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Secretion of Heat-Labile Enterotoxin by Porcine-Origin Enterotoxigenic *Escherichia coli* and Relation to Virulence

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SECRETION OF HEAT-LABILE ENTEROTOXIN BY PORCINE-ORIGIN ENTEROTOXIGENIC *ESCHERICHIA COLI* AND RELATION TO VIRULENCE

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SECRETION OF HEAT-LABILE ENTEROTOXIN BY PORCINE-ORIGIN ENTEROTOXIGENIC *ESCHERICHIA COLI* AND RELATION TO VIRULENCE

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Heat-labile enterotoxin (LT) is an important virulence factor secreted by some strains of porcine-origin enterotoxigenic *Escherichia coli* (pETEC). The prototypic human-origin strain H10407 secretes LT via a type II secretion system (T2SS), but its presence or importance in pETEC has not been established. Exposure of pETEC to glucose has been shown to result in different secretion levels of LT. Furthermore, the relationship between the level of LT secreted and the virulence potential of the respective pETEC strain has not been established. To determine the relationship between the capacity to secrete LT and virulence in wild-type (WT) pETEC, 16 strains isolated from cases of severe diarrheal disease were analyzed by monosialoganglioside-ELISA to measure LT concentrations in culture supernatants. All strains had detectable LT in culture supernatants; however, 3030-2, which was particularly virulent, had the highest LT level. Collectively, in gnotobiotic piglets inoculated with one of three strains varying in LT secretion level, *viz.*, 3030-2, 2534-86, or G58-1 , LT secretion correlated with 92% of the variation in time-to-a-moribund-condition ($R^2 = 0.92$, $P<0.0001$). Further examination of the 16 WT pETEC strains revealed the presence of genes for a T2SS. Bioinformatic analysis of 4 pETEC strains for which complete genomic sequences were

available revealed a T2SS with a high degree of amino acid sequence identity to that of H10407. Protein modeling of the ATPase gene (*gspE*) suggested a direct relationship between the predicted ATP-binding capacities and LT secretion levels. When grown in casamino acid-yeast extract medium with varying glucose levels (0, 0.25, 0.5, 1.0 or 2.0%), only media containing 0.25% glucose resulted in increased adherence and cAMP levels. Furthermore, LT^+ pETEC strains adhered to porcine epithelial IPEC-J2 cells more than LT^- strains. These studies support the hypothesis that glucose, at a concentration optimal for LT expression, enhances bacterial adherence through the promotion of LT production, and demonstrate a direct relationship between the predicted ATP-binding capacity of GspE and LT secretion, and between the latter and virulence. Hence, these results establish the physiological relevance of the effects of glucose on LT production, and provide a basis for how glucose intake may influence the severity of ETEC infection.

DEDICATION

This work is dedicated to my mother, Nandanee Wijemanne, and my father D. S. Wijemanne, who taught me the value of education and gave me all the opportunities, resources and support to fulfil my goals.

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CHAPTER 1

LITERATURE REVIEW

Enterotoxigenic *Escherichia coli* (ETEC)

Introduction

Escherichia coli is a Gram-negative, rod-shaped, non-sporulating, facultative anaerobe, belonging to the *Enterobacteriaceace* family. *E. coli* is a very diverse organism with regard to its living environments and the niches it occupies. *E. coli* strains may exist as mutualists, commensals or pathogens in both humans and animals, and may occupy intestinal and extraintesinal niches (Nataro & Kaper, 1998; Crossman *et al.*, 2010). *E. coli* also exist in external environments after being shed from the animal host. Harsh environmental conditions, which include such factors as nutrient deprivation, may force it to enter a "viable-but-nonculturable" (VBNC) state. In this state, *E. coli* is alive but undetectable by known laboratory culture methods (Ravel *et al.*, 1995). In certain tropical ecosystems, *E .coli* can be found as a member of the normal bacterial flora (Jimenez, 1989). These environments can support the growth of some specific saprophytic strains of *E. coli* depending on nutrient availability and temperature (Solo-Gabriele *et al.*, 2000; Power *et al.*, 2005).

