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Title:
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Date:
2018

Persistent Link:
https://hdl.handle.net/11343/219875

The effect of *tonB* deletion on the expression of the genes encoding Shiga toxin, TonB-dependent receptors and fimbriae in the 16001 oedema disease strain of *E. coli*

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Thesis submitted in total fulfillment of the requirements of the degree of Master of Veterinary Science at The University of Melbourne

April, 2018

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Abstract

Oedema disease is caused by Shiga toxin producing *Escherichia coli* (STEC) and generally affects piglets at one to two weeks after weaning. The stress related to dietary change and loss of lactogenic immunity after weaning are believed to contribute to the onset of the disease. Affected piglets typically have oedematous lesions in multiple organs, including the stomach, colon and eyelids, and also neurological signs, such as ataxia and paralysis. Occasionally, the disease can result in sudden death without any apparent clinical signs. The Shiga toxin and fimbriae are the two key virulence factors and they play an essential role in the pathogenesis of the disease. Multiple attempts have been made to develop active, passive and maternal approaches to immunization. These studies have mainly focussed on attenuating the organism by manipulating these virulence factors at the molecular level. Although the disease is endemic and has a considerable economic impact, there is no commercial vaccine available in Australia. Control measures mainly focus on antibiotic treatment but this has been challenged by the emergence of drug resistance in the strain.

Iron is one of the most important micronutrients for the growth of bacteria and its availability to pathogens is restricted both inside and outside the host. Pathogenic bacteria respond by elaboration of siderophores in order to transport iron across the outer membrane, and the TonB protein supplies the necessary energy for this transport. The aims of this research project were to develop *tonB* mutants of an Australian STEC strain (16001) isolated from pigs with oedema disease by deleting the *tonB* gene using the lambda Red recombination

system and to investigate whether the transcription of the virulence factors Shiga toxin 2e (*stx2e*) and the F18 fimbriae and TonB-dependent transporter genes was influenced by the *tonB* deletion. The lambda Red recombination system was used to replace the target gene with an antibiotic resistance cassette, and successful mutagenesis was confirmed by molecular and phenotypic characterisation of the mutant. Sequencing studies showed that the *tonB* gene was replaced with a kanamycin resistance gene. The mutant had a slower growth rate and produced greater concentrations of siderophores on chrome azurol S (CAS) agar. In general, phenotypic characterization of the *tonB* mutant indicated that it had reduced intracellular iron levels as a result of the *tonB* deletion. The aim of deleting *tonB* was to reduce intracellular iron levels, which was anticipated to attenuate the organism and also upregulate the iron regulon to increase the production of protective antigens. During iron shortage, siderophore synthesis and expression of siderophore transporters is increased, thus eliciting an enhanced immune response against these antigens. Upregulation of the genes for some outer membrane receptors, as well as those for the key virulence factors Stx2e and F18 fimbriae, was demonstrated in this study. In conclusion, the deletion mutant generated in this study may be able to be used as the basis for development of a vaccine candidate for control of this important disease of pigs.

Declaration

The work described here was performed in the Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Australia and was my original work. Due acknowledgement has been made of work conducted by other authors and elsewhere. This thesis complies with The University of Melbourne requirements in being less than 50,000 words in length, excluding tables, maps, bibliographies and appendices.

Aga Edema Gelgie

Acknowledgements

I would like to thank all people who have made direct and indirect contributions to this work. Before anyone, I wish to express my sincere gratitude to my supervisors Prof. Glenn F. Browning, Dr. Marc S. Marenda and Dr. Kelly A. Tivendale, for giving me the chance to do this work. Your guidance, support and patience were enormous. Thank you for taking me through the journey and I assure you what you seeded in me will grow and bear more seeds. I am thankful to my committee chair, Prof. James Gilkerson, for facilitating my progress review meetings and all your encouragement in the meetings.

I am grateful to the Department of Economic Development, Jobs, Transport and Resources for providing us with the oedema disease field isolate.

To everyone in the veterinary microbiology research group, thank you for your company and help in the laboratory and the office. Special thanks to Ms. Joanne Allen and Nino Ficorilli (former virology laboratory manager) for you were sources of answers for many of my questions and showed me shortcuts in experiments. To Anna Kanci, thank you for your help in the lambda Red recombination experiment. I would like to thank Ms. Cynthia Brown for meeting all my sterile supply needs. Special thanks go to Mesula Geloye Korsa for his friendship and for always being there for me for advice on microbiological issues and recently on gene expression. I also need to thank Mesula's family, his wife Mrs. Ayantu

Bekele Sike and sons Melka and Nimo for your friendship and support. I would also like to thank Jose A. Quinteros for his unreserved support in troubleshooting the gene expression experiment.

I am grateful to the University of Melbourne for financial support through a Melbourne International Research Scholarship and a Melbourne International Fee Remission Scholarship.

I would like to say thank you to my family in Ethiopia, my father Edema Gelgie Bariso, my mother Shore Edema Guye and my elder brother Buno Edema Galge and all the members of my family for their encouragement and love.

Finally, I would like to thank all members of the Rehoboth Oromo Christian Church Australia for helping me mature spiritually and for all their support, in particular, the elders and the choir.

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List of Abbreviations

AIDA	adhesin involved in diffuse adherence
APEC	avian pathogenic *Escherichia coli*
bp	base pairs
CAS	chrome azurol S
cDNA	complementary DNA
ddH_2O	double-distilled water
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ETEC	enterotoxigenic *E. coli*
EHEC	enterohaemorrhagic *E. coli*
µF	microfarad
Fur	ferric uptake regulator

Gb3	globotriasylceramide
Gb4	globotetraosylceramide
HCl	hydrochloric acid
HUS	haemolytic uraemic syndrome
H_2O_2	hydrogen peroxide
IgA	immunoglobulin A
IgG	immunoglobulin G
IgY	immunoglobulin Y
kb	kilobase
kv	kilovolt
LB	Luria Bertani
M	molar
mg	milligram
$MgCl_2$	magnesium chloride
ml	millilitre
MM9	minimal medium 9
ng	nanogram

OD	optical density
16001ΔtonB:km	*tonB* deficient 16001
PCR	polymerase chain reaction
rpm	revolutions per minute
RNA	ribonucleic acid
RT	reverse transcription
rRNA	ribosomal RNA
SS III	SuperScript III
STEC	Shiga toxin producing *E. coli*
Stx	Shiga toxin synthesised by *Shigella dysenteriae*
Stx1	Shiga toxin 1
Stx2	Shiga toxin 2
Stx2e	Shiga toxin 2e
SLT IIv	Shiga toxin 2e
S. dysenteriae	*Shigella dysenteriae*
TBDTs	TonB-dependent receptors
TCA	tricarboxylic acid

U	unit
UPEC	uropathogenic *E. coli*
V/cm	volts per centimetre
WT	wild type
Ω	ohms
µl	microlitre
µg	microgram

1. Literature review

1.1. Oedema disease

Oedema disease, also known as ‘bowel oedema’ or ‘gut oedema’, is a fatal disease of weaner and grower pigs characterised by sudden death and oedema in the submucosa of the stomach, spiral colon and eyelids, and neurological dysfunction, including ataxia, paralysis and an inability to stand (Imberechts *et al*., 1992). The disease is caused by β-haemolytic Stx2e producing strains of *E. coli* (STEC) (Osek *et al*., 1999) possessing F18ab fimbriae, which facilitate bacterial adherence to host epithelial cells (Duan *et al*., 2012).

The disease was first reported in Ireland in 1938 (Shanks, 1938) and occurs worldwide, causing considerable economic loss (Fairbrother *et al*., 2005). It has a moderate morbidity (16%), but a high mortality (90%) (Imberechts *et al*., 1992). A high prevalence of serum antibodies against this pathogen has been detected in several countries, including Belgium (Verdonck *et al*., 2003), Japan (Kusumoto *et al*., 2016), Denmark (Aarestrup *et al*., 1997) and the Republic of Korea (Kwon *et al*., 2002). The disease commonly occurs as an outbreak preceded by factors such as vaccination, weaning, dietary change, transportation or overstocking (Radostits *et al*., 2006).

The disease is usually triggered by weaning, when changes in food composition and loss of lactogenic immunity occur, and clinical signs are seen about one week after oral inoculation of pigs (Imberechts *et al*., 1992). Deprivation of colostrum has been shown to induce the disease artificially (Sato *et al*., 2017) and a high protein diet is also reported to favour the occurrence of the disease (Bosworth *et al*., 1996). The first event in the infection process is fimbrially mediated adherence of the bacteria to the epithelial cells and rapid multiplication in the mid-jejunum and ileum (Bertschinger and Pohlenz, 1983), followed by production and systemic absorption of the toxin (Nagy and Fekete, 1998). Erythrocytes, which have high levels of globotetraosylceramide (Gb4) on the cell surface, bind the toxin and carry it to the organs, where lesions are seen (Boyd *et al*., 1993; Matise *et al*., 2003). Stx2e is chiefly responsible for the clinical signs and lesions, including the collapse of the blood-brain-barrier and infarction and malacia in the brain stem due to endothelial cell damage, which elicits neurological signs and sudden death (Marques *et al*., 1987; Meisen *et al*., 2013; Kausche *et al*., 1992). Moreover, endothelial cell damage is aggravated by hypertension resulting from oedema (Clugston *et al*., 1974; Imberechts *et al*., 1992). The toxin binds to endothelial cells of the colon, ileum, spinal cord, cerebellum, eyelid and liver, and to vascular smooth muscle cells and red blood cells, where it causes the lesions associated with the disease, including oedema and smooth muscle necrosis (Matise *et al*., 2003).

A history of an outbreak of disease after weaning, and after dietary changes or other stressful events, with the typical neurological signs and necropsy findings, such as subcutaneous oedema of the eyelids, abdomen and mesocolon, are strong indications of the diagnosis (Imberechts *et al*., 1992). PCR of cultured isolates is quite commonly used for the detection

of the *stx2e* and *F18ab* genes (Barth *et al*., 2011; Byun *et al.*, 2013; da Silva *et al*., 2001). Development of multiplex PCR assays has facilitated diagnosis of the disease, as this enables detection of multiple genes in a single assay (Casey and Bosworth, 2009). PCR detection of O-antigen genes specific for serotype O139 (Wang *et al*., 2005), one of the three (O138, O139 and O141) typical serogroups responsible for oedema disease (Gannon and Gyles, 1990) and a PCR-based DNA microarray of O-antigen genes have also been shown to be accurate and highly sensitive methods for screening the disease (Han *et al*., 2007). A Gb4 based enzyme-linked immunosorbent assay (ELISA), which utilises isolates from culture, has been developed (Togashi and Sasaki, 2015), and recently a group from Japan developed an immunochromatographic test trip that specifically detects the Stx2e toxin in a culture supernatant (Arimitsu *et al*., 2016). Pulsed-field gel electrophoresis of genomic DNA after digestion with restriction endonucleases that cleave the genome infrequently has been useful for investigation of clonal relationships between strains in outbreaks (Aarestrup *et al*., 1997).

