The primers suggested by primer-BLAST, both for the lpf_{B1} operon and the $lpf_{B1}C$ gene, were then inspected and analysed in Oligo. The designed primer pairs were found without hairpin loop structures, optimum internal stability, and the difference between melting temperatures of forward and reverse primers were not more than 1°C. The characteristics and the nucleotide sequences of the primers are described in Table 6.

Table 6: List and characteristics of primer pairs for the lpf_{B1} operon and the $lpf_{B1}C$ gene

Operon /gene	Type of primer	Length (nt)	Primer Sequences (5' to 3')	T_a* (°C)	Product length	Hair-pin loop	ΔTm
$lpf_{B1}C$ gene	Forward	20	5' CGGCTATACGGTCAAGACAC 3'	55.6	141 bp	No	>1°C
	Reverse	20	5' GCTTTAACCACTGCACCACG 3'			No	
lpf_{B1} operon	Forward	20	5' AACTGCTGTCGAAGGTTGAG 3'	58.6	6,303 bp	No	>1°C
	Reverse	23	5'AAGAAATGGGTGAGATGACAA CG 3'			No	

T_a*: Annealing temperature of the primer pair. ΔTm: Difference of melting temp. between forward and reverse primer.

4.1.2 Agarose gel profile of *lpf$_{B1}$* operon and *lpf$_{B1}$C* gene PCR products

In the electrophoresis gel four clear bands of a size between 100 bp and 200 bp were seen in the lanes with strain FHI11, FHI13, FHI15, and FHI21 respectively, corresponding with the expected size of 141 bp for the *lpf$_{B1}$C* amplification product. No bands were seen in the lanes with strain SO-ECO 13-1, SO-ECO 13-2 and SO-ECO 10-1 (Fig.11).

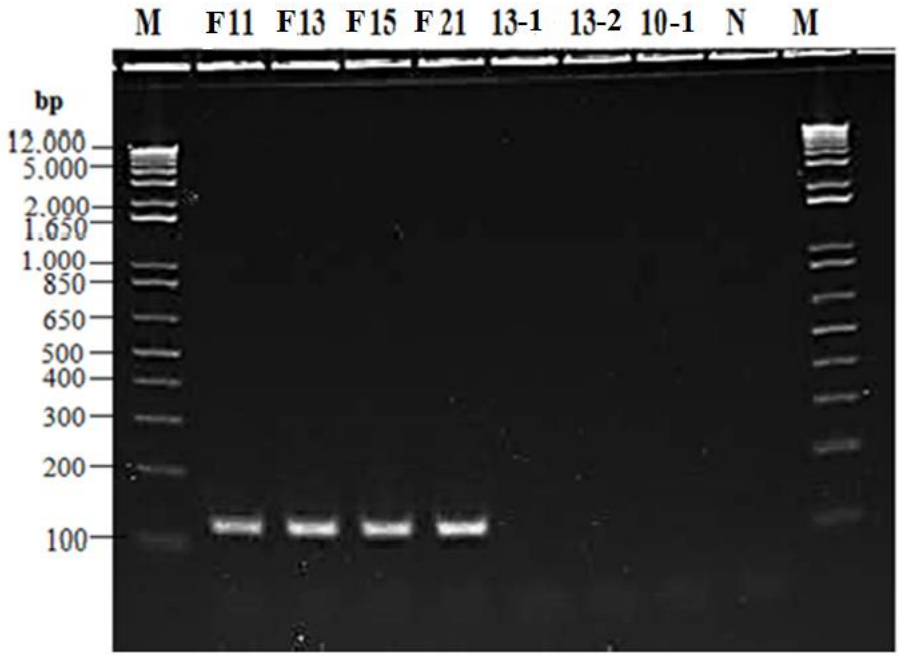

Figure 11: Agarose gel profile of the *lpg$_{B1}$C* gene-PCR products of seven clinical STEC strains. F11, F13, F15, F21, 13-1, 13-2 and 10-1 correspond to strain FHI11, FHI13, FHI15, FHI21, SO-ECO 13-1, SO-ECO 13-2 and SO-ECO 10-1 respectively. Strain identification is given above each lane. M: DNA ladder 1 kb plus as marker. N: negative control. Note: The dim bands seen at the bottom of the gel are most likely due to primer-dimers, and these bands are not called as 'bands' in this study.

4.1.3 Agarose gel profile of the *lpf$_{B1}$* operon

PCR of the *lpf$_{B1}$* operon done using the standard PCR protocol (Table 4) gave multiple non-specific amplification products. Expected size of bands were not seen on the gel (Figure 12 a). However, when PCR reaction was optimized using touchdown principle only a single amplification product was achieved. Genomic DNA from strain FHI11, FHI13 and FHI15 were amplified using touchdown PCR. On the gel of the *lpf$_{B1}$* operon touchdown-PCR product (figure 12 b) clear bands of less than 12,000 bp and higher than 5,000 bp were present. The expected band size was 6303 bp. The separation of the marker bands was somewhat unclear. Some nonspecific bands were also observed which were very dim and few small bands were seen at the area below 100 bp. The amplification products were further confirmed by Sanger sequencing (section 4.1.4).

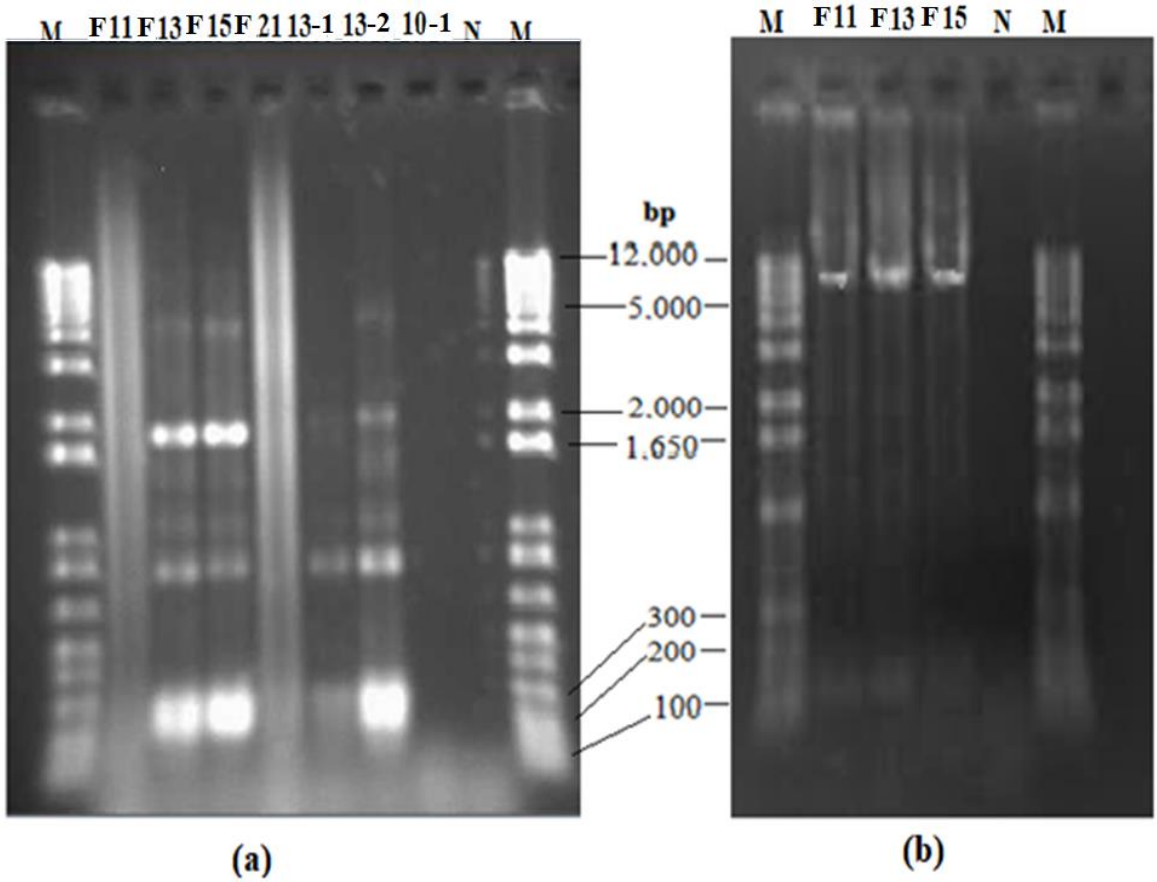

Figure 12: Agarose gel analysis of *lpf*$_{B1}$ operon PCR products amplified using a) the standard PCR protocol, and b) the touchdown PCR protocol. F11, F13, F15, F21, 13-1, 13-2 and 10-1 correspond to strain FHI11, FHI13, FHI15, FHI21, SO-ECO 13-1, SO-ECO 13-2 and SO-ECO 10-1 respectively. Numbers above the lanes indicate isolate ID. M = DNA ladder 1 kb plus as marker. N indicates negative control without DNA template.

4.1.4 Sequencing of the *lpf*$_{B1}$ operon-PCR product

The product obtained from long range PCR was confirmed by Sanger sequencing. The *lpf*$_{B1}$ operon primer pair was used for the sequencing reaction. After the sequencing reaction was completed, the sequence analysis showed many overlapping nucleotides (Fig. 13), most likely due to the presence of non-specific PCR products in the mixture of amplification products that had been submitted to sequencing. However, at one end part of sequencing chain some non-overlapped bases with non-overlapped single peaks representing single bases were found. About 60 identifiable nucleotides were extracted and compared with the reference sequence of the *lpf*$_{B1}$ operon (Fig. 14). The nucleotide bases were found as: CCGTATTGGGTTCGGTAGCATAAAAAGACGTACTGATATCATCGGTATTCAGTATCCCCA. These bases were found to be complementary to a sequence within the *lpf*$_{B1}$ operon, confirming that the amplification product from the touchdown PCR was included an amplification product from the *lpf*$_{B1}$ operon.