E. coli is a normal inhabitant of the lower intestines of warm-blooded animals, birds and reptiles (Gordon & Cowling, 2003). The intestinal microbiome typically consists of more than 500 species of mostly anaerobic bacteria at concentrations of 10^{10} - 10^{11} cells per g, that are estimated to outnumber *E. coli* by 100:1 to 10,000:1 (Berg,

1996). The prevalence and density of *E. coli in vivo* depends on a number of characteristics, such as host body size, gut morphology, diet, digesta retention times, and native microbiota of the host intestine. As a result of these characteristics, the prevalence can vary from 0 to 100% and density by over 6 orders of magnitude among host species. Prevalence rates among humans, wild animals, birds and reptiles are >90%, 56%, 23% and 10% respectively (Mitsuoka & Hayakawa 1972; Penders *et al.*, 2006; Gordon & Cowling, 2003). The concentration per g of feces in human beings and domestic animals is typically 10^7 - 10^9 and 10^4 - 10^6 colony-forming units (CFU), respectively (Mitsuoka & Hayakawa 1972; Penders *et al.*, 2006; Slanetz & Bartley, 1957).

In the digestive tract, commensal or mutualistic *E. coli* strains are in greatest numbers in the large intestine, especially in the cecum and colon. They reside in the mucus layer that covers the epithelial cells throughout the intestinal tract, being shed into the lumen with the degraded mucus component and then excreted in the feces (Poulsen *et al.*, 1994). The mucus defines a nutritional ecological niche to which *E. coli* metabolism has adapted (Freter *et al.*, 1983). Strains that are isolated from the large intestine grow on nutrients acquired from mucus, including more than 7 mucus-derived sugars; gluconate seems to have a predominant role (Chang *et al.*, 2004). Although the concentrations of these sugars in the intestine are low (Peekhaus & Conway, 1998), *E. coli* maximizes its growth by using microaerobic and anaerobic respiration in the intestine (Jones *et al.*, 2007). This results in a 30-min generation time *in vitro* on intestinal mucus (Licht *et al.*, 1999) compared to 40-80 min in the intestines of streptomycin-treated mice, in which the cells in the luminal content are static (Poulsen *et al.*, 1994). The generation time is 120 min when the mice are ‘conventionalized’ by removing the streptomycin and feeding

them with mouse cecal content (Rang *et al.*, 1999). This change in growth rate in the presence of other species illustrates the fact that *E. coli* effectively competes with other microbial flora normally found in the intestinal tract. However, these interactions are complex and sometimes mutually beneficial, as *E. coli* may benefit from anaerobe-mediated degradation of mucosal polysaccharides and dietary fibers. Growth of these other anaerobes may also help by limiting the oxygen content of the intestine (Jones *et al.*, 2008).

E. coli is among the first bacterial species to colonize the intestine during infancy, reaching cell densities higher than 10^9 CFU per g of feces (Mitsuoka & Hayakawa, 1972; Penders *et al.*, 2006) before the expansion of anaerobes (Syed *et al.*, 1970). After two years of age in the human being, the *E. coli* levels stabilize and remain at around 10^8 CFU per g of feces until they gradually decrease in the elderly (Mitsuoka & Hayakawa 1972). The initial strains may originate from the maternal fecal microbiota and maternity nursing staff (Bettelheim & Lennox-King, 1976). In fact, increased hygiene in hospitals and families living in industrial countries has reduced early colonization by *E. coli* (Nowrouzian *et al.*, 2003; Jaureguy *et al.*, 2004).

The relationship between non-pathogenic *E. coli* and the host should be defined as mutualism, in which both organisms benefit from the interaction between them, or commensalism, whereby one (i.e., *E. coli*) benefits without an effect on the host. *E. coli* strains derive from their host a steady supply of nutrients (Conway *et al.*, 2004), a stable environment, and protection against some stresses, as well as transport and dissemination. Colonization of the gastrointestinal tract by non-pathogenic *E. coli* usually also benefits the host, preventing colonization of pathogens through competitive exclusion, which is

effected by bacteriocins and other products and mechanisms (Rastegar *et al.*, 1990; Vollaard *et al.*, 2004; Hudault *et al.*, 2001).

Phylogenetic groups of *E. coli*

E. coli isolates found in human or animal hosts, whether commensals, mutualists or pathogens, can be assigned into one of four main phylogenetic groups (Herzer *et al.*, 1990). The phylogenetic grouping is determined by multilocus enzyme electrophoresis or ribotyping, to infer or ascertain the presence or absence of genetic markers *chuA*, *yjaA,* and DNA fragment TspE4.C2 (Clermont *et al.*, 2000; Carlos *et al.*, 2010). There are four main phylogenetic groups, A, B1, B2 and D, which are further subdivided into seven subgroups, A_0, A_1, B1, $B2_2$, $B2_3$, D_1 and D_2. Isolates that belong to different phylogenetic groups have distinct ecological niches, life-cycles, abilities to exploit different sugar sources, antibiotic-resistance profiles, growth rates and virulence factors (Gordon & Cowling, 2003; Gordon, 2004).