In humans, O157:H7 enterohaemorrhagic *E. coli* (EHEC) synthesise Shiga toxin 1 (Stx1) and cause haemolytic uraemic syndrome (O'Brien *et al*., 1983). Cattle are thought to be the major reservoir for these *E. coli*, but are resistant to its effects because they lack the globotriaosylceramide (Gb3) receptor for the toxin (Pruimboom-Brees *et al*., 2000). This serotype is commonly associated with disease in humans, but has also been isolated from pigs (Johnsen *et al.*, 2001). Although there is good evidence that the Stx2e toxin is not associated with disease in humans (Beutin *et al*., 2008), recent studies indicate that pigs can be an important reservoir for strains of *E. coli* that cause disease in humans (Cha *et al*., 2018). In a study that compared Stx2e producing *E. coli* from diarrhoeic human patients with those

isolated from pigs with oedema disease and post-weaning diarrhoea, the majority of the pig strains were found to contain the gene encoding the F18 adhesin and the adhesin involved in diffuse adherence (AIDA), while the human strains did not possess these adhesins (Sonntag *et al*., 2005), which might partially explain the host specificity of these strains.

Historically, antimicrobial drugs have typically been used to treat and prevent oedema disease (Radostits *et al*., 2006). A range of antimicrobial agents, including trimethoprim, quinolones (ciprofloxacin, norfloxacin and nalidixic acid) and furazolidone have been shown to induce an SOS response which in turn upregulates the *stx2* gene, enhancing toxin production in O157 STEC strains (Kimmitt *et al*., 2000). Pigs susceptible to infection with $F18^+$ *E. coli* infections express $FUT1^{A/A}$, the F18 receptor, in their small intestine (Meijerink *et al*., 2000). This has prompted the suggestion that breeding of $F18^+$ infection resistant pigs could be a potential approach to prevent oedema disease (Coddens *et al*., 2008). Egg yolk IgY antibodies have also been suggested as an alternative therapy for the disease (Feng *et al.*, 2013). Some non-pathogenic *E. coli* have also been shown to inhibit the growth of STEC strains and thus may be able to be used as probiotics for prevention and treatment (Reissbrodt *et al*., 2008).

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

Escherichia coli are one of the most common inhabitants of the intestinal tract and belong to the family *Enterobacteriaceae* within the order *Enterobacteriales*. Shiga toxin producing *E. coli* are a group of *E. coli* consisting of organisms capable of synthesising a cytotoxin known as Shiga toxin (O'Brien *et al.*, 1982) and this group includes the strains that cause oedema disease (Marques *et al*., 1987). Some porcine STEC strains can produce one of the enterotoxins (which are produced by enterotoxigenic *E. coli* (ETEC) strains, which cause diarrhoea) and Shiga toxin (Kusumoto *et al*., 2016) and this may explain the inconsistent association between diarrhoea and oedema disease (da Silva *et al*., 2001). Based on a literature search and analysis of data on pathotypes, serotypes, serogroups of *E. coli*, country of origin and age of pigs infected, numbers and percentage of positive samples and isolates, Abubakar *et al* reported that STEC comprise 7.9% of the *E. coli* characterised in pigs (Abubakar *et al.*, 2017), and were first characterised in the 1980s and named 'shiga-like toxin producing *E. coli* (STEC)' at the time (O'Brien *et al*., 1984), but more recently have been referred to as Shiga toxin producing *E. coli* because of the similarity of the toxin they produce to the Shiga toxin produced by *Shigella dysenteriae* (Gyles, 2007). The serogroups typically associated with oedema disease in pigs are O138, O139, and O141 (Gannon and Gyles, 1990), but serogroup O147 and O149 *E. coli* have also been reported to be associated with oedema disease in the United States (Helgerson *et al*., 2006) and South Africa (Kanengoni *et al*., 2017).

1.3. Virulence factors

Adherence of the bacteria to enterocytes and production of the toxin are the main events in the pathogenesis of oedema disease (Imberechts *et al*., 1992). Intravenous injection of the toxin has been shown to reproduce the disease experimentally (MacLeod *et al*., 1991) which indicates its importance in pathogenesis, while flagella have been shown to contribute to adhesion, invasion, biofilm formation and IL-8 production *in vitro* (Duan *et al*., 2012).

1.3.1. The F18 fimbriae

F18 fimbriae is a colonisation factor found on both STEC and ETEC, with two well-known variants, known as F18ab and F18ac, known to exist (Rippinger *et al*., 1995), as well as and another more recently recognised serotype, F18 new variant (Byun *et al*., 2013). These two variants have recently been shown to bind to the same receptor (Tiels *et al*., 2017). F18ab fimbriae are predominantly expressed by oedema disease strains (Rippinger *et al*., 1995), while F18ac are mostly expressed by ETEC (Nagy *et al*., 1997). F18 fimbriae are encoded by a plasmid-borne gene *fed* (Mainil *et al*., 2002) and it binds to receptors known as F18R on the surface of the enterocytes. In some strains the F18 fimbriae are not expressed *in vitro* (Bertschinger *et al*., 1988) or are only expressed when the organisms are cultured microaerophilically or on alizarin-yellow agar (Wittig *et al*., 1994). Porcine STEC also express the AIDA adhesion, which is a plasmid-encoded afimbrial adhesin that is also likely

to contribute to the pathogenesis of oedema disease as a mutation in the gene encoding AIDA (*orfB*) has been associated with the absence of disease (Niewerth *et al*., 2001; Oanh *et al*., 2010; Zhao *et al*., 2009). The *orfB* gene is found on the same plasmid encoding the *fed* gene, and this plasmid is also found in some non-STEC strains (Mainil *et al*., 2002). The intimin gene (*eae*), which is harboured by attaching and effacing *E. coli* (AEEC), is not found in oedema disease strains (Gannon *et al*., 1993).

1.3.2. Stx2e toxin

Shiga toxin (Stx2e), which is also known as verotoxin because of its toxicity for Vero cells (Gyles, 2007), is a heat-labile toxin expressed by oedema disease strains of *E. coli* (da Silva *et al*., 2001; Marques *et al*., 1987). All the Shiga toxins (Stxs), including that of *S. dysenteriae*, Stx1, Stx2 and the plant toxin ricin have AB_5 structures (Melton-Celsa, 2014), with an enzymatically active A subunit (32 kDa) and five B subunits (7.5 kDa each), which bind to glycolipids on the host target cells (Ling *et al*., 2000). The A subunit possesses N-glycosidase activity, which cleaves an adenosine in the 28S rRNA of the 60S ribosomal subunit, inhibiting protein synthesis in the target cells (Endo and Tsurugi, 1987; Saxena *et al*., 1989). The B subunits bind to Gb4 receptors on the target cells, enabling the holotoxin to be internalised via endocytosis (DeGrandis *et al.*, 1989).

Unlike other Stx variants, Stx2e is not toxic to HeLa cells (Marques *et al*., 1987) and its B subunits have a specific tropism for the Gb_4 receptor (Paton and Paton, 1998). However, Keusch *et al* have shown that Stx2e can also utilize Gb3 as a functional receptor (Keusch *et al*., 1995). The sequence of the B subunit of Stx2e has similarity to those of the other Stx toxins than they have with each other, which may be correlated with the differences in the tropism of this Shiga toxin for glycolipid receptors (Weinstein *et al*., 1988b).

The Shiga toxins and ricin toxin can induce apoptosis both *in vitro* and *in vivo* (Tesh, 2011). Stx2 has also been shown to activate intracellular stress pathways, including the endoplasmic stress and the ribotoxic stress response pathways, which may lead to apoptosis via the activation of various cell death signals (Bernedo-Navarro and Yano, 2016).

1.3.2.1. Regulation of Shiga toxins

The Shiga toxins are divided into two groups; Stx1 (Stx1 and Stx1c) and Stx2 (Stx2, Stx2c, Stx2d, Stx2e and Stx2f) (Melton-Celsa, 2014). The phage-encoded Shiga toxin Stx2e (Muniesa *et al*., 2000; Recktenwald and Schmidt, 2002) is encoded by an operon composed of the A and B subunit genes, which are separated by an intergenic space of 12 nucleotides (Weinstein *et al*., 1988b). While the gene encoding the B subunit of Stx expressed by *S. dysenteriae* is transcribed autonomously (Habib and Jackson, 1992), the gene encoding the B subunit of Stx2 lacks an independent promoter (Sung *et al*., 1990). The transcription of

stx1 is under the control of the ferric uptake regulator (Fur) protein, which was first demonstrated by the detection of a 'fur box' in the promoter region of the operon (Calderwood and Mekalanos, 1987). The expression of the oedema disease toxin Stx2e has been shown to be attenuated by zinc *in vitro*, resulting in a significantly smaller zone of haemolysis on agar plates supplemented with zinc (Uemura *et al*., 2017). Incubation temperature affects the production of Shiga toxin by *S. dysenteriae*, but has no effect on Stx1 synthesis by enterohaemorrhagic *E. coli* (Weinstein *et al*., 1988a). The *stx2* gene is carried by bacteriophages, the replication of which results in concomitant replication of the toxin genes (Mühldorfer *et al*., 1996). The replication of the bacteriophages is induced by the SOS response (Little and Mount, 1982), a global response to DNA damage that involves cell cycle arrest and DNA repair. Some antimicrobial agents, including quinolones, trimethoprim, furazolidone and metronidazole, have been shown to upregulate the expression of *stx2* by inducing the SOS response (Kimmitt *et al*., 2000). A study performed on an O157:H7 STEC strain demonstrated that H_2O_2 induced oxidative stress resulted in significant upregulation of *stx1* and *stx2* (Mei *et al*., 2015). Another study showed that salt-induced expression of *Rec*A mediated activation of the prophage encoding Stx2, resulting in enhanced production of Stx2 by an O157:H7 strain (Harris *et al*., 2012). Deletion of the *luxS* gene, which is involved in quorum sensing, in a porcine O139:H1 *E. coli* has been shown to attenuate production of the Stx2e toxin (Yang *et al*., 2014).