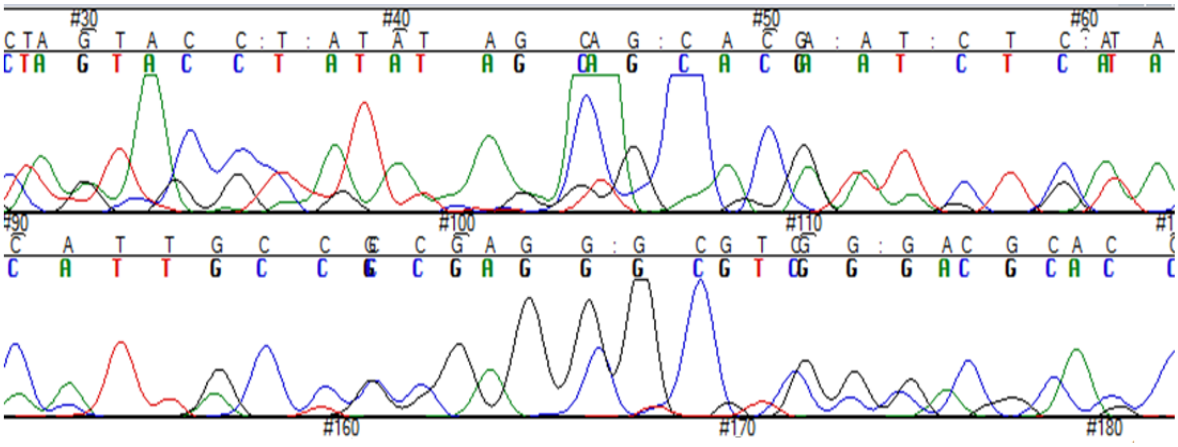

Figure 13: Some overlapping bases with overlapping peaks found in a sequencing chain analyzed from sequencer

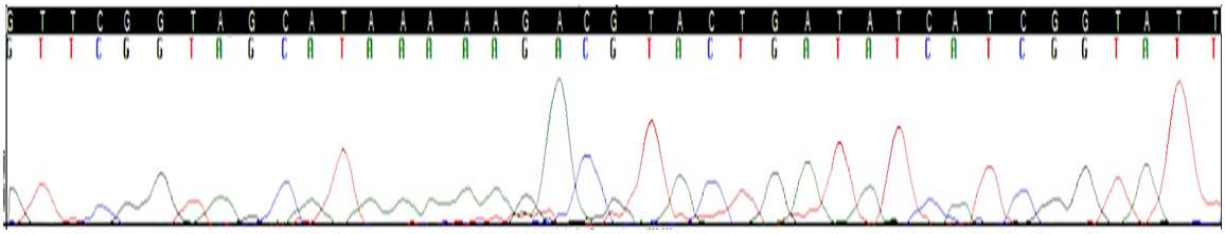

Figure 14: A part of sequencing chain with non-overlapping bases with non-overlapping peaks

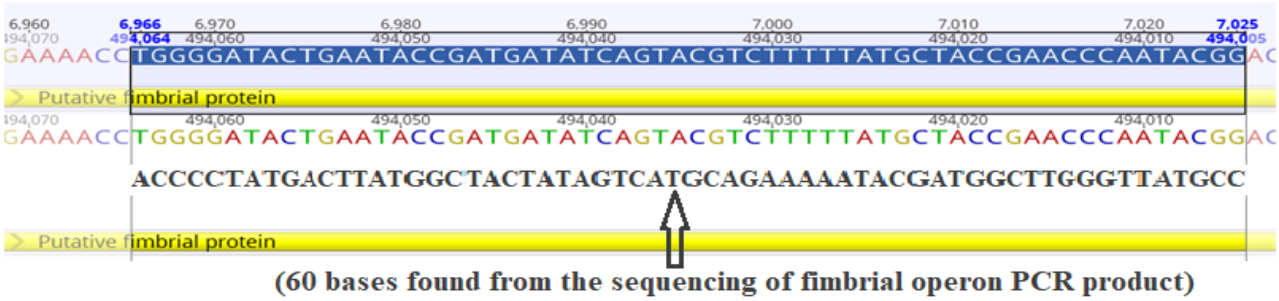

Figure 15: Comparing of the 60 non-overlapping bases found from sequencing with the *lpf*$_{B1}$ operon sequence of the reference strain FHI6.

4.2 Cloning of the *lpf*$_{B1}$ operon

4.2.1 Ligation of the lpfB1 operon into the pCR 4 TOPO vector

Ligation between pCR 4-TOPO vector with PCR product was observed with 4 possible results. At first case, there was possibility of occurrence of no ligation reaction. In second case, there was possibility of cloning of *lpf*$_{B1}$ with vector within *lacZ* site. The plasmid thus formed was given as name pDA09 plasmid. Third possibility was formation of self-ligated vector which was named as pDA00 plasmid. And, at last, there was possibility of cloning of non-specific PCR products within *lacZ* site of vector (Fig. 16).

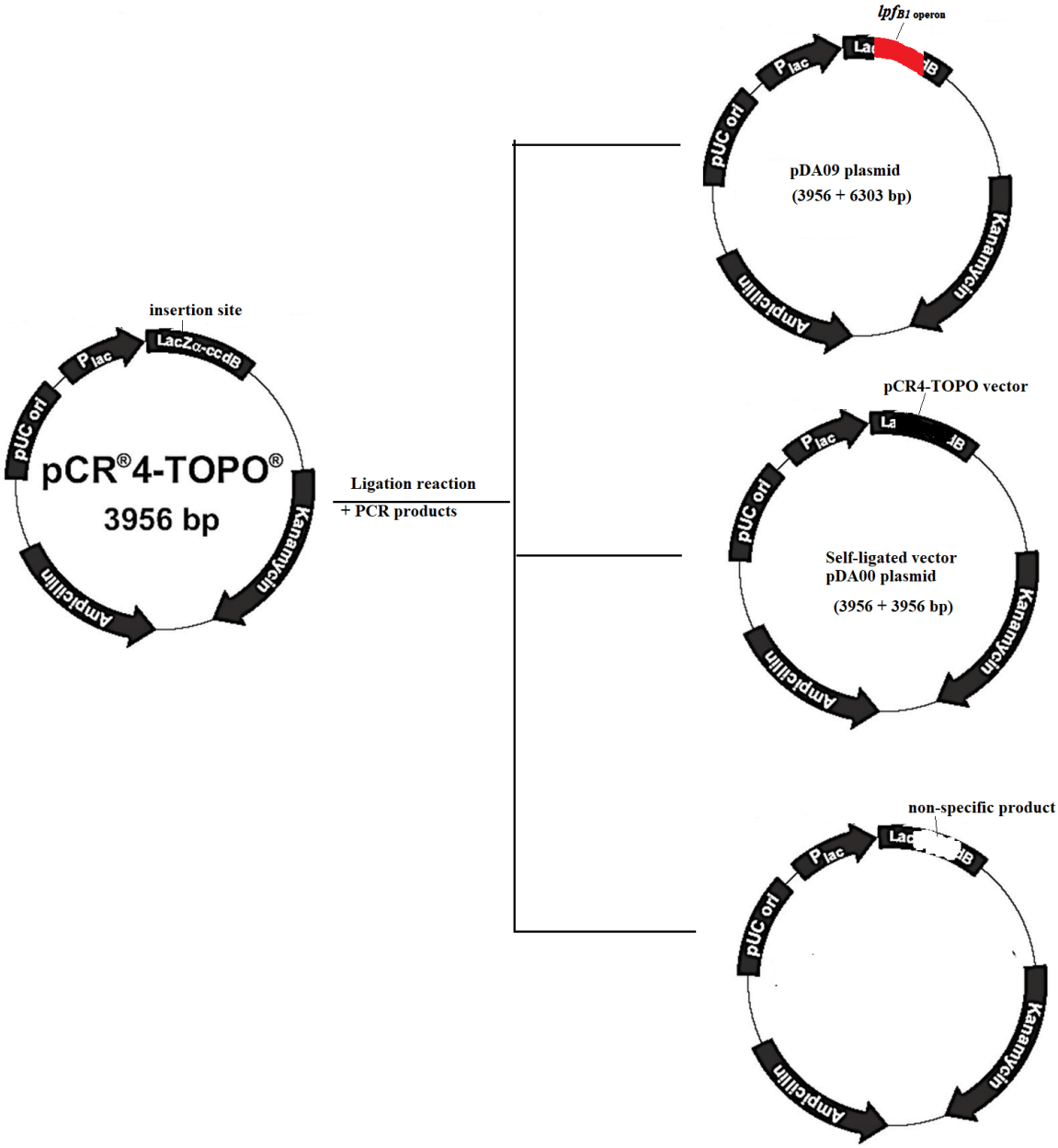

Figure 16: Possible results of ligation reaction between PCR products and pCR 4-TOPO vector. Although the length of *lpf*$_{B1}$ operon is larger than vector, in figure it is represented as shorter fragment.

4.2.2 Transformation of the *lpf*$_{B1}$ operon into TOP10 competent *E. coli* cell

After that transformation of TOP10 competent *E. coli* cells plasmid pDA009 and pDA00 vector was attempted, the transformed cells were inoculated on LB agar with 50 µg ampicillin. More than 20 colonies grew on the agar plate in which transformed cells with Topo-vector-*lpf*$_{B1}$-PCR products were cultured, and five colonies were found on the agar plate in which transformed cells with Topo-vector (without operon products) were cultured (Fig. 17). The fewer colonies that grew on the on the negative control-agar plate could represent plasmid vectors that were self-ligated and transformed into competent cells, while the higher number of colonies on

sample plate were more likely to represent strains transformed with plasmid vector with insert, possibly together with transformants with self-ligated molecules without insert and incorrectly ligated molecules. The presence of a higher number of colonies of the sample plate indicate that the transformation reaction and cloning were successful.