1.3.3. Other virulence factors

The *E. coli* causing oedema disease have been reported to form biofilms which may confer an ability to evade the host immune response and render them less susceptible to antibiotics and disinfectants (Cocchi *et al*., 2017). They also possess the *E. coli* type III secretion system 2 (ETT2) pathogenicity island (Prager *et al*., 2004), which plays roles in pathogenesis and virulence (Ideses *et al*., 2005). Some STEC strains are able to survive several months under freezing conditions and tolerate pH as low as 2.5, which facilitates colonisation of the gastrointestinal tract (Castro *et al*., 2017).

1.4. Iron and Gram-negative bacteria

Iron is an indispensable micronutrient for almost all forms of life, including bacterial pathogens. Its availability determines their survival inside their host, because it plays a role in gene regulation, in the tricarboxylic acid (TCA) cycle and in DNA biosynthesis, among many other functions (Andrews *et al*., 2003; Ratledge and Dover, 2000). It is not readily available in aerobic environments, as ferrous (Fe^{2+}) iron is easily oxidised to ferric iron (Fe^{3+}), which has low solubility at neutral pH and hence is difficult for pathogens to acquire in the host (Escolar *et al*., 1999; Köster, 2001). Anaerobic bacteria, however, take advantage of the high solubility of Fe^{2+} in anaerobic conditions and therefore don't face the iron shortage that aerobic bacteria do (Braun and Killmann, 1999). Furthermore, animals restrict

iron availability as part of their innate immunity by forming iron-lactoferrin and iron-transferrin complexes (Andrews *et al*., 2003). This is a cue for many bacterial pathogens that they are in a vertebrate host and they start to express virulence factors, such as siderophores and associated outer membrane proteins (Klebba *et al.*, 1982). Siderophores are low-molecular weight molecules with a high affinity for iron that facilitate transport of Fe^{3+} across the cell membrane via outer membrane proteins (Köster, 2001). The synthesis of the siderophores is transcriptionally activated by an extra-cytoplasmic sigma factor FecI, the expression of which is repressed by the Fur protein (Visca *et al*., 2002). Bacteria also have a mechanism for accumulating iron during the lag phase to withstand iron shortage as a result of host sequestration in the later stages of growth (Bertrand, 2014).

Siderophores are categorised as catecholates, hydroxamates and carboxylates, based on the ligand used to chelate iron (Miethke and Marahiel, 2007). Their high affinity for ferric iron (Hider, 1984) has been exploited for isolation and identification of bacteria (Louden *et al*., 2011). The chrome azurol S (CAS) assay has been shown to be useful for quantifying production of siderophores (Schwyn and Neilands, 1987). The assay utilizes chrome azurol S and hexadecyltrimethylammonium bromide (HDTMA) as an indicator which complexes with iron to form a blue substrate on the CAS agar plates. When siderophores remove the iron from the complex, the indicator turns orange, which helps to quantify the level of siderophore production (Louden *et al*., 2011).

1.4.1. The TonB-ExbB-ExbD system

The TonB-ExbB-ExbD energy transduction system plays a key role in siderophore-mediated active iron transport across the cell membrane; TonB is cell membrane-anchored and ExbB and ExbD are cell membrane-embedded proteins (Miethke and Marahiel, 2007). Structurally, the TonB protein spans the periplasmic space, linking the outer and inner membrane components of the TonB dependent transporters (Evans *et al*., 1986). It is composed of N-terminal domain which forms an energy transducing-complex with the ExbB and ExbD proteins, a C-terminal domain, which makes contacts with the outer membrane proteins, and an intermediate domain (Pawelek *et al*., 2006). The Ton complex is composed of a pentamer of ExbB, a dimer of ExbD and one TonB (Celia *et al*., 2018). The TonB-dependent process is enhanced by ExbB, as it physically stabilizes TonB (Fischer *et al*., 1989). The protein is also an important energy source for the transport of phages across the outer membrane (Hancock and Braun, 1976). The essentiality of this protein for virulence has been demonstrated in avian pathogenic *E. coli* (APEC) (Holden *et al*., 2012), uropathogenic *E. coli* (Torres *et al*., 2001) and other Gram negative organisms, including *Pasteurella multocida, Pseudomonas aeruginosa* and *Salmonella* Typhimurium (Bosch *et al*., 2002; Takase *et al*., 2000; Tsolis *et al*., 1996).

Table 1.1 TonB-dependent transporters (TBDTs)

Gene	Function	Fur regulation	Reference
fhuE	Iron-coprogen uptake	Yes	Hantke, 1983
cirA	Colicin uptake	Yes	Buchanan *et al.*, 2007
fecA	Ferric-citrate uptake	Yes	Visca *et al*., 2002
fhuA	Ferrichrome bound iron transport	Yes	Noinaj *et al*., 2010
fepA	Enterobactin transport	Yes	Hunt *et al*., 1994
iroN	Salmochelin transporter gene	-	Baumler *et al*., 1998; Feldmann *et al*., 2007
iutA	Aerobactin transporter gene	-	Johnson, 1991

‘ - ‘ no evidence from a literature search that the gene is regulated by Fur

TonB-dependent iron transport genes (Table 1.1) are repressed by the Fur protein, as iron is toxic for cells when it is imported in excess (Noinaj *et al*., 2010). In the presence of ample iron in the bacterial cell, iron binds to Fur which, in turn, binds to the ‘Fur boxes’ in the

promoter regions of the iron-uptake genes to outcompete RNA polymerase and thus prevent transcription (Carpenter *et al*., 2009). This protects cells from production of iron-triggered oxygen radicals (Touati *et al*., 1995). Free radicals are capable of initiating DNA damage and are generated as a result of the Fenton reaction, in which hydrogen peroxide is formed through iron autoxidation (Loeb *et al*., 1988). The 'Fur box' has a consensus Fur-Fe^{2+} binding sequence of GATAATGATAATCATTATC, which is present in all Fur-regulated promoters (Hantke, 2001). This regulation is complemented by the RyhB sRNA, which downregulates iron storage and iron-using proteins when intracellular iron levels are limited and is itself under the control of Fur (Massé and Gottesman, 2002). It acts through RNase-mediated degradation of mRNAs from the genes encoding iron-using and iron-storing proteins (Massé *et al*., 2003).

1.4.2. The Fur regulon

Fur (ferric uptake regulator) is a 17 kDa protein, the N-terminal of which binds to DNA and the C-terminal of which interacts with metal ions, mainly Fe^{2+} (Coy and Neilands, 1991). It is a global iron-dependent regulator that mediates iron metabolism (Andrews *et al*., 2003). The *fur* gene is conserved across several bacterial species and uses the ferrous iron (Fe^{2+}) (Carpenter *et al.*, 2009) and manganese ion (Mn^{2+}) (Brickman *et al*., 1990) as co-factors. Fur influences the expression of more than 90 genes in *E. coli* (Andrews *et al*., 2003), 60 of which are genes associated with biosynthesis and transport of siderophores (Hantke, 2001). The control Fur exercises over many genes is direct, however it operates through the extra-

cytoplasmic sigma factor FecI in the case of the *fecA*, the ferric-citrate uptake gene (Visca *et al*., 2002).

The *fur* gene transcriptionally represses genes such as those encoding TonB (Postle, 1990), all the TonB-dependent ferric-siderophore transporters, the cytoplasmic membrane proteins ExbB and ExbD (Noinaj *et al*., 2010), and the metalloprotein superoxide dismutase A (Niederhoffer *et al*., 1990). It also represses RyhB, a 90 nt small RNA which, in turn, downregulates the expression of iron-using and iron-storage proteins in the event of iron scarcity (Massé and Gottesman, 2002). Fur also regulates the outer membrane adhesin Iha in O157:H7 EHEC (Rashid *et al*., 2006). However, the *acnA* gene, which encodes aconitase A, *fumA*, which encodes the aerobic iron-sulphur containing fumarase (Gruer and Guest, 1994), and *sodB*, which encodes the iron superoxide dismutase (Dubrac and Touati, 2000), are derepressed by Fur indirectly through the RyhB sRNA (Massé and Gottesman, 2002). The *fur* gene, itself, is autoregulated by its product and the catabolite-activator protein (CAP) (Lorenzo *et al*., 1988). Apart from iron metabolism, Fur also influences cellular processes such as acid tolerance (Hall and Foster, 1996), adhesion (Karjalainen *et al*., 1991), toxin production (Litwin and Calderwood, 1993), haemolysin secretion (Lebek and Gruenig, 1985) and bioluminescence (Makemson and Hastings, 1982).

1.5. The lambda Red system

The lambda Red recombination system can be used to replace the DNA sequence of a targeted gene in the *E. coli* genome with an antibiotic resistance marker gene using flanking homologous sequences generated by PCR (Yu *et al*., 2000). The method utilises an easily curable low copy number plasmid, pKD46, that carries the *bet*, *exo* and *gam* genes, which encode the Bet, Exo and Gam proteins, respectively (Datsenko and Wanner, 2000). The Bet (ssDNA binding protein) and Exo (dsDNA exonuclease) proteins promote the integration of the linear DNA generated by PCR into the *E. coli* genome, while the Gam protein protects the linear DNA against RecBCD and SbcCD nuclease activities, which would otherwise degrade the linear DNA (Sawitzke *et al*., 2007). The system has been used to make site-specific deletions, insertions and point mutations in a number of strains of *E. coli* (Datsenko and Wanner, 2000).

A number of models have been proposed to explain lambda Red recombination. Mosberg and colleagues suggest that the Exo protein degrades one strand of the linear PCR product, leaving the other strand intact and that recombination of this strand then occurs through Bet-mediated annealing with the lagging strand at the replication fork (Mosberg *et al*., 2010). In an earlier model, it was proposed that Exo degrades the linear DNA at both 5' ends leaving 3' overhangs which are then bound by Bet, which promotes annealing to complementary sequences in the genome (Yu *et al*., 2003), while Poteete argued that the incoming 3' end displaces the original template for the leading strand polymerase and a non-specified

nuclease makes a single-stranded cut, releasing a lagging strand arm as the replication fork continues in the same direction on its new template (Poteete, 2008).