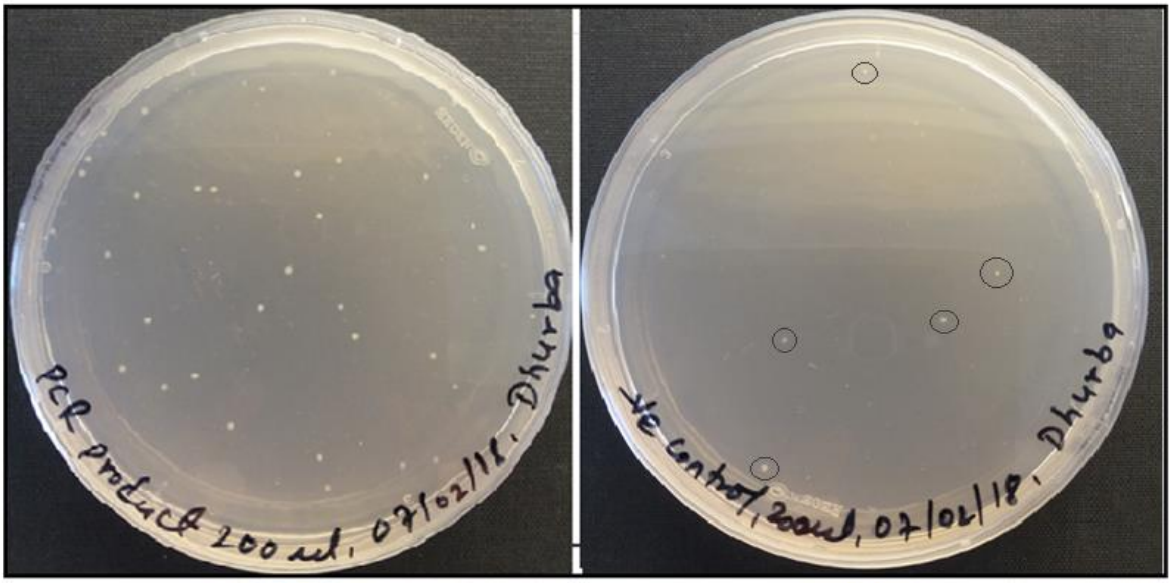

Figure 17: Growth characteristics of transformed TOP10 competent *E. coli* cells on the LB agar plate containing 50µg/ml ampicillin: a) Growth of TOP10 competent cell with pDA09 plasmid (sample plate) and b) Growth of TOP10 competent cell with pDA00 plasmid (negative control plate). Colonies are circled in plate (b).

4.2.3 Verification of the presence of the *lpf$_{B1}$C* gene and *lpf$_{B1}$* operon in plasmid pDA09

The presence of the insert with the *lpf$_{B1}$C* gene and *lpf$_{B1}$* operon was then verified by plasmid extraction, amplification of plasmid with *lpf$_{B1}$C* and *lpf$_{B1}$* PCR, and agarose gel electrophoresis of PCR products.

Among sixteen colonies which were used in the extraction of plasmid, eight colonies (colonies 1, 2, 6, 7, 9, 11, 15 and 16) were found to contain an amplification product for *lpf$_{B1}$C* of expected size between 100 to 200 (expected 141) bp on gel electrophoresis. Plasmid-PCR products from colony number 3, 4, 5, 6, 10, 12, 13 and 14 were found with no bands (Fig. 18). Three of the plasmids which showed an amplification product in the *lpf$_{B1}$C*-PCR, were further tested in the PCR targeting the *lpf$_{B1}$* operon. All three PCR gave amplification products of a size between 5000 and 12000 (expected 6303) bp on the gel. Unspecific bands of smaller size less than 5000 bp were also found on the gel, but they were found dimmer (Fig. 19).

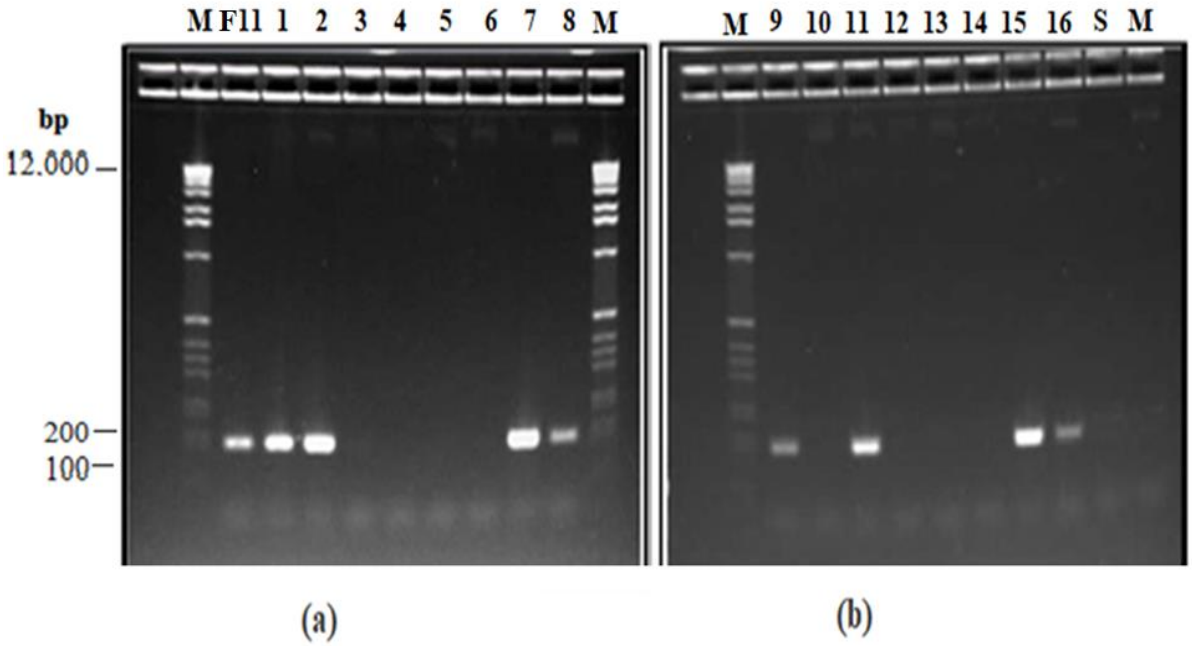

Figure 18: Result of agarose gel electrophoresis of amplification products from extracted plasmid from transformants. Numbers above the lanes indicate isolate/colony number. M = 1 kb plus DNA ladder. F11 (strain FHI11) is used as positive control in this experiment. S: plasmid extraction from colony from negative control plate. N indicates negative control without DNA template.

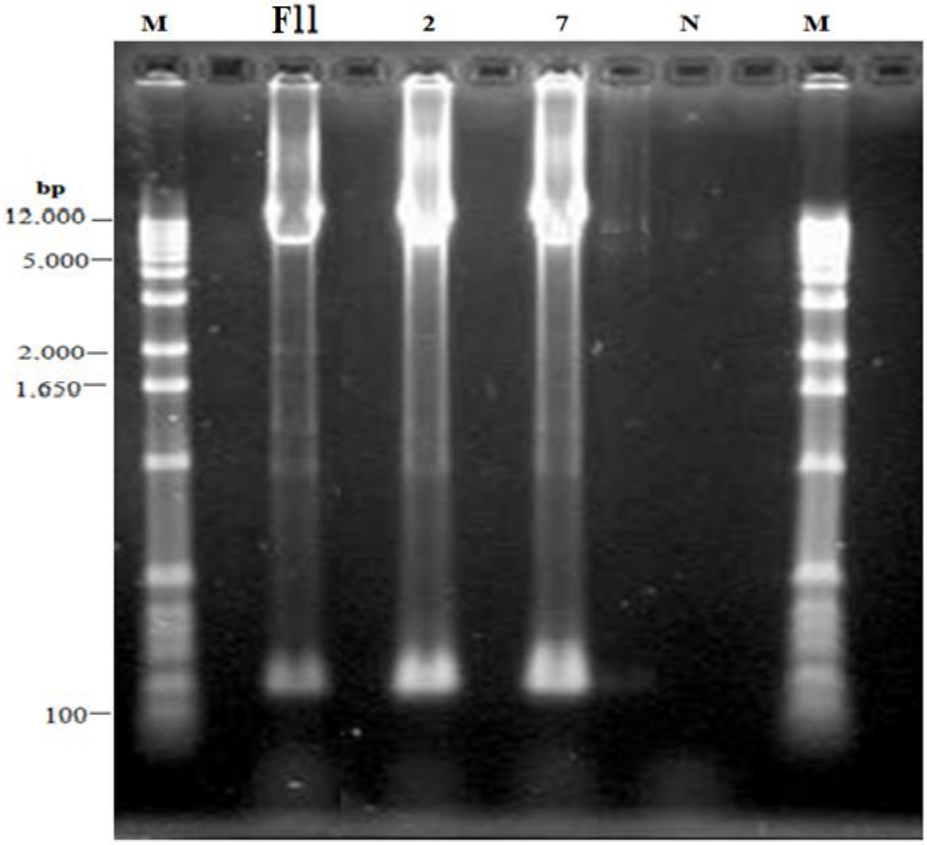

Figure 19: Result of agarose gel electrophoresis of amplification products from extracted plasmid from transformed cells. Numbers above the lanes indicate isolate/colonies ID. M = DNA ladder 1 kb plus as marker. The first top band of the marker is 12,000 bp long and the bottom band is of 100 bp long. F11 (strain FHI11) is used as positive control in this experiment. N indicates PCR-negative control without DNA template.

Hence, plasmids with an insert of the amplification product with the *lpf$_{B1}$C* gene did as expected also contain the whole *lpf$_{B1}$* operon. Growth of transformed cells on ampicillin containing LB media, the presence of the *lpf$_{B1}$C* gene and *lpf$_{B1}$* operon in plasmid confirmed that the transformation reaction with the pDA009 plasmid had been successful. The two plasmids with (pDA09) and without (pDA00) the *lpf$_{B1}$* operon (Table 7) were then used in the transformation reaction with a wild type competent non-O157 STEC strain.

Table 7: Feature of plasmid isolated from transformed cells

Name of plasmid	***lpf$_{B1}$C* gene**	***lpf$_{B1}$* operon**	**Components of plasmid**
pDA00	-	-	Self-ligated PCR^{TM}4-Topo vector
pDA09	+	+	PCR^{TM}4-Topo vector + *lpf$_{B1}$* operon-PCR product

4.2.4 Transformation of pDA00 and pDA09 into competent wild type *E. coli*

pDA00 and pDA09 were transformed into competent the wild type *E. coli* strain SO-ECO 13-1, and cultured on containing LB agar containing ampicillin 50µg/ml (Fig. 20). After overnight incubation three colonies were found on both the LB agar with cells containing plasmid pDA09 (Fig. 20a) and on the agar containing pDA00 (Fig. 20b). Satellite growth was observed around the colonies on both agar plates.

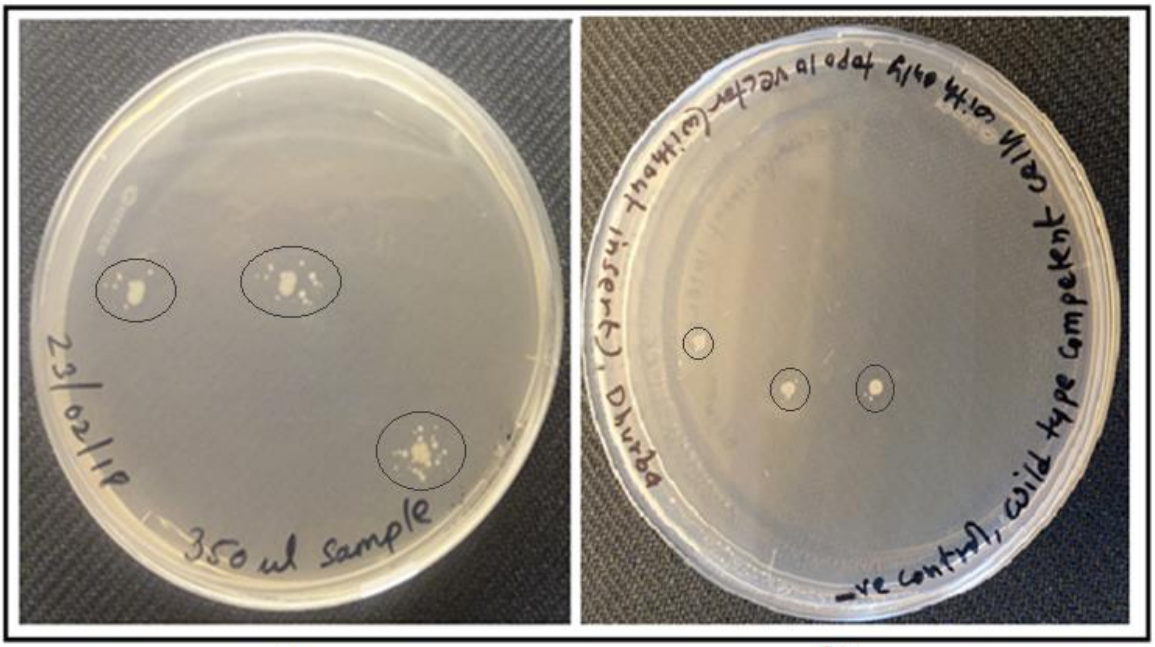

Figure 20: Growth of transformed cells on 50 µg/ml ampicillin-LB agar plate: a) colonies from transformed cells with plasmid pDA09 (with *lpf$_{B1}$* operon insert); b) negative control with plasmid pDA00 (without fimbrial operon insert). Black background is used to make the pictures clearer. Colonies are circled in both plates.

All big colonies without the surrounding satellite growth from the agar plate with cells containing pDA09 were then used to extract plasmid pDA09. All three plasmids were confirmed to contain the *lpf*$_{B1}$*C* gene and *lpf*$_{B1}$ operon by PCR and agarose gel electrophoresis as above (Fig. 21).

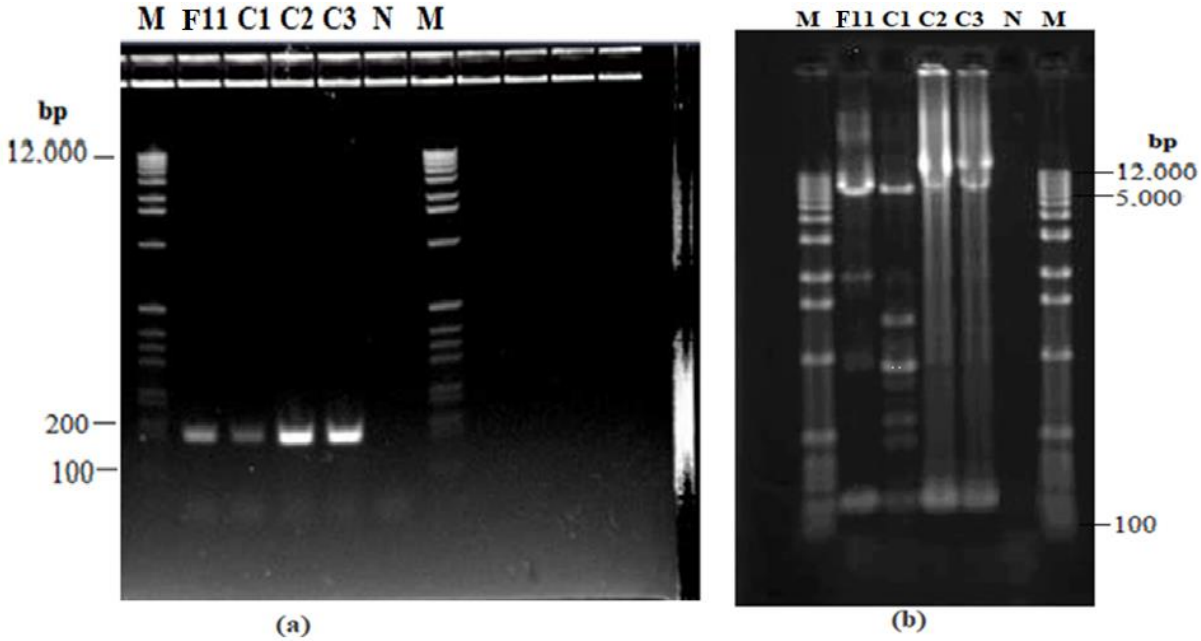

Figure 21: Result of agarose gel electrophoresis of (a) the *lpf*$_{B1}$*C* gene PCR product of extracted plasmids from transformants and (b) the *lpf*$_{B1}$ operon PCR products of extracted plasmids from transformants. Numbers above the lanes indicate strain/colony ID. M = DNA ladder 1 kb plus as marker. Strain FHI11 (F11) was used as positive control. N indicates PCR-negative control without DNA template.

4.3 Comparing expression of the *lpf*$_{B1}$*C* gene under different growth conditions

4.3.1 Amplification efficiency of *lpf*$_{B1}$*C* and *rpoA* real time PCR

Efficiency of the primers for *lpf*$_{B1}$*C* gene and the reference gene *rpoA* (encoding RNA polymerase α-subunit) was tested in real-time PCR (qPCR) using Eva-Green. The plasmid pDA09 was used as template for the efficiency test. 10-fold serial dilutions of DNA extract of the plasmid, from 10^{-1} to 10^{-6} dilution, were amplified with respective PCRs. Each dilution was assayed in triplicate. An amplification curve was generated by the qPCR-software of the PCR instrument (Fig. 22). A standard curve was then constructed by plotting the dilution factor against the mean C_T value (of three replicates in each dilution) obtained during amplification of each dilution. The slope of the regression line was -3.2023 and -3-347 for the *lpf*$_{B1}$*C*- and the *rpoA*-PCR, respectively. R^2 of the regression line, that implies the fitting of experimental data in regression line, was found >0.980 in both cases. R^2>0.980 indicates the higher efficiency of

PCR. Using the slope method, the efficiency was calculated to 105% and 99% for the *lpf*$_{B1}$*C*-PCR and *rpoA*-PCR, respectively (Fig. 22 a and b). Thus, both primer pairs were found suitable to for real time PCR. In addition, melting curve analysis of *lpf*$_{B1}$*C* primer pair and *rpoA* primer pair was done to distinguish potential non-specific products. Only one melt peak was found for both PCRs, at 79ºC for the *lpf*$_{B1}$*C*-PCR and 84°C for the *lpf*$_{B1}$-PCR, respectively (Fig. 24). Thus, high efficiency was confirmed for both PCRs.

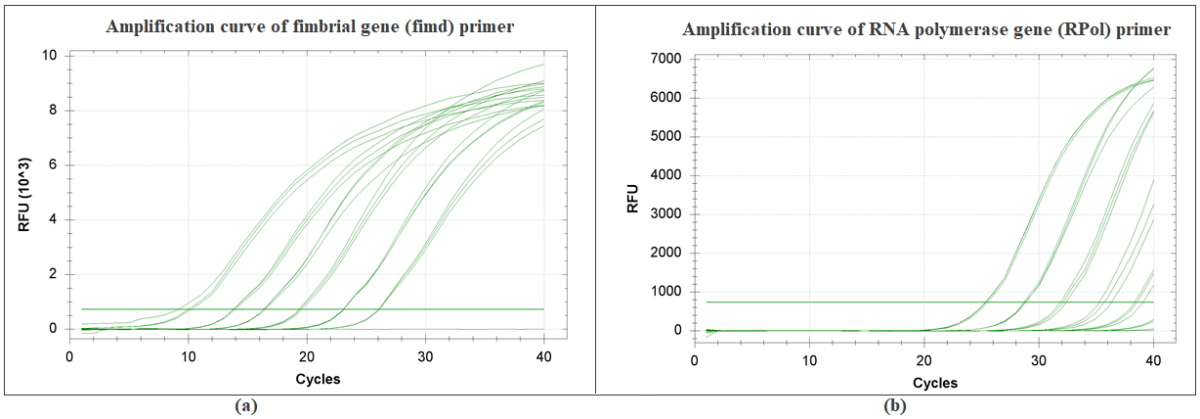

Figure 22: Amplification curve of the dilution series of PCR for plasmid pDA09 with: a) *lpf*$_{B1}$*C* PCR and b) *rpoA* PCR. Each dilution was tested in triplicate. The green horizontal line indicates the cycle threshold (C_T) that indicates the cycle in which the fluorescence reaches the threshold value.