1.6. Research aims

The aim of the studies described in this thesis were to investigate whether a *tonB* gene deletion would influence the expression of the Stx2e toxin, the TonB-dependent receptors and the fimbriae in the 16001 oedema disease strain. Oedema disease poses a significant challenge to the pig industry, as it occurs one to two weeks after weaning and targets the fastest growing groups of pigs (Imberechts *et al.*, 1992). Infections with *E. coli*, overall, are one of the driving reasons for the use of antimicrobials in the Australian pig industry (Jordan *et al.*, 2009) this use has the potential to escalate antimicrobial resistance, thus reducing the efficacy of antimicrobials in treatment of clinical disease in Australia and other countries (Hart *et al*., 2004; Uemura *et al*., 2003). Treatment of oedema disease is problematic because of the acute onset of the disease. While feed management practices, principally by restricting protein content in the feed, may help prevent the disease, this has an adverse effect on weight gain (Kim *et al*., 2012). Therefore, development of an effective vaccine that can be used by the pig industry to control this disease would have considerable impact on both animal health and production and on public health. The studies described here aimed to develop *tonB* deletion mutants to provide more insights into the association between Fe^{2+} levels and Shiga toxin secretion, TonB-dependent receptors and fimbriae expression and to serve as a platform for developing vaccine candidates for the control of oedema disease in the future.

2. Deletion of the *tonB* gene

2.1. Introduction

Deletion of *tonB* has been shown to reduce intracellular iron levels and hence growth and infectivity in avian pathogenic *E. coli*. The lower intracellular iron levels trigger production of siderophores, outer membrane receptor proteins and other virulence factors that are likely to elicit a protective humoral immune response (Hantke, 2001; Holden *et al*., 2012). The deletion of this gene in the 16001 porcine STEC strain might be expected to have a similar effect.

2.2. Materials and Methods

2.2.1. Bacterial strains, culture conditions and reagents

The 16001 STEC strain of Oedema disease isolate was obtained from the Department of Economic Development, Jobs, Transport and Resources (DEDJTR) on October 19, 2016. APEC strains E3 and E956 (Ginns *et al*., 2000) were used as a source of intact *tonB* gene sequences. The *E. coli* strains were cultured on Luria-Bertani (LB) agar or CAS agar (Louden

et al., 2011), or in LB broth or minimal medium 9 broth at 37 ºC for 18-24 h unless stated otherwise. The OD_{600} of the cultures was measured using an Eppendorf BioPhotometer. Kanamycin, ampicillin and chloramphenicol were used at a concentration of 40 µg/ml, 100 µg/ml and 12 µg/ml, respectively.

2.2.2. Antimicrobial susceptibility test

The Calibrated Dichotomous Sensitivity (CDS) test (Bell, 1975) was employed to test whether the field isolate had developed resistance to antimicrobial drugs. Sensitest agar was stored at 4 ºC and dried at 37 ºC for 2 h immediately before use. A colony of the 16001 WT was touched with an inoculating wire and the organisms transferred to 2.5 ml of sterile normal saline. The whole surface of the Sensitest plate was covered with the bacterial suspension and after excess suspension was removed the plate was allowed to dry. Kanamycin (30 µg), chloramphenicol (30 µg), ampicillin (25 µg), and ceftiofur (30 µg) discs were placed on the plate and it was incubated overnight at 37 ºC. The *E. coli* NCTC 10418 strain was included in the test as a quality assurance standard.

2.2.3. DNA isolation and quantitation

Plasmid DNA (Table 2.2) was extracted using the Wizard Plus SV DNA Purification System (Promega) according to the manufacturer's instructions. DNA purity and concentration was measured using a NanoDrop Spectrophotometer ND-1000 and Qubit 3.0 Fluorometer. PCR products were purified using an MO Bio Ultraclean PCR clean-up kit or a QIAEX® II Gel Extraction kit according to the manufacturer's instructions.

2.2.4. PCR

PCR was used to amplify the *tonB, fur, stx2e, fed, cirA, fepA, fur, fhuE, fhuA, iroN, fyuA* and *iutA* genes using the primer pairs listed in Table 2.1. The forward and reverse primers for the *stx2e* (M21534) and *fed* (Z26520) genes *were* designed based on *E. coli* K-12 and *E. coli* strain 107/86 sequences (NC_000913.3 and Z26520) obtained from Genbank using Geneious 7.1.9 (Java Version 1.7.0_51-b13). PCR reactions contained 1 × Green Go *Taq* Flexi Buffer (Promega), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of each primer, 1.25 U Go *Taq* polymerase (Promega) and ddH_2O to a total volume of 23 µl. Template DNA (2 µl) was added to each reaction. Template DNA was plasmid DNA, an overnight LB broth culture or an *E. coli* colony resuspended in 20 µl of ddH_2O. Wild type APEC strain E3 and E956 (Ginns *et al*., 2000) positive controls and no-template negative controls were included in all PCR reactions.

2.2.5. Agarose gel electrophoresis

PCR amplicons were separated in 1% agarose gels at 4.5 V/cm for 1-2 h, and plasmid DNA was separated in 0.7% agarose gels at 1.25 V/cm for 5-6 h. A HyperLadder^{TM} 1kb DNA (Bioline) ladder was used for molecular size markers in all the PCRs.

2.2.6. Sequencing

Reactions were sent to the Australian Genome Research Facility (AGRF) for Sanger sequencing. Each 20 µl sequencing reaction contained 1.5 µl of BDTV3.1 terminator, 1 × BDT dilution buffer, 5 µM primer and 200-300 ng template DNA. The DNA cycle sequencing was performed using a BioRad T100 Thermal Cycler with reactions incubated at 96 °C for 1 min and then through 30 cycles of 96 °C for 10 secs, 50 °C for 5 secs and 60 °C for 4 min. The resultant DNA sequence was compared with a reference using Geneious 7.1.9. BLAST, at https://blast.ncbi.nlm.nih.gov/, was then used to compare the resultant DNA sequences with those in the GenBank database.

Table 2.1 Primers used in this study

Primers	Sequence	Reference
TonBF1	AGCTGCTTCCAGCGACTCAA	Holden *et al*., 2012
TonBR1	CCGCCGATACCAATGCCAAT	Holden *et al*., 2012
KchΔtonBF1	CGGAACAGTAAGCCAATGGA	Holden *et al*., 2012
YciBΔtonBR1	GGTCGTCTTCGGTGGCTTGA	Holden *et al*., 2012
CATF1+	GCATCTCGAGACGCACTTTGCGCCGAATAA	Holden *et al*., 2012
CATR1+	CCTCGGCTCGAGGAGTTGGTAGCTCAGAGAA	Holden *et al*., 2012
FedF	ATGCGTTTAAAATATATCTT	This study
FedR	TTACTGTATCTCGAAAACAA	
FurF1	CGCACCATTCACCATCTTCT	Holden *et al*., 2014
FurR1	AACTGACCGCTGAACGTGTA	
IutAF	AGTAACGGGCTGAATTCCG	Holden *et al*., 2014
IutAR	GGAGGTAAAGCGCTCATGAT	
FhuEF	ATTGTCGGCGGCTACCAGAA	Holden *et al*., 2014
FhuER	ACGTGCGCCGAGGATCAG	
FecAF	ATTTACCCTCTCGGTTGACGCCAG	Holden *et al*., 2014
FecAR	GATAGAGCACGTTCAACGCCGGA	
CirAF	TACGGTGAACACTGGAGTCCGC	Holden *et al*., 2014
CirAR	GGTTTCCACGCCCTGAATACG	
FyuAF	AGTAAGTTCAACCTGAGCGG	Holden *et al*., 2014
FyuAR	ACTGCTTAAATAAGGCATTGTCGG	
FhuAF	ATTGTCGGCGGCTACCAGAA	Holden *et al*., 2014
FhuAR	ACGTGCGCCGAGGATCAG	

FepAF	TCCGCTTGTATGGCAACCTCGA	Holden *et al*., 2014
FepAR	TCAGCTCCATGTTGTTTTCGGCAA	
IroNF	TAACATTGAGCCGGTTCCTGGCACGA	Holden *et al*., 2014
IroNR	TGGACAGCCATTGCCTTTCGAGT	
Stx2eF	GGCGGTCCATTATCTGCATCAT	This study
Stx2eR	CAAAGCCTGAGCCTGAACTG	

Table 2.2 Plasmids used in this study

Plasmids	**Description**	**Reference**
pKL1	pGEM-T Easy vector containing a 1.533 kb fragment encoding *tonB* from APEC strain E3	Holden *et al*., 2012
pinvKL1	PCR product of pKL1 generated with primers invtonBF1+ and invtonBF1+, digested with *Xho*I and then self-ligated	Holden *et al*., 2012
pKL1Km	pinvKL1 containing kanamycin resistance cassette	Holden *et al*., 2012
pCNS	Plasmid carrying chloramphenicol resistance gene	Holden *et al*., 2012
pKD46	Lambda Red helper plasmid	AY048746

Table 2.3 PCR conditions used to amplify the genes of interest

Gene/PCR product	**Primers**	**Expected size (bp)**	**Annealing Temperature (ºC)**	**Extension time (s)**
Chloramphenicol resistance gene (*cat*)	CATF1+ and CATR1+	1100	41	55
tonB	tonBF1 and tonBR1	1533	56	110
fed	FedF and FedR	903	41	60
Linear DNA fragment (pKL1km)	tonBF1 and tonBR1	2020	56	110
kch and *yciB*	kchΔtonBF1 and yciBΔtonBR1	2700	56	110
stx2e	Stx2eF and Stx2eR	969	57	60
*iut*A	IutAF and IutAR	294	55	60
*fhu*E	FhuEF and IutAR	753	55	60
*fec*A	FecAF and FecAR	1433	58	120
*cir*A	CirAF and CirAR	432	55	60
*fyu*A	FyuAF and FyuAR	585	55	60
*fhu*A	FhuAF and FhuAR	355	55	60
*fep*A	FepAF and FepAR	696	55	60
*iro*N	IroNF and IroNR	217	55	60

2.2.7. Ligation

Ligation reactions contained 1 × T4 DNA ligase reaction buffer, 100-120 ng of vector DNA, 80 ng of insert DNA, 400 U of T4 DNA ligase and ddH_2O to a total volume of 20 µl. The reaction was incubated at 4 °C overnight and heat inactivated at 65 °C for 10 minutes.

2.2.8. Electrocompetent cells

Electrocompetent cells were prepared as previously described (Datsenko and Wanner, 2000). An overnight culture of WT 16001 *E. coli* was chilled on ice for 10 mins and aliquoted into 1.5 ml microfuge tubes. The cell suspensions were centrifuged at 4000 g for 10 mins at 4 °C. The pellet was resuspended in 1 ml of ice-cold 10% glycerol and again centrifuged for 10 mins at 4000 g and 4 °C. The resultant pellet was resuspended in 50 µl of ice-cold 10% glycerol and maintained at -80 °C.

2.2.9. Lambda Red mutagenesis

The lambda Red recombineering system was employed to knock out the *tonB* gene from a wildtype 16001 porcine STEC isolate and replace it with the kanamycin resistance gene

cassette (Datsenko and Wanner, 2000; Holden *et al*., 2012). Electro-competent 16001 cells were transformed with the Red helper plasmid pKD46 (Table 2.2) using a Bio-Rad electroporator set at 2.5 kV, 200 Ω and 25 μF, recovered in 1 ml of LB broth and incubated at 30 °C for 1 h with shaking at 220 rpm. The culture was spread on LB agar containing 100 μg ampicillin/ml and incubated at 30 °C overnight. The presence of pKD46 was confirmed by purifying the plasmid from an overnight culture of pKD46 transformed 16001 using the Wizard Plus SV DNA Purification System (Promega). LB broth was inoculated from a colony of 16001 strain containing pKD46 and the culture incubated at 30 °C until it reached an OD_{600} of 0.27. The Red system was then induced using 100 mM L-arabinose and the culture then incubated further until the OD_{600} reached 0.89. The cells were then chilled on ice for 10 minutes, centrifuged at 5000 g for 10 minutes to remove the medium and washed with 10% glycerol at 5000 g for 10 minutes, before resuspending the cells in 50-100 μl of 10% glycerol. A linear DNA fragment (570 ng) containing homologous regions of the *tonB* gene and the kanamycin resistance gene cassette was amplified from plasmid pKL1Km using the primers tonBF1 and tonBR1 and purified using the MO Bio Ultraclean PCR clean-up kit. The ~2 kb fragment was introduced into the cells by electroporation as described above. A 1 ml volume of LB broth was added to the electroporated cells and they were allowed to recover for 4 h at 37 °C. The suspension was spread onto LB agar containing 40 μg kanamycin/ml and the plate was incubated at 37 °C for 24-48 h. Colonies were screened for the *tonB* deletion by PCR using the primers KchΔtonBF1 and YciBΔtonBR1 and the conditions described in Table 2.3.

2.2.10. Growth characteristics in an iron-replete medium

The 16001 WT and the *tonB* mutant were inoculated into separate 3 ml volumes of LB broth and incubated overnight. The overnight culture was subcultured into 10 ml LB broth and the initial OD_{600} was measured. The culture was then incubated at 37 °C with shaking and the OD_{600} was measured 4 times at intervals of 20 mins. This was repeated three times.

2.2.11. Culture on chrome azurol S (CAS) agar

The CAS agar assay was performed as described previously (Louden *et al*., 2011; Schwyn and Neilands, 1987). All glassware was cleaned with 6 M HCl and rinsed with deionised water to remove any trace of iron. Only deionised water was used throughout the process. Minimal medium 9 (MM9) broth was made as described previously (Murugappan *et al*., 2011). Kanamycin was included in the MM9 broth used to culture the mutants. The MM9 broth was inoculated with the *tonB* deletion mutants and the wildtype 16001 and the cultures incubated overnight at 37 °C. The CAS plates were streaked with the overnight culture to compare the halo zone between the wildtypes and the mutants.

2.3. Results

2.3.1. Antibiotic susceptibility test

The inhibition zones around the discs were measured and those around the kanamycin, chloramphenicol, ampicillin and ceftiofur discs had annular radii of 7.5, 11, 7 and 10 mm with the 16001 WT strain and 8, 11.5, 8.5 and 11 mm with the *E. coli* NCTC 10418 control strain. A zone of inhibition with an annular radius of 6 mm was considered the cut-off. The annular radius of zone of inhibition for the control strain was confirmed to lie within the acceptable range (Bell *et al*., 2006). Based on this, the WT strain 16001 was susceptible to all four antibiotics tested – kanamycin, chloramphenicol, ampicillin and ceftiofur.

2.3.2. Detection of virulence and iron metabolism genes

The porcine STEC isolate was shown to carry the *tonB*, *fur*, *stx2e* and *fed* genes, with products of 1533, 1296, 969 and 903 bp obtained in PCRs using the primers listed in Table 2.1 and shown in Figure 2.1. All the positive controls were positive for the genes tested and the no-template controls were negative.

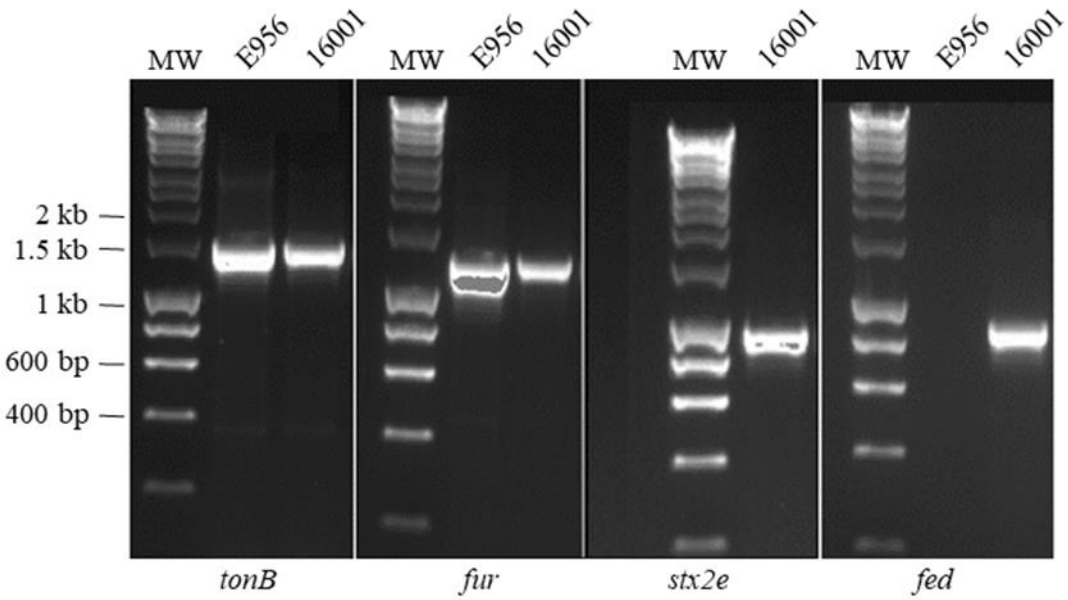

Figure 2.1 PCR amplification of *tonB*, *fur*, *stx2e* and *fed* sequences from the 16001 porcine STEC strain.

MW: Bioline 1 kb hyperladder molecular weight marker. APEC strain E956 used as a positive control. The assays in the 3rd panel (from the left) were performed without a positive control as it was difficult to obtain other *stx2e* carrying *E. coli* strains.

The 16001 WT was also shown to carry sequences for five TonB-dependent outer membrane receptors, which transport iron-siderophore complexes, out of the eight tested for. It was positive for *cirA* (ferric dihydroxybenzoylserine), *fepA* (enterobactin), *fhuA* (ferrichrome), *fhuE* (ferric-rhodotoluric acid and ferri-coprogen) and *fecA* (ferric di-citrate) and negative for *fyuA* (yersiniabactin), *iroN* (salmochelin) and *iutA* (aerobactin) (Fig. 2.2). The E956 APEC strain was used as positive control. The *fecA* was tested separately (the far right panel) because of the higher annealing temperature of the primers.

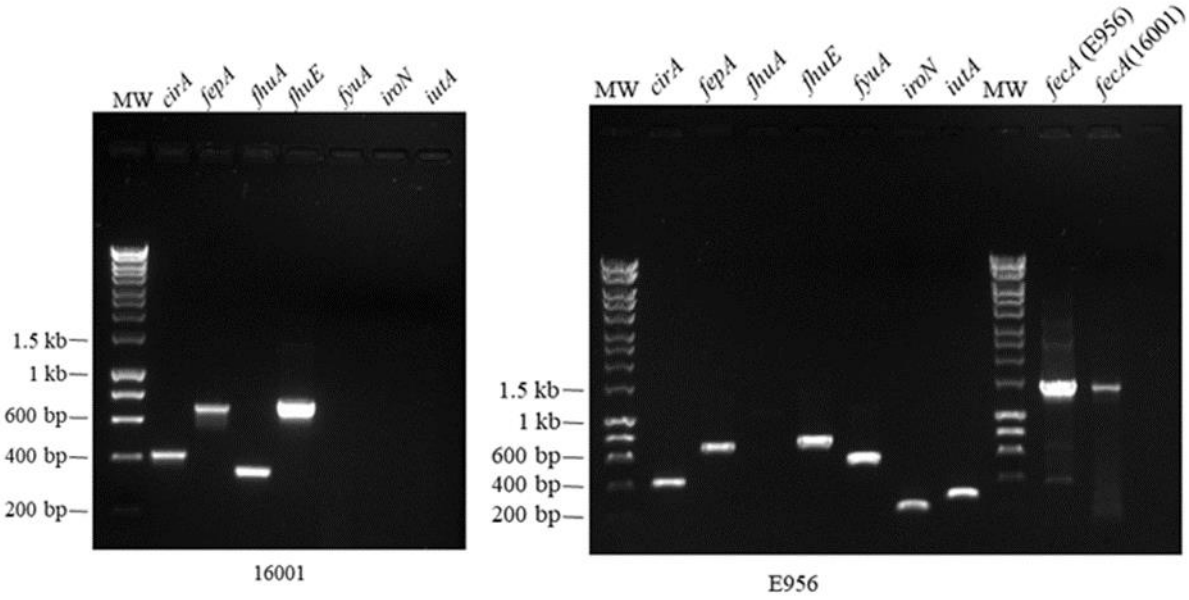

Figure 2.2 Detection of genes encoding TonB-dependent outer membrane receptors in the porcine 16001 STEC strain. E956, APEC strain (Holden *et al.*, 2014) used as a positive control.

2.3.3. Mutagenesis of *tonB*

The porcine 16001 STEC isolate served as a parental strain to develop a *tonB* deletion mutant using the lambda Red recombination system. The *tonB* gene was successfully deleted from the genome of the wild type porcine 16001 STEC strain. A PCR reaction using primers KchΔtonBF1 and YciBΔtonBR1, which annealed to sequences either side of *tonB* (Table

2.1), and the porcine 16001 STEC wild type or the candidate 16001ΔtonB:km knock-out mutant as templates revealed a size difference between the two strains in the targeted region, indicating insertion of the kanamycin resistance gene cassette in the mutant (Fig. 2.3). A 455 bp portion of the gene was confirmed to have been deleted by sequencing the PCR products. Five attempts to delete *fur* using the same approach were unsuccessful.