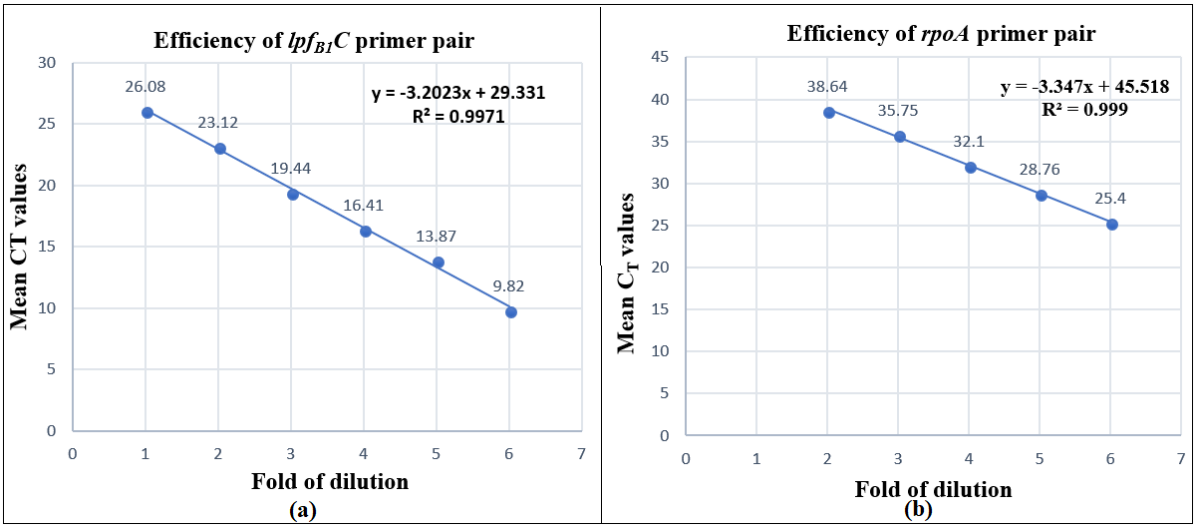

Figure 23: Standard curve with C_T plotted against fold dilution. The equation for the standard curve (also called as regression line) and R^2 value are shown at the top left side in the graph. R^2 value of standard curve shows how well the experimental data fit the regression line.

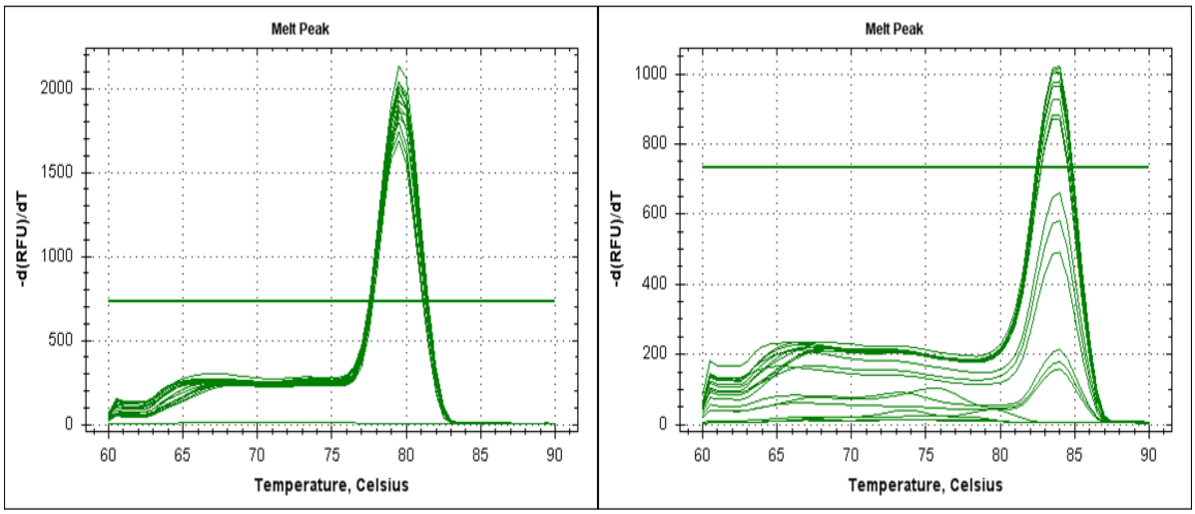

Figure 24: Melt-curve analysis of amplification product from qPCR: a) melt curve analysis of the *lpf*$_{B1}$*C*-PCR product showing a single melt peak at Tm 79ºC; b) melt curve analysis of *rpoA*-PCR product showing a single melt peak at 84ºC.

4.3.2 Isolation of RNA and quality check of RNA

One of the objectives of this study was to analyze the expression of *lpf*$_{B1}$*C* gene in the STEC transformed with plasmid pDA009 in two different growth conditions, in LB and SILAC broth. LB broth is a widely used enriched medium to culture recombinant *E. coli* cells. SILACE medium is an optimized SILAC medium for culture of *E. coli* for quantitative proteomics analyses.

The concentration of total RNA was found in the range of 67.5 to 546.1 ng/µl in the samples tested. The lowest concentration of RNA was found in SILACE broth inoculated with the transformant with plasmid pDA00 (here labelled RNA SIL-). For assessment of the purity of extracted RNA, the ratio of absorbance at 260nmn and 280 nm, as well as 206/230, was analyzed in a NanoDrop Spectrophotometer. An A_{260}/A_{280} ratio of~2.0 is generally considered as "pure" for RNA, whole the 260/230 ratio commonly are in the range of 2.0-2.2. Bothe the ratio of A_{260}/A_{280} and $A_{260/230}$ were found to be in the acceptable range for three of the four samples. RNA from SILACE broth with pDA00 (RNA SIL-) did however not fulfill the criteria for acceptable purity. Of note is that the A_{260}/A_{230} ratio seemed to decrease with lower concentration of RNA (Table 8).

In the gel electrophoresis of total RNA three very similar major bands were seen (Fig.). The size of all RNA-bands was found above the length of 650 bp. The expected size of RNAs in *E. coli* depend on type of RNA, 5S rRNA is ~120 nt, 16S rRNA is ~1.5 kb, and 23S rRNA is ~2.9 kb, while tRNA is ~90 nt and mRNA has a varying in size usually in the range of

~1000nt. The size of the two major bands observed most likely corresponds to that of mRNA (~1000nt) and 16S rRNA (~1.5 kb). The strongest bands were found in the lanes of RNA extracted from the bacterial cells cultured on LB broth, while less strong bands were found in the lanes of RNA extracted from bacterial cells cultured in SILAC broth (Fig. 25).

Table 8: concentration of total cell RNA in different growth conditions

Sample ID	ng/µl	A_{260}	A_{280}	260/280	260/230
RNA-LB-pDA09	546.05	13.651	6.707	2.04	2.49
RNA-LB-pDA00	456.61	11.415	5.509	2.07	2.39
RNA-SIL-pDA09	143.04	3.576	1.760	2.03	1.82
RNA-SIL-pDA00	67.51	1.688	0.928	1.82	1.04

RNA-LB-pDA09: RNA extracted from bacterial cells with pDA09 cultured in LB broth
RNA-LB-pDA00: RNA extracted from bacterial cells with pDA00 cultured in LB broth
RNA-SIL-pDA09: RNA extracted from bacterial cells with pDA09 cultured in SILACE broth
RNA-SIL-pDA00: RNA extracted from bacterial cells with pDA09 cultured in SILACE broth

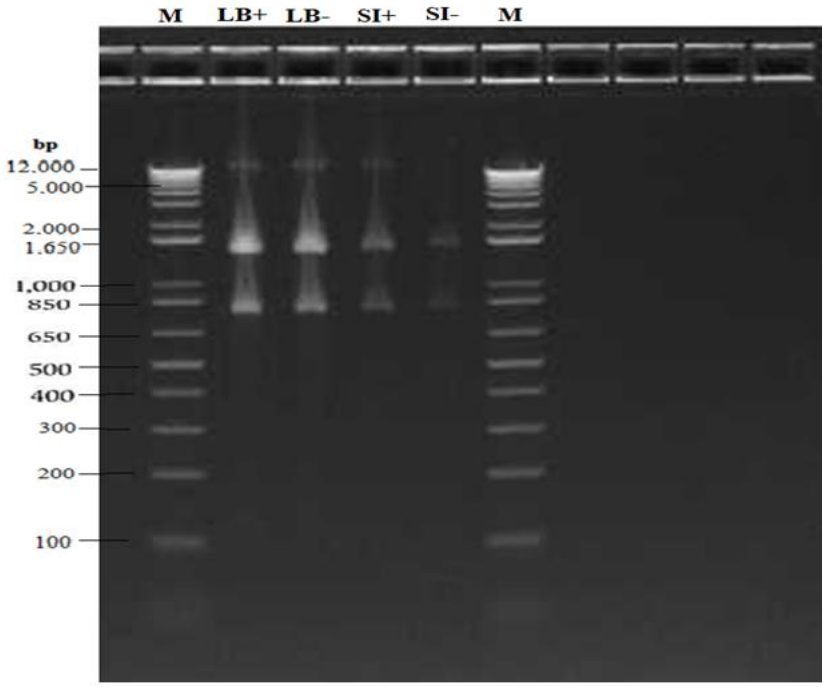

Figure 25: Results of agarose gel electrophoresis of the RNA from transformed wild type *E. coli*: with pDA09 cultured in LB broth (LB+), with pDA00 cultured in LB broth (LB-), with pDA09 cultured in SILAC broth (SILAC+) and with pDA00 cultured in SILAC broth (SILAC-). M = DNA ladder 1 kb plus as marker.