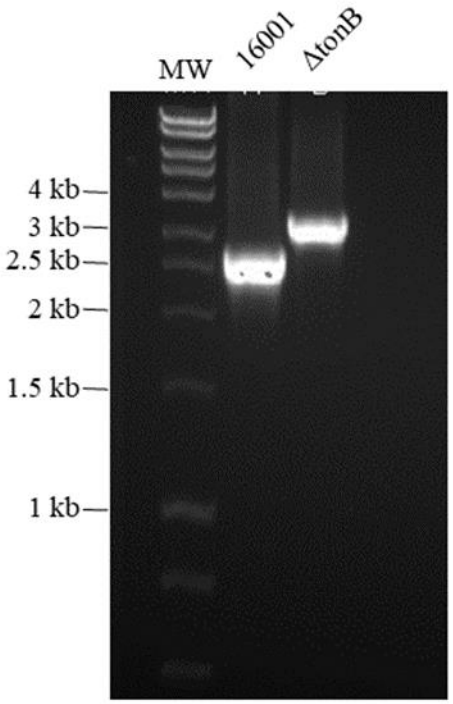

Figure 2.3 PCR confirmation of *tonB* deletion in the 16001. ΔtonB is the *tonB* deletion mutant of the 16001.

2.3.4. Growth characteristics on iron-replete medium

The *tonB* mutant colonies appeared smaller than the WT on LB agar (Fig. 2.4).

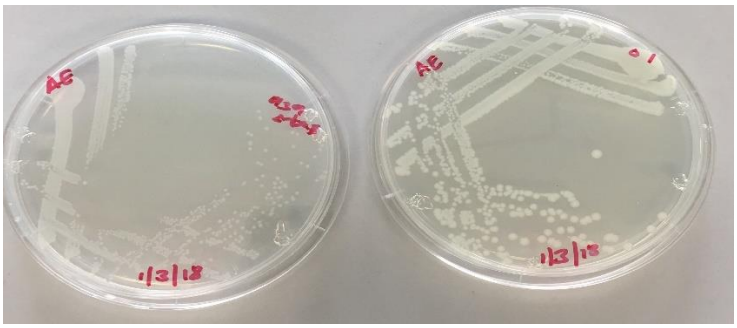

Figure 2.4 Comparison of colony characteristics between the WT (right) and the mutant (left) on LB agar.

The growth rates of the mutant and the WT were also compared in LB broth. The OD_{600} was measured in three independent experiments and the data were analysed using Prism (GraphPad Software). The mutant grew slower than the wild type, as shown in Fig 2.5.

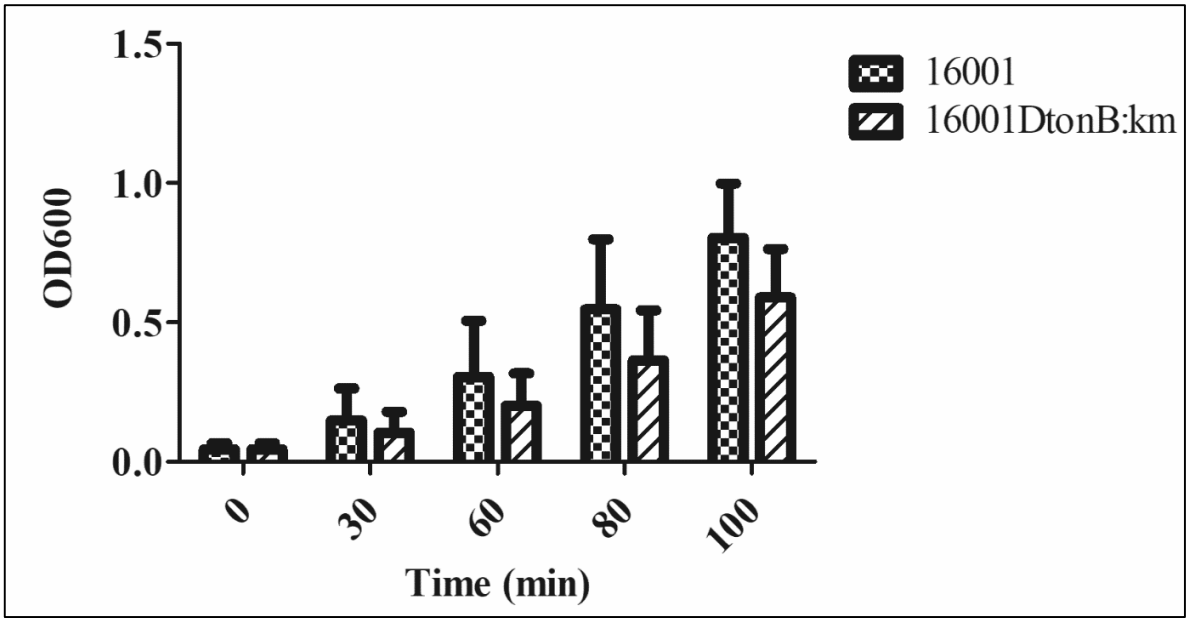

Figure 2.5 Graph showing growth characteristics of the 16001ΔtonB:km mutant and the 16001 WT in an iron-replete medium. The mean and standard deviation for three replicate experiments are shown.

2.3.5. Growth on CAS agar

The *tonB* mutant was phenotypically characterised and compared to the wildtype by examining its growth on CAS agar. The *tonB* mutant generated a larger orange halo than the wildtype on CAS agar (Fig. 2.6), indicating that it produced greater concentrations of siderophores.

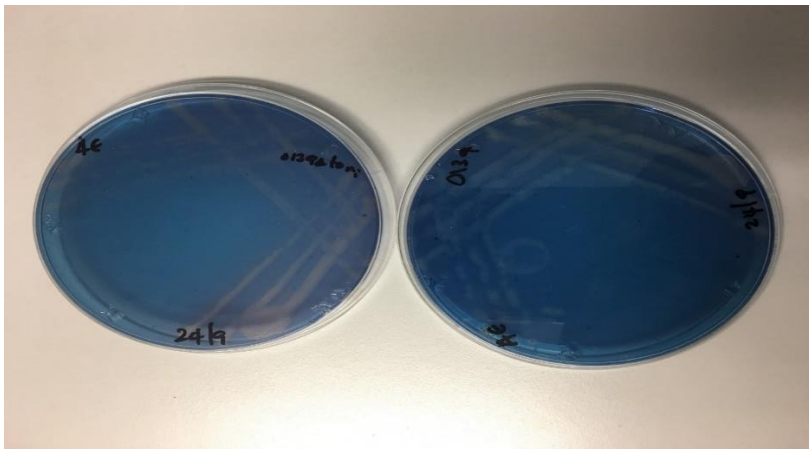

Figure 2.6 Growth characteristics on CAS agar Right: 16001 WT. Left: 16001ΔtonB:km (knock-out mutant).

2.4. Discussion

The studies described in this chapter generated a *tonB* deletion in the porcine 16001 STEC strain using the lambda Red recombination system to develop an iron transport deficient mutant.

The first swine vaccine targeted against erysipelas was created by Pasteur and Thuillier in 1882, followed by one for *Salmonella* in 1886. This pursuit for protection of the infectious diseases of swine also opened a door to a new kind of inactivated vaccine in other bacterial diseases (Haesebrouck *et al*., 2004). Control of oedema disease has historically relied on antimicrobial treatment and use of lower concentrations of protein in the feed (Kempf *et al*.,

2013). In recent decades, passive transfer of hyperimmune sera and active immunization have increasingly been used to control the disease. Vaccines for the disease started being developed in the 1990s, when pigs of various ages were actively immunised with SLT-IIv (Stx2e) toxoid and received immunoglobulin purified from porcine antisera against purified shiga-like toxin variant SLT-IIv (MacLeod and Gyles, 1991). A Shiga toxin with an A subunit in which the enzymatic activity has been suppressed by site-directed mutagenesis has been shown to induce protection against clinical cases (Bosworth *et al.*, 1996; Makino *et al*., 2001). A trial of passive immunization with Stx2e-specific antisera prepared by inoculating horses with Stx2e toxoid showed that passive immunization could be considered as a prevention option, however the incidence of adverse effects shortly after injection could limit its applicability (Johansen *et al*., 2000). A DNA vaccine combining the gene for Stx2e and the *fedF* gene has also been shown to be effective against oedema disease in mice (Ren *et al*., 2013). Maternal antibodies against Stx2e can be transferred to suckling piglets and have shown effective protection against oedema disease (Oanh *et al*., 2012). ECOPORC SHIGA is a genetically modified and recombinantly produced Stx2e commercial vaccine that has been found to be effective in a field trial (Fricke *et al*., 2015).

In Australia, a report from the Pork CRC in 2014 suggested that oedema disease was a problem during the 1980s and was controlled using live autogenous *E. coli* vaccines. In 2010 and 2011, it recurred in Australian pig farms, particularly in Queensland and Victoria, and was unresponsive to treatment. The report focused on passively immunising piglets for oedema disease using exogenous antibodies from horses administered with Stx2e toxoid. However, production of the wildtype toxin, to make the toxoid from, was problematic as no

assay was available to assess whether the protein extract from the bacteria contained sufficient Stx2e toxin. The exposure of piglets to live *E. coli*, recovered from sick pigs, as a vaccine was also ineffective.

In the past, the wild type toxin has been inactivated using various approaches, including glutaraldehyde (MacLeod and Gyles, 1991) and formaldehyde (Gordon *et al*., 1992), although the latter was unsuccessful. Plasmid DNA with a coding region of *stx2eB* and *FedF* as a DNA vaccination (Ren *et al.*, 2013) and site-directed mutagenesis (Bosworth *et al*., 1996; Makino *et al*., 2001) have also been used.

The establishment of kanamycin susceptibility in the 16001 strain was important as a kanamycin resistance gene cassette was used to generate mutants and kanamycin resistance was used for screening for these mutants. This study has once again shown that the Lambda Red system is a powerful tool for developing deletion mutants in *E. coli*.

Bacterial pathogens need iron for survival and infection (Andrews *et al*., 2003). However, this requirement is at odds with the insolubility of iron in the aerobic environments. Pathogens overcome this problem by excreting siderophores, which are capable of binding iron and facilitating its transport via dedicated outer membrane proteins (Köster, 2001). The TonB-ExbB-ExbD system is essential for generation of the necessary energy for this active process (Klebba, 2016) and therefore, it is not surprising that TonB is essential for virulence

(Holden *et al*., 2012). The approach used in this study was to delete *tonB* so that intracellular iron concentrations would be reduced, attenuating the organism while retaining its ability to elicit an immune response. Multiple attempts to delete *fur* were unsuccessful in this study, but it has been shown that the *tonB* deletion alone can sufficiently reduce the intracellular pool of iron in APEC to attenuate virulence sufficiently to allow a deleted strain to be safely used as a trial vaccine (Holden *et al*., 2014). The PCR and sequencing results demonstrated successful insertion of the kanamycin cassette into the *tonB* region. The colony characteristics, the retarded growth rate of the mutant in the iron-replete medium, and production of relatively higher amounts of siderophores by the mutant suggests a reduction in the intracellular iron levels as a result of the *tonB* deletion. The low intracellular iron level is anticipated to upregulate the iron regulon which consists of several genes expressed during iron scarcity. This includes, among many others, genes encoding siderophores and outer membrane receptors (Noinaj *et al*., 2010). The increased production of these proteins is expected to upsurge the amount of antigens available to elicit the humoral immune response.

3. Analysis of the effect of the *tonB* deletion on the expression of Stx2e, outer membrane receptors and the fimbriae in the 16001 STEC strain

3.1. Introduction

The TonB protein plays a vital role in the active transport of siderophore-bound Fe^{3+} by outer membrane proteins (Klebba, 2016) and the deletion of *tonB* has been shown to reduce the intracellular iron pool in this and another study (Holden *et al*., 2012). It has also been shown to be important for virulence in many other organisms (Hsieh *et al*., 2008; Takase *et al*., 2000; Torres *et al*., 2001; Tsolis *et al*., 1996). In the face of iron scarcity, such as that generated by the *tonB* deletion, the repression of a number of genes, including *stx1* in other STEC strains, by Fur is relieved (Calderwood and Mekalanos, 1987). Therefore, the aim of the studies described in this chapter was to determine whether the expression of *stx2e* and other genes in porcine STEC was influenced by the low intracellular iron levels generated by the *tonB* deletion.

3.2. Materials and Methods

3.2.1. RNA extraction

E. coli strains 16001 WT and 16001ΔtonB:km were grown in LB broth (containing 50 µg kanamycin/ml for 16001ΔtonB:km). The overnight cultures were further subcultured into LB broth at a 1:500 dilution and incubated until they reached an OD_{600} of 0.60 (~4.8×10^8 cells/µl). The RNAprotect Bacteria Reagent kit was used according to the manufacturer's instructions to lyse the bacterial cells. The RNeasy® Mini Kit (250) was used according to the manufacturer's instructions, with minor changes in the number of bacteria from which the RNA was extracted and the volume of lysozyme buffer used. The RNAs were stored at -80°C.

3.2.2. DNase treatment

The TURBO DNA-free™ kit (ThermoFisher Scientific) was used according to the manufacturer's instructions to ensure the RNA was free of DNA contamination. Four reactions were prepared for DNase digestion, including for the reverse transcriptase (RT) negative control. Each reaction contained 0.88× TURBO DNase buffer, 1 µl TURBO DNase and 6 µg (16001) or 3 µg (16001ΔtonB:km) of the RNA in a total volume of 28.5 µl. After

30 min incubation at 37°C, the enzyme was inactivated using 2.5 µl of the DNase inactivation reagent. The resulting RNA was aliquoted and stored at -80^{o}C until reverse transcription.

3.2.3. Reverse transcription PCR

The SuperScript^{TM} III First-Strand Synthesis System was used according to the manufacturer's instructions to prepare cDNA from the RNA samples. A total of four reactions were prepared. The primer annealing mixture contained 50 ng of random hexamers, 0.5 mM of each dNTP and 3.8 µg of the WT RNA and 2.2 µg of the mutant RNA. The mixture was incubated at 65°C for 5 mins and was immediately chilled on ice for 2 mins. This mixture was added to 1× First-Strand buffer, 5 mM DTT, 40 U RNaseOUT and 200 U SSIII RT enzyme, resulting in a total reaction volume of 20 µl. A 1 µl volume of RNase free water was used for the RT negative control samples instead of the SSIII RT enzyme. The reaction was briefly spun and incubated at 25°C for 10 mins, at 50°C for 50 mins and then at 85°C for 5 mins and immediately chilled on ice. The resulting cDNAs were stored at -20°C for further use.

3.2.4. Gene expression

The resulting cDNAs were used as templates in PCR assays to detect gene expression. The samples were tested for expression of the *stx2e*, *fur*, *fed*, *fepA*, *fhuE*, *fhuA* and *cirA* genes

using the primers listed in Table 2.1 and the PCR conditions shown in Table 2.3. The PCR master mix components were as described in Chapter 2. A 10 µl sample of the PCR amplicon was loaded onto a 1% agarose gel for electrophoresis, with the HyperLadder™ 1kb BIOLINE markers used to determine the molecular size of the products. The ChemiDoc™ XRS+ System and Image Lab (Version 5.2.1 build 11 Nov 2014, Bio-Rad Laboratories) were used to capture the gel images.

3.3. Results

The total amount of RNA extracted from strain 16001 was 6 µg and the amount extracted from strain 16001ΔtonB:km was 3 µg. After DNAse treatment 3.8 µg of RNA remained from strain 16001 and 2.2 µg from strain 16001ΔtonB:km.

The expression of seven genes, *fur*, *stx2e*, *fed*, *fepA*, *cirA*, *fhuE* and *fhuA*, was assessed (Figure 3.1 and 3.2) based on the appearance of the bands. Genomic DNA from the parent strain 16001 was used as a positive control. Expression of *fur* was not detected in either the WT or the mutant. Expression of the *stx2e*, *fed*, *fepA*, *cirA* and *fhuA* genes was detected in both the WT and the mutant, whereas *fhuE* expression was only detected in the mutant. The *stx2e* and *fed* genes appeared to be slightly more abundantly transcribed in the mutant than the WT. The *fepA* and *cirA* genes were considerably more abundantly expressed in the mutant than the WT. The *fhuA* gene appeared slightly more abundantly expressed in the WT than in the mutant. The no-template controls were negative.

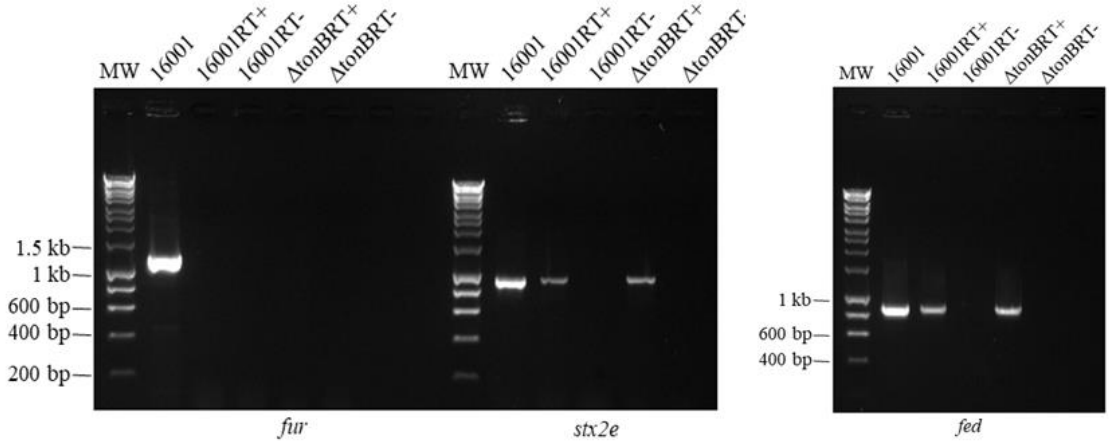

Figure 3.1 RT-PCR assays to detect *fur*, *stx2e* and *fed* mRNA in WT 16001 and the 16001ΔtonB:km mutant:

16001, WT 16001 DNA; 16001RT+, WT cDNA with reverse transcriptase (RT); 16001RT-, WT cDNA without RT; ΔtonBRT+, mutant cDNA with RT; and ΔtonBRT-, mutant cDNA without RT.

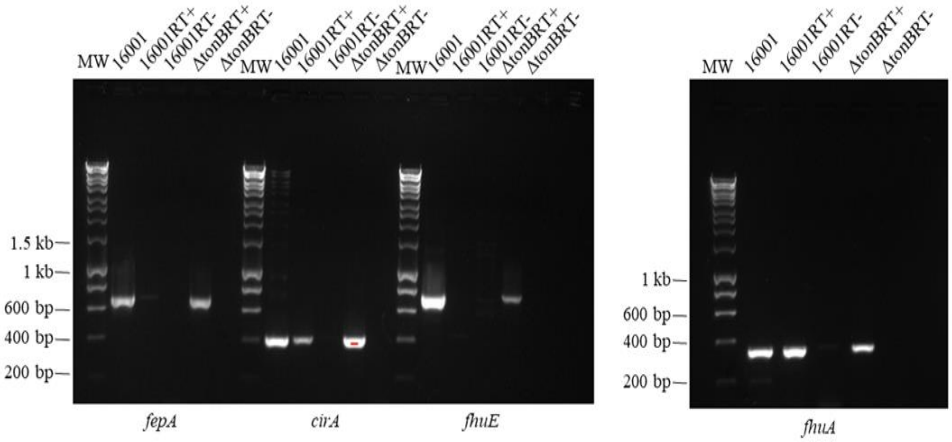

Figure 3.2 RT-PCR assays to detect *fepA, cirA, fhuE* and *fhuA* mRNA in WT 16001 and the 16001ΔtonB:km mutant:

16001, WT 16001 DNA; 16001RT+, WT cDNA with reverse transcriptase (RT); 16001RT-, WT cDNA without RT; ΔtonBRT+, mutant cDNA with RT; and ΔtonBRT-, mutant cDNA without RT.

3.4. Discussion

The study described in this chapter examined the influence of the *tonB* deletion on the expression of the *fur, stx2e, fed, fepA, cirA, fhuE* and *fhuA* genes. The aim was to establish whether there was any relationship between the intracellular iron levels and expression of these genes. Upregulation of these genes would suggest that this mutation would be a useful inclusion in a potential attenuated vaccine, as products of these genes might be expected to induce protective humoral immunity.

Pathogenic bacteria have an absolute need for iron for gene regulation, TCA cycle function and DNA biosynthesis (Ratledge and Dover, 2000). However uncontrolled influx is detrimental for cells, as they are exposed to free radical intoxication (Noinaj *et al*., 2010; Touati *et al*., 1995). The *fur* gene is the central regulator of iron import and utilization, regulating expression of a number of proteins, including siderophores (Hantke, 2001; Visca *et al*., 2002), transport receptors (Hunt *et al*., 1994; Noinaj *et al*., 2010), the Ton complex (Postle, 1990), iron storage proteins, and proteins that use iron (Massé and Gottesman, 2002), so that iron starvation and intoxication are controlled. When intracellular iron concentrations are high, Fe^{2+} serves as a co-factor with Fur to shut down the transcription of iron acquisition genes (Carpenter *et al*., 2009), whereas these genes are constitutively expressed in the absence of Fur (Hantke, 1981). Most oxygen metabolizing microorganisms respond to iron starvation by producing and excreting siderophores (Lankford and Byers, 1973), followed by their transport into the cells through their outer membrane receptors (Noinaj *et al*., 2010).