4.3.3 Gene expression analysis

After cDNA synthesis as described before (section 3.10.3), expression of the *lpf*$_{B1}$*C* gene was analysed under two different growth conditions, LB broth and SILACE broth, using qPCR, with expression of the *rpoA* gene as control. cDNA samples extracted from RNA samples were amplified in real time PCR. There were 4 samples of cDNA (Table 9)

Table 9: List of samples used in qPCR for gene expression analysis

cDNA sample	Extracted from
cDNA-LB-pDA09	RNA-LB-pDA09
cDNA-LB-pDA00	RNA-LB-pDA00
cDNA-SIL-pDA09	RNA-SIL-pDA09
cDNA-SIL-pDA00	RNA-SIL-pDA00

qPCR of cDNA for *rpoA* gave an amplification curve for all four samples, with C_T values between 16 to 19 (Table 10, Fig. 26). There was minimal difference between triplicates of each sample (Table 10). It was clear that *rpoA* gene was expressed evenly between samples cultured in LB broth, and also in SILACE broth, but possibly at lower level in the latter (Table 10, Fig. 26b). Hence, amplification of housekeeping gene was found not controlled by type of growth media as well as presence or absence of gene insert.

In contrast to the results from qPCR for *rpoA*, PCR for $lpf_{B1}C$ gave not amplification product for any of the four samples (Table10, Fig. 26a), indicating that the $lpf_{B1}C$ gene was not expressed under any of the two different growth conditions.

Table 10: C_T values generated during amplification of cDNAs in different growth conditions to anlayse gene expression

Primer Pair for gene	Sample	C_T values (triplicate)			Mean C_T values
$lpf_{B1}C$	cDNA LB+	N/A	N/A	N/A	0.00
	cDNA LB-	N/A	N/A	N/A	0.00
	cDNA SIL+	N/A	N/A	N/A	0.00
	cDNA SIL-	N/A	N/A	N/A	0.00
	-ve	N/A	N/A	N/A	0.00
rpoA	cDNA LB+	18.96	18.62	18.62	18.73
	cDNA LB-	17.95	18.07	17.84	17.95
	cDNA SIL+	16.47	16.58	16.51	16.52
	cDNA SIL-	16.78	16.77	16.69	16.75
	-ve	N/A	N/A	N/A	0.00

N/A: not applicable

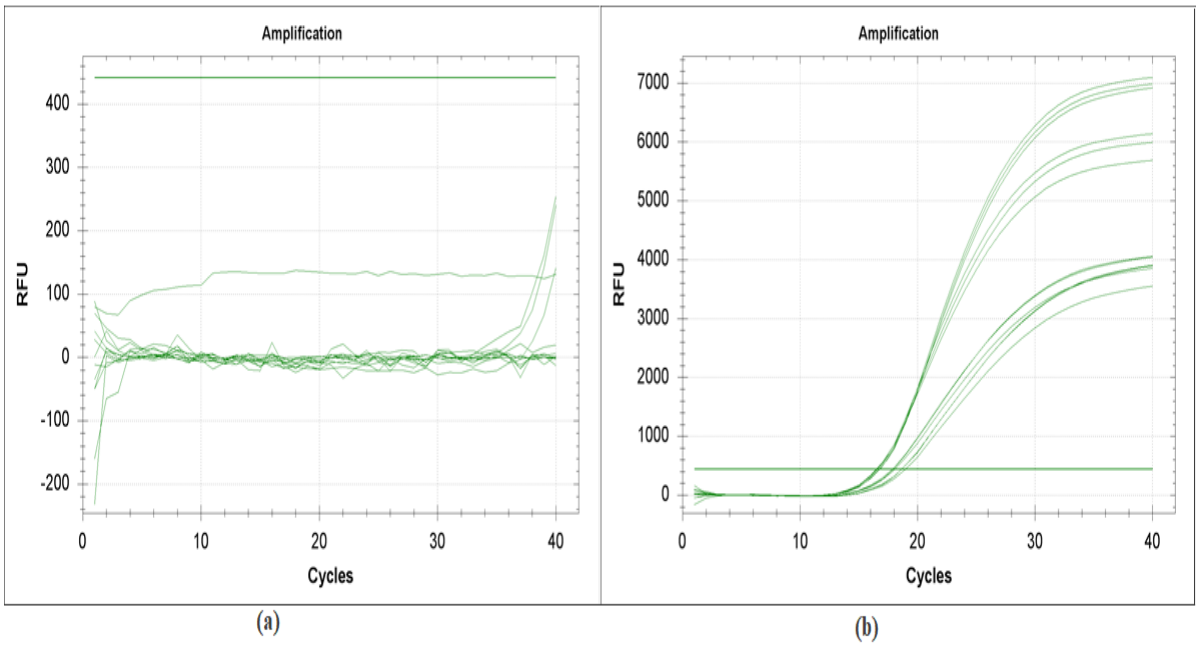

Figure 26: Amplification curves generated from cDNA samples: a) with PCR for *lpf*$_{B1}$*C* ; b) with PCR for *rpoA*. Green horizontal line indicates cycle threshold (C_T) value.

5 Discussion

STEC cause human illnesses such as diarrhea, bloody diarrhea, hemorrhagic colitis and hemolytic uremic syndrome. Shiga toxins are essential virulence factors of STEC, but also other factors have been reported to be involved in virulence of such bacteria. Adherence to the target intestinal epithelial cell is an important initial step in establishing STEC infection. Intimin is the major adhesin of most variants of STEC. In addition, *E. coli* bacteria express fimbrial proteins such as long polar fimbriae (LPF) which may also have a role in adhesion. For some fimbrial and putative fimbrial proteins the function in virulence is still not clear. The aim of this study was to establish PCR for the whole putative fimbrial operon *lpf*$_{B1}$ as well as for detection and expression analysis of one of the genes within the operon, clone the operon into a wild type STEC non-O157 strain and analyse the expression of the fimbrial operon in this strain. These clones could then be utilized in future studies, e.g. cell culture adhesion assays, to clarify the functional role of the operon.

PCRs for the *lpf*$_{B1}$ operon and the *lpf*$_{B1}$*C* gene were developed and optimized, and the operon was first successfully transformed into TOP10 competent *E. coli* cells, and then into the selected wild type strain. However, the *lpf*$_{B1}$*C* gene was not found to be expressed in the transformed wild type strain under two different growth conditions when analyzed by qPCR. There could be several reasons for this lack of expression, which will be discussed in the following sections.

When planning the study, we chose to identify and include the native promoter for the *lpf*$_{B1}$ operon in the PCR product, so that this promoter could be used for expression of the operon. For identification of the promoter the PromoterHunter tool was used. The PromoterHunter is a "Tool for search in prokaryotic genomes" (REF: http://www.phisite.org/main/index.php?nav=tools&nav_sel=hunter). There are however few references for the quality of this tool in correct identification of promoters in prokaryote genomes. On the other hand, in our study we were more interested in identifying the region of possible promoters so that we could ensure that the native promoter was included with the fimbrial operon in the amplification product. Using PromoterHunter possible promoters were found within the 250 upstream-bases from the first structural gene *lpf*$_{B1}$*A* of the operon. The selected forward primer site of the operon targeted a sequence more than 1000 bases upstream from the first structural gene of the operon. Hence, due to the long distance between forward primer site and operon, it is most likely that the site of the native promoter was included in the PCR product even though the PromoterHunter software might not identify the correct promoter

sequence. It is therefore unlikely that the reason for lack of expression of the *lpf*$_{B1}$ operon was that the native promoter was not present in the vector insert.

The choice of cloning vector is important. In this study, we used the pCR 4-TOPO plasmid vector which is a cloning vector. Alternatively we could have selected an expression vector which is useful if you what to produce high amounts of protein. Expression vectors are based on strong promoters that are recognized by the transformed cell [90, 101]. Hence, choosing an expression vector, instead of the pCR 4-TOPO cloning vector and native promoter, might have increased the chance that the *lpf*$_{B1}$ operon had been expressed. Arenas-Hernández et al. used the highly expressive pEXT5-CT/TOPO vector with an LPF2 operon insert with its native promoter site to study expression of a operon gene. In that study gene expression was under the control of the vector-promoter instead of operon-promoter [102].

The wild type *E. coli* strains chosen for use in the cloning experiment were strains that had lost their *stx* genes making it possible to work with live strains in a BSL2 laboratory environment. These strains were also chosen because they 1) belonged to a non-O157 serogroup, 2) to phylogroup B1 to which many STEC containing the *lpf*$_{B1}$ operon belong, and 3) to serotype O103:H2 which was known from a previous study to contain strains lacking the *lpf*$_{B1}$ operon. Level of gene expression may vary considerably between different strains . Hence, the properties of the strains used in a cloning experiment might be one of the possible reasons that a cloned gene is not expressed. The wild type strains used in this study were considered by the clinical laboratory to have lost its *stx* genes. Loss of *stx* genes can happen after ingestion, during the course of infection, or in the laboratory [103, 104]. Bielaszewska et al. mention that excision of the *stx* converting bacteriophage from STEC is the most likely mechanism for loss of *stx* [105]. So, absence of *stx* in the wild type strain used in the cloning experiment implies that the reference strain and wild type strain are genetically different, at least with respect to the stx bacteriophage. In addition, the strains were found by genomic analysis to belong to clearly different clusters of the B1-phylogroup even though they belonged to the same serogroup [72]. Differences in genetic background between strains may include differences in their regulatory mechanisms for gene expression. Such differences may have a role in the lack of expression of the *lpf*$_{B1}$*C* gene in this study.

Global regulators play the major role in expression of gene. The histone-like protein H-NS, one the intensively studied global regulators in *E. coli*, has a major role in the structuring of the bacterial chromosome [106]. In addition to this, H-NS can influence the regulation of genes with changing environment such as pH and anaerobiosis [107]. Several regulatory genes are

under the control of H-NS. H-NS has been reported to be a transcriptional silencer for several genes that have been involved in virulence such as adhesion caused by long polar fimbriae. In case of EHEC, Ler proteins are found to have a role as anti H-NS factor that may activate the expression of other genes on the LEE island. Torres et al. verified the role of Ler protein in regulation of a fimbrial gene in EHEC [108]. Hence, one possible mechanism of not observing any expression of the *lpf*$_{B1}$*C* gene in this study might be due to negative regulation by a global regulator.