In the studies described in Chapter 2, the mutant was found to have colony characteristics and a growth rate indicating impaired growth as a result of the *tonB* deletion. This difference was also reflected in a lower total RNA yield from the mutant.

In the study described in this chapter, expression of the *fur* gene was not detected in the WT strain, which is probably attributable to its ability to suppress its own expression in the presence of iron by blocking binding of the sigma70 factor-dependent RNA polymerase on the *fur* promoter (Maeda *et al*., 2000; Tronnet *et al*., 2017). Its expression was not expected in the *tonB* deletion mutant, in which iron levels are low, as it is only expressed when iron levels are high (Carpenter *et al*., 2009).

Fur also regulates transcription of certain virulence factors that are not directly involved in iron metabolism, including the *stx* and *stx1* genes (Dubos and Geiger, 1946; Calderwood and Mekalanos, 1987; Weinstein *et al*., 1988a). However, unlike the *stx* and *stx1*, the *stx2e* promoter lacks sequences to bind Fur and hence its expression is not influenced by Fur (Sung *et al*., 1990; Weinstein *et al*., 1988b). Sung *et al* compared the level of Stx2 expression in low and high iron concentrations and detected no effect of low iron on the regulation of *stx2*, which has an identical promoter to that of *stx2e*. Furthermore, they showed that the *stx*, *stx1*, *stx2* and *stx2e* promoters performed in similar manner under low iron growth conditions. In the studies described in this chapter, *stx2e* appeared to be transcribed slightly more abundantly in the *tonB* deletion mutant, in which intracellular iron levels can be presumed to be low, than the parent strain.

In other *E. coli* strains, such as uropathogenic *E. coli* (UPEC) and enterotoxigenic *E. coli* (ETEC), iron has a repressive effect on the production of the fimbrial protein, an effect mediated by Fur (Karjalainen *et al*., 1991; Kurabayashi *et al*., 2016), and this is also the case for *Klebsiella pneumoniae* (Wu *et al*., 2012). Expression of an IrgA homologue adhesion (Iha), the adherence-conferring outer-membrane protein of an O157 EHEC strain, has also been shown to be repressed by Fur (Rashid *et al*., 2006). In the study of Karjalainen *et al*, addition of an iron chelator to the medium abolished the repressive effect of Fur and also significantly increased the activity of the fimbrial gene promoter. They also demonstrated that deletion of *fur* or iron depletion increased adhesion and invasion by UPEC because production of the fimbrial protein was increased (Kurabayashi *et al*., 2016). Similarly, the studies described in this chapter, found that transcription from *fed* product was more abundant in the *tonB* mutant, albeit slightly.

The *cirA* gene was shown to be carried by the 16001 strain in the study described in Chapter 2. It is one of the monocistronically transcribed TonB-dependent transporters (Noinaj *et al*., 2010) and its expression is repressed by Fur when intracellular iron levels are high (Griggs and Konisky, 1989). It is mainly involved in colicin transport (Buchanan *et al*., 2007), and its role in iron metabolism has not been defined (Griggs *et al*., 1987; Klebba *et al*., 1982; Neilands, 1982). It has been shown that CirA does not play a role in the establishment of infection in some *E. coli* strains (Garcia *et al*., 2011). The study described in this chapter showed that, as expected, *cirA* was expressed more abundantly in the *tonB* deletion mutant than the WT.

Enterobactin (enterochelin) is a cyclic triester of 2,3-dihydroxybenzolyserine (O'Brien and Gibson, 1970) that binds extracellular ferric iron for its transport across the outer membrane by FepA (Buchanan *et al*., 1999; Raymond *et al*., 2003). The enterobactin system requires the product of the *entD* gene for biosynthesis (Armstrong *et al*., 1989) and that of *fepA* for transport (Pettis *et al*., 1988). These genes are encoded on the same transcript and their expression is induced when the iron available to cells is depleted (Brickman *et al*., 1990; Fleming *et al*., 1983; Pettis *et al*., 1988; Shea and McIntosh, 1991). As might be expected, the study reported in this chapter found that *fepA* mRNA was more abundant in the iron-uptake deficient strain. Similarly, the minimal level of expression of *fepA* in the WT strain is in agreement with previous reports (Higgs *et al*., 2002). This is presumably due to the fact that the medium used in this study was iron-replete and thus that the WT bacteria did not face any shortage of iron.

FhuA is a TonB-dependent transporter of ferrichrome (Noinaj *et al*., 2010; Schoffler and Braun, 1989). In addition to ferrichrome transport, it is also involved in the uptake of peptide antibiotics, such as microcin, colicin and albomycin, and also bacteriophages (Bonhivers *et al*., 1996; Salomón and Farías, 1993). When intracellular iron is scarce, such as that generated by *tonB* deletion, the Fur-repressed Ryhb downregulates iron-storage and iron-using proteins to save iron for essential proteins (Massé and Gottesman, 2002). The *fhuA* gene has been shown to be downregulated upon over-expression of Ryhb in *E. coli* (Masse *et al*., 2005), and this may explain the less abundant transcription of *fhuA* in the *tonB* deletion mutant.

FhuE is another TonB-dependent transporter under the control of Fur (Chen *et al*., 2007). It mediates the transfer of iron complexed with coprogen and rhodotoluric acid (Sauer *et al*., 1987). Its expression under low iron conditions has been reported previously (Hantke, 1983; Tuntufye *et al*., 2012). In the study described in this chapter, expression of *fhuE* was not expressed at detectable levels in the WT strain, in contrast with other receptors, such as FepA and CirA. This gene was expressed in the *tonB* deletion mutant, suggesting that it was upregulated when intracellular iron concentrations were low.

In the studies described in this chapter, transcription of *stx2e*, and possibly *fed*, may be influenced by intracellular iron levels in a Fur independent manner. It is possible that previous studies on the influence of iron concentrations on *stx2e* expression did not expose the *E. coli* to the very low intracellular iron concentrations that result from deletion of *tonB*. The apparent increased transcription of *fepA* and *cirA* suggests lower intracellular iron levels. This is also supported by the higher siderophore concentration produced in the mutant than the WT which was described in Chapter 2.

4. General Discussion

The studies described in this thesis report the development of a deletion mutant in the Australian 16001 oedema disease strain of *E. coli*. The *tonB* deletion mutant was successfully generated as a first step in the development of an attenuated vaccine for this disease. The WT and the mutant were compared with regard to growth characteristics and *tonB* sequence length to confirm the mutation. Subsequent expression of the genes encoding the virulence factors Stx2e and the fimbrial protein Fed, as well as TonB-dependent receptors, was also analysed.

It is not debatable that vaccination is the most plausible approach to control many microbial diseases, as it minimizes morbidity and associated production loss and also reduces selection for antimicrobial resistance. Vaccines and drugs take advantage of the critical needs of organisms for nutrients and target the systems that are important for their uptake. Iron is one such nutrient required by pathogens to establish infection in their host (Andrews *et al*., 2003). Multiple systems work in harmony to meet the demand for iron, including the TonB system (Klebba *et al*., 1982; Miethke and Marahiel, 2007; Noinaj *et al*., 2010), which has previously been shown to be important for virulence in APEC (Holden *et al*., 2012).

Oedema disease is an economically important disease in piglets worldwide (Fairbrother *et al*., 2005). The disease reduces productivity through morbidity and, more importantly,

through high mortality, which sometimes occurs without clinical signs. Moreover, the time of the infection is one to two weeks after weaning, when pigs have lost most of the passively acquired immunity from their dam (Imberechts *et al*., 1992). The Australian pig industry mainly depends on antimicrobial drugs, rather than a vaccine, for control of this disease (Jordan *et al*., 2009) and this has contributed to growing antimicrobial resistance in pig pathogens (Smith *et al*., 2010; Hart *et al*., 2004). Vaccine-based approaches to control this disease include active, passive and maternal vaccination approaches (MacLeod *et al*., 1991; Makino *et al*., 2001; Oanh *et al*., 2012; Ren *et al*., 2013). However, these previous approaches have not been commercialised. Several deletion mutants have been generated in the laboratory and suggested as vaccine candidates, but have not been assessed in field trials. Oedema disease is prevalent in Australia, with losses of up to 20% seen in weaners. Work funded by the CRC for High Integrity Pork attempted to immunize piglets passively, but was unsuccessful. Recent outbreaks have not been controlled by live autogenous *E. coli* vaccines developed in the 1980s. Because Stx2e is absorbed into the circulation, active and passive immunization approaches to increase IgG levels have been thought to be likely to be successful.

This study aimed to generate a *tonB* deletion mutant of the Australian porcine STEC field isolate 16001 using lambda Red mutagenesis, which has been widely used to construct deletion mutants in different organisms (Thaler and Stahl, 1988; Yamamoto *et al*., 2009). The deletion of *tonB* is likely to reduce virulence by compromising the iron scavenging capacity of the strain. The low intracellular iron concentrations that resulted from the deletion are likely to boost production of outer membrane proteins that can enhance induction of IgG

against the iron transporters of this organism. This simultaneous enhancement of the immune response against multiple proteins is likely to have a synergistic effect and is likely to be more effective than targeting a single virulence factor. In the studies described here, the two key virulence factors, the toxin and the fimbriae, were overexpressed after deletion of *tonB* in the 16001 Australian porcine STEC strain. This may have resulted in an enhancement of virulence. The introduction of an additional mutation into the *tonB* mutant could help to circumvent this risk. Deletion of *stx2e* or *fed* might be expected to eliminate any risk that the *tonB* mutant had enhanced virulence (Bosworth *et al*., 1996; Makino *et al*., 2001; Ren *et al*., 2013), but might also reduce the immunogenicity of the strain. However, deletion of just the gene encoding the A subunit might be expected to result in a mutant that still overproduced the receptor binding component of the toxin, thus inducing antibody against the toxin, but with no capacity to cause disease.

A more effective vaccine against oedema disease is clearly needed by Australian pig farmers. This study is just a beginning in the long journey to development of a vaccine for this disease. The next step would be to proceed to an animal trial to evaluate the immune response to the wild type and the vaccine candidate. Quantitation of the level of expression of these genes would also be useful to further consolidate the results in this study. Further molecular level manipulations of iron metabolism and related genes could also be studied to ensure a better humoral immune response. Briefly, this study will serve as a foundation upon which future vaccine development work could be built.

5. References

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