Gene expression may vary depending on which growth condition and growth phase the study is done. In this study, transformed cells were cultured until an exponential phase (OD_{600} 0.4) in both LB and SILACE broth. These culture media were chosen to study the difference in expression of the fimbrial gene in a nutrient rich condition and a nutrient-limiting condition. LB broth is composed as an enriched medium to grow recombinant *E. coli*, while the SILACE is a chemically defined medium with glucose, amino acids and M9 salts optimized for culture of non-auxotrophic *E. coli*. Such a nutrient-limiting growth medium might to some degree reflect the nutrient-limiting growth condition in the lower intestines where *E. coli* have to compete with other normal flora for nutrients.

Analysis of gene expression in an early exponential phase was chosen because we assumed that the fimbrial genes might have the role in the early phase of adhesion, and because study of its role in later stages of adhesion would be difficult due to the dominating role of intimate adhesion due to LEE and intimin. Gabrielsen et al. reported the expression of genes within the *lpf*$_{B1}$ operon at an early exponential phase of growth (OD_{600} 0.3 for 1.5 hr. culture) in SILAC medium [73]. In contrast, Arenas-Hernández et al. demonstrated that the LPF is highly expressed in culture at an OD_{600} of 1.0 to 1.2 in Dulbecco's Modified Eagle's Medium (DMEM) with iron and bile salts. They used an O157:H7 strain in their study [109]. Similarly, Torres et al. also support that the rate of transcription of LPF is high during the late exponential growth phase, using EHEC O157:H7 and LB media. They found stimulated transcription in LB media even in iron depleted growth condition, which is in contrast to the study by Arenas-Hernández et al. [110]. Another factor that might have had a role in repression of gene expression is low-density growth of bacteria in the culture medium. Despite being an enriched medium, cell growth stops at relatively low density in LB broth because it contains low amount of carbon sources and divalent cations. To ensure sufficient growth of bacteria carbon sources can be added to the culture medium (e.g. glucose, lactose) [111]. These results show that the expression of fimbrial genes may vary depending on the growth and growth condition [112].

When performing quantitative analysis of gene expression, chose of reference gene for comparison is important. Housekeeping genes are commonly used as the reference genes. Housekeeping genes are genes that encode functions essential for basic functions needed for survival of the organisms. Expression of gene products from such genes are required at all times and is expressed at a more or less constant level in a cell. Examples of such genes include genes encoding DNA polymerase, RNA polymerase, ribosomal RNA and enzymes for central metabolic pathways [113]. There is no validated system for which housekeeping genes are most suitable for use as reference genes in quantitative analyses. However, it is important that the reference gene should have a stable expression under different experimental conditions, and the efficiency of PCR- amplification should be over 90% (and max. 105%). Some researchers suggest using two stable housekeeping gene as reference [114].

In our study the RNA polymerase α subunit gene *rpoA* was used as reference gene for the study of gene expression. Different studies of gene expression in different bacteria have proved *rpoA* as a suitable reference gene. In a study done by Ritz et al., expression levels of different housekeeping genes in *Campylobacter jejuni* were analysed under different growth phases (exponential and stationary) as well as stress conditions (cold shock, oxidative stress). The *rpoA* was found as the best reference gene in that study [115]. Galisa et al. tried to validate the expression of reference genes in *Glucocnobacter diazotophicus* grown in carbon sources other than source- sugarcane. Here also, *rpoA* was found as one of the best stable reference genes [114]. In our study, RT-qPCR analysis showed that the *rpoA* gene was expressed both in LB and SILACE broth. The C_T values were somewhat higher in samples from LB than in SILACE broth, which might indicate difference in expression of the *rpoA* gene between the two-culture media. However, the experiment was not designed to study this aspect, and as firm conclusion cannot be made about how stable the *rpoA* gene is expressed in these two culture conditions.

For analyses of gene expression, it is important that the quality of RNA is optimal, since degradation of RNA may influence on the results of expression analysis. In this study RNA was extracted using the RNeasy Mini Kit (Qiagen). The manufacturer recommends use of a RNA-protect bacterial reagent to stabilize RNA (Qiagen). However, we did not have such stabilizing reagents available for this study. Different types of RNAs may have different degradation rate under different growth conditions. Without using the stabilizer there is a risk for degradation of RNA that may eventually affect the synthesis of cDNA and gene expression. In this study we tried to minimize RNA degradation by using RNeasy mini kit which has RNeasy silica-membrane that removes most of the DNA. In addition to this, isolation of RNA was done in

ice, gloves were used, and isolated RNA was immediately transferred to reverse transcription step to synthesize cDNA. And, we tested the quality of total RNA extracted using Nanodrop and agarose gel electrophoresis. We found acceptable ratios of absorbance A_{260}/A_{280} and A_{260}/A_{230} for three of the four samples, and the gel profile of RNA samples also showed bands as expected. We could therefore confirm that the quality of RNA as good, for at least three of the four samples tested. Nanodrop and gel electrophoresis method does however have some limitations. All nucleic acid types such as single stranded and double stranded DNA and RNA are absorbed at 260nm, so the absorption ratio might be affected if there is any contaminant (e.g. DNA) and nucleic acid concentration can be overestimated. In addition, changes in RNA integrity cannot be predicted since degraded RNA is also absorbed at 260nm. Likewise, agarose gel also cannot determine the presence of amplification inhibitors. These problems could be overcome by using of alternative analyses e.g. the Bioanalyzer. Using a Bioanalyzer (Agilent Genomic) it is possible to measure RNA integrity as well as to resolve small RNAs [116]. However, this is quite expensive and due to lack of sufficient funds we were not able to use this method. The fact that we were able to show expression of the reference gene *rpoA* shows that the quality of extracted RNA in this study was acceptable.

Presence of bacterial DNA together with cDNA might influence on PCR results. DNase, which could be used to degrade bacterial DNA [117], was not used in this study. However, the RNeasy mini kit contains a RNeasy silica membrane that removes most of the DNA without DNase treatment. The negative result for amplification of the *lpf*$_{B1}$*C* gene in the expression experiments shows that there must have been very little intact DNA present in the samples after extraction.

In this study, cDNA was prepared from RNA by reverse transcriptase with the help of random hexamer primers. Random primers contain all possible nucleotide combinations and they can randomly bind to all RNAs present. Because of this, truncated cDNAs can be synthesized and there may be a possibility for lacking binding sites on cDNAs for PCR primers [100, 118]. This might affect amplification efficiency and subsequently gene expression. We did not have any way to check the efficiency of the cDNA synthesis from extracted total RNA. So, we were not able to confirm what sufficient lengths of cDNAs were made, and that we had obtained the target cDNA product. However, the fact that we were able to show expression of the reference gene *rpoA* indicates that the quality of cDNA in this study was acceptable.

An essential element of relative quantitative real-time PCR is to compare the C_T-values of the target gene with that of a reference gene [119]. This is done to normalize results between different experiments e.g. with respect to bacterial concentration. To be able to compare C_T

values between different PCRs it is essential that amplification efficiency is optimal and similar between the two methods. Using the slope method, we were able to show that the amplification efficiency was acceptable for use in qPCR for both the target gene *lpf*$_{B1}$*C* (105%) and the reference gene *rpoA* (99%).

6 Conclusion

We had STEC O103:H25 strain with over expression of putative fimbrial operon *lpf*$_{B1}$-genes that were found 42-63 % similarity to the long polar fimbrial operon genes of O111:H-. So, it was expected that *lpf*$_{B1}$ gene would show the expression and would prove its role in adhesion. However, expression of *lpf*$_{B1}$ genes could not be found in this study. Based on the aim of the study, PCR amplification and cloning of the *lpf*$_{B1}$ operon were successful. Furthermore, plasmid pDA09 containing *lpf*$_{B1}$ operon was cloned successfully into the wild type STEC strain. But, fimbrial gene expression could not be observed in the wild type STEC at different growth conditions. Exact reasons for not showing gene expression could not be found as there might be many unknown regulating factors. However, nutrition, growth density, global regulators, environmental condition, nature of host cells, genetic factors and nature of plasmid vectors and quality of RNA and cDNA were discussed as possible reasons in regulation of fimbrial gene expression.

There were some limitations in our study which probably affected the outcomes of the study. One of the limitations was related to extraction of RNA and cDNA synthesis. In addition to this, funding and study period were other limitations. Nevertheless, these limitations have expanded the possibilities of further improvements and perspectives in the study.

7 Future perspectives

Depending on the results from this study there would be different further perspectives. It could be interesting to modify the experimental conditions to observe the fimbrial gene expression and find out the role of fimbriae in adhesion and virulence. Instead of Stx-/STEC strains, fimbrial expression can be studied in Stx+/STEC of same background and same phylogenic group. To see the expression of gene, instead of cloning vector, expression vector could be used. The expression of gene could be seen in different growth and nutritional conditions. In this study, RNA synthesis and cDNA synthesis were done once. So, in future repetition of RNA and cDNA synthesis could be useful to validate the reliability of the experiment. Gene specific primers could be used instead of random primers during cDNA synthesis. Native promoter of the study strain could be tagged with reporter gene so that we can see when the promoter is activated. Likewise, expression of fimbrial gene can be studied in animal cell culture.

8 References

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9 Appendices

9.1 Appendix I

List of equipment and kit used in the study

Equipment/Kits/Reagents	**Manufacturer**
Instruments	
CEQ genetic analysis system (for sequencing)	Beckman Coulter, USA
E-Gel with SYBR safe	Invitrogen, ThermoFisher Scientific, USA
Electrophoresis-Gel cast	Bio-Rad, USA
Gel-doc system	Bio-Rad, USA
Microcentrifug, model 5415R	Eppendorf, Germany
Nano.drop reaction system	ThermosFisher Scientific, USA
PCR thermocycler -T100	Bio-Rad, USA
PCR tube Spinner	Fisher Scientific, USA
Real time system CFX-96	Bio-Rad, USA
Techne Dri-block, heater model DB-2A	Nerliens, Norway
Thermomixer, comfort	Eppendorf, Germany
UV-visible spectrophotometer, UV-1700	SHIMADZU, BioNordika, Norway
Water bath, GFL (97-254)	Pharma Spec, UK
Kits	

AmpliTaq gold DNA polymerase with 10x PCR buffer	ThermoFisher, Scientific, USA
DNA ladder 1kb+	Invitrogen, USA
DNeasy blood and tissue kit	Qiagen, Germany
Long Amp*Taq* 2X Master mix	BioLabs, USA
Plasmid Miniprep kit	Qiagen, Germany
Quick start kit	BioLabs, USA
RNeasy mini kit	Qiagen, Germany
TOPO TA cloning kit	Invitrigen, USA
Biological reagents	
0.5xTBE buffer	Made in laboratory
Agarose	Sigma-Aldrich, USA
cDNA synthesis mix	Prepared in laboratory
Gel red	Biotium, USA
Lysozyme	ThermoFisher Scientific, USA
S.O.C media	Made in laboratory
TE buffer	Made in laboratory
TSS broth	Made in laboratory
Culture media	
Blood agar base	Sigma-Aldrich, USA
Luria agar base	Sigma-Aldrich, USA
Luria broth base	Sigma-Aldrich, USA
SILACE medium	Made in laboratory

9.2 Appendix II

Composition and preparation of culture media and antibiotic solution used in the study are as follows:

1. **Blood Agar (BA)**

Ingredients	Grams/litre
Meat extract	10.000
Peptone	10.000
Sodium chloride	5.000
Agar	15.000
Final pH (at 25℃)	7.3±0.2

Preparation: 40.0 grams was suspended in 1000 ml distilled water. The medium was heated to boiling to dissolve completely. The medium was then sterilized by autoclaving at 15 lbs pressure (121℃) for 15 minutes. Then medium was cooled to 45-50℃ and aseptically added 5% v/v sterile defibrinated blood. Mixture was mixed well and poured into sterile Petri plates.

2. Luria broth/Agar (LB/LA)

Ingredients	Grams/litre
Tryptone	10.000
Yeast extract	5.000
Sodium chloride	10.000
Agar**	15.000
Final pH (at 25℃)	7.0±0.2

** LB does not contain agar.

Preparation of LB: 25 grams was suspended in 1000 ml distilled water. Solution was heated to dissolve the medium completely. The medium was then sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Medium was poured into sterile petri plates.

Preparation of LA: 40.0 grams was suspended in 1000 ml distilled water. The medium was heated to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Medium was mixed well and poured into Sterile Petri plates.

3. SILACE medium

Ingredients	**mg/litre**
Inorganic salts	
$Na_2HPO_4.12H_2O$	17100
KH_2PO_4	3000
NaCl	580
$MgSO_4$	120
$CaCl_2$	11
Amino Acid	
L-Serine	400
L-Threoninr	200
L-Valine	150
L-Arginine	100
L-Phenylalanine	50
L-Isoleucine	30
L-Leucine	30
L-lysine	30
L-Tyrosine	30
L-Histidine HCL	20
L-Methionine	20
L-Tryptophan	20
Others	
Glucose	10000
Biotin	0.085
Calcium Pantothenate	17

Folic Acid	0.085
Inositol	85
Niacin	17
Paminobenzoic acid	8.5
Pyridoxine hydrochloride	17
Riboflavin	8.5
Thiamine hydrochloride	17
Thiamine HCL	1
Adenine sulfate	20
Uracil	20

4. Preparation of Antibiotic solution

In this study ampicillin was used in a concentration 0f 50µg/ml. Antibiotic stock solution was made by using the Antibiotic tablet which weight is 3.2 mg. according to the instruction:3.1 mg od tablet is dissolved in 3.05 ml of distilled water. Then the solution was supposed as 1 mg/ml. 0.05 ml antibiotic solution was added to 1 ml LB/LA to obtain the working solution: 50µg/ml.

9.3 Appendix III

Biological reagents used in the study

1. Preparation of TE (Tris-EDTA) buffer

TE buffer is commonly used in solubilization of DNA or RNA to protect it from degradation. TE was prepared with mixing of Tris-Cl and EDTA with water.

Reagent	Amount to add	Final concentration
Tris-Cl (1M)	1 ml	10mM
EDTA (0.5 M)	200 µl	1mM
H2O	98.8 ml	
pH 7.4 to 8.0		

2. Preparation of 0.5X TBE buffer

A stock solution of 1 litre 10X TBE buffer was made. 108 g Tris and 55 g Boric acid was dissolved in 800 ml distilled water with the use of magnetic stirrer. Then 40 ml 0.5 M Na_2EDTA

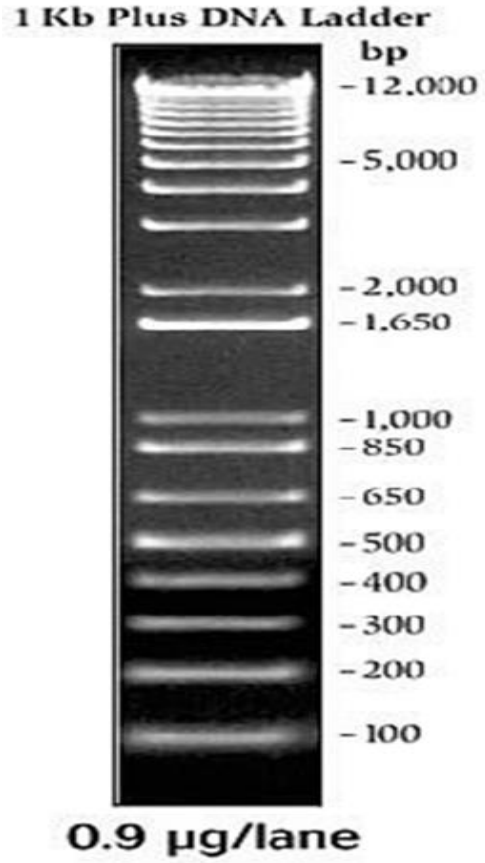
(pH 8.0) was added and volume was adjusted to 1 litre with distilled water. To make 0.5X TBE buffer working solution, 1:20 dilution was done.

3. Preparation of GelRed solution

GelRed^{TM} 10,000X stock solution was diluted 3,300-fold to make a 3X staining solution in H_20. To make 1 litre GelRed solution, 0.3 ml (300 µl) GelRed solution was added to 1 litre of solution.

4. DNA ladder

Working solution of DNA ladder 1 kb plus solution was made as 1:10 with TE buffer working solution.



5. TSS (Transformation and Storage solution) broth

To make 50 mL TSS buffer: 5g PEG 8000, 1.5 mL 1M MgCl2 (or 0.30g MgCl2*6H20), 2.5 mL DMSO was mixed then LB was added to 50 ml. Then the solution was filter-sterilized (0.22 µm filter)

6. SOC medium (200 ml)

SOC medium

Trypton	4 g
Yeast extract	1 g
NaCl	0.1 g
H_2O	To 190 ml, shake until dissolved
are added with 250 mM KCl (1.86 gm KCl to 100 ml water)	2 ml
pH is adjusted to 7.0, add H_2O to 200 ml, and is autoclaved	
Then the above solution is added with 2 M $MgCl_2$ (40.6 gm $MgC_{l2}.6H_20$ to 100 ml water) And is autoclaved	1 ml
Then solution is cooled down to < 60℃ then	
+ 1 M glucose (filtered) (7.2 gm to 40 ml water) Is added	4 ml
Is kept at freeze at – 20℃	

9.4 Appendix IV

One Shot TOPO 10 competent *E. coli* cells

- One Shot® TOP10 *E. coli* cells are similar to the DH10B™ strain, and offer the following features:
- hsdR for efficient transformation of unmethylated DNA from PCR amplifications
- mcrA for efficient transformation of methylated DNA from genomic preparations
- lacZΔM15 for blue/white color screening of recombinant clones
- endA1 for cleaner DNA preparations and better results in downstream applications due to elimination of nonspecific digestion by Endonuclease I
- recA1 for reduced occurrence of nonspecific recombination in cloned DNA **Genotype:** F- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ *lac*X74 *rec*A1 *ara*D139 Δ(*araleu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G

9.5 Appendix V

Nanodrop spectrophotometer

Principle

Nanodrop Spectrophotometer is used to measure the concentration of nucleic acids. RNA, ssDNA, and dsDNA absorb at 260nm and they will contribute the total absorbance of the sample. Two types of ratio are considered while measuring the absorbance.

260/280 ratio: This ratio is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as "pure" for DNA; a ratio of ~2.0 is generally accepted as "pure" for RNA. If the ratio is lower than standard, it may indicate the presence of protein, or other contaminants that absorb strongly near 280 nm (ThermoFisher Scientific, USA).

260/230 ratio: This ratio is used as a secondary measure of nucleic acid purity. The 260/230 values for "pure" nucleic acid are often higher than the respective 260/280 values. Expected 260/230 values are commonly in the range of 2.0-2.2. If the ratio is lower than expected, it may indicate the presence of contaminants that absorb at 230 nm (ThermoFisher Scientific, USA).

At first, the upper and lower optical surfaces of the nanodrop spectrophotometer sample retention system was cleaned by pipetting 2 to 3µl of deionized water onto the lower optical surface. Then lever arm was closed to make the contact of upper pedestal with the water. Lift the lever arm was lifted and wiped off both optical surfaces with a clean, dry, lint-free lab wipe. Nanodrop software was then opened and select the Nucleic Acid menu was selected. Blank measurement with deionized water was performed first by dispensing 1 µl of water onto the lower optical surface. The lever warm was then lowered and "Blank" was selected in the nuceic acid application. After completion of blank measurement, the both optical surfaces were cleaned. Then 1µl of DNA/RNA sample was dispensed onto the lower optical surface and lever arm was closed. From software "measure" menu was selected, and the nucleic acid concentration was shown in the screen **[120].**