Pathogenic *E. coli*

Even though mutualistic or commensal *E. coli* are usually sequestered within the intestinal lumen, given the opportunity, they may become pathogenic. Examples of conditions which give rise to disease include a compromise in host immunity or intestinal barriers. However, most cases of disease that originate in healthy humans and animals are caused by *E. coli* strains that have acquired virulence factors enabling them to become primary pathogens. These primary pathogens have been classified into several highly adapted *E. coli* pathovars. Pathogenic *E. coli* infections may be limited to the

mucosal surfaces or disseminated systemically through the body, resulting in three general clinical syndromes, including urinary tract infection, sepsis or meningitis, and diarrheal disease. A broader categorization is diarrheagenic and extraintestinal clinical syndromes, in which eight pathovars have been described. The diarrheagenic pathovars include enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC). The two extraintestinal pathovars are uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC).

Pathovars whose mechanisms have not been adequately elucidated include necrotoxigenic *E. coli* (NTEC), cell-detaching *E. coli* (CDEC), and adherent-invasive *E. coli* (AIEC) (Kaper *et al.*, 2004). NTEC, isolated from human urinary tract infections, and CDEC, which cause diarrhea in children, both secrete cytotoxic compounds. AIEC is found in 36% of patients with Crohn's disease, but has not been established to be the cause of this disease (Rolhion & Darfeuille-Michaud, 2007).

Pathogenic *E. coli* usually arise when a normal commensal strain acquires extrachromosomal DNA containing virulence genes, *e.g.*, plasmids or bacteriophage, or chromosomal virulence operons (Crossman *et al.*, 2010). Other mechanisms by which a pathogenic *E. coli* strain may arise are 'black hole' gene deletions that directly render the organism pathogenic (Maurelli, 2007) or random point mutations that help strains adapt to pathogenic environments (Duriez *et al.*, 2001). The loss and gain of mobile genetic elements has a pivotal role in shaping the genomes of pathogenic bacteria. Horizontal gene transfer (HGT) is an important mechanism that rapidly disseminates new traits to recipient organisms. Acquiring these new traits is crucial in promoting the fitness and

survival of a pathogen while it co-evolves with its host (Shames *et al.*, 2009). Large groups of pathogenetically related groups of virulence operons, called pathogenicity islands (PAIs), are carried on plasmids or the chromosome in pathogenic bacteria, but are not found in non-pathogenic bacteria. Traits acquired by HGT may allow the recipient bacterium to colonize a new niche and survive selective pressures. ETEC obtain the ability to produce enterotoxins when they acquire large plasmids called Ent (Smith & Halls, 1968; Skerman *et al.*, 1972). These plasmids carry genes for either or both LT and ST, and are acquired by conjugal transfer from other bacteria. In addition, they carry genes for adhesins that mediate attachment to intestinal epithelial cells (Smith *et al.*, 1983; McConnell *et al.*, 1981).

A typical *E. coli* isolate has approximately 2,200 genes that can be considered its core, but may have a total of >13,000 genes (Rasko *et al.*, 2008; Touchon *et al.*, 2009). Non-pathogenic *E. coli*, *e.g.*, K-12 and related strains, have considerably fewer genes than their pathogenic counterparts, the latter which usually contain >5,000. Most genes in pathogenic strains are non-core, which means they were acquired from different sources (Lloyd *et al.*, 2007; Iguchi *et al.*, 2009; Maurelli, 2007). The number of genes in different pathovars varies greatly. For example, *E. coli* O157:H7, the prototype of EHEC, contains a greater number of genes than strains of the UPEC pathovar (Croxen & Finlay, 2010).

Characteristics of ETEC

ETEC infection results in an acute onset of symptoms, with the most notable manifestation being watery, non-bloody diarrhea. The severity of diarrhea can range from mild and self-limiting to severe and purging, and in the latter cases are frequently fatal in

both humans and animals. There are an estimated 200 million cases of ETEC related diarrhea in the world, mostly in developing countries. ETEC is a common cause of mortality among children under 5 years of age in the developing world (Wenneras & Erling, 2004), and the main cause of traveler's diarrhea among adults in developing countries (Nataro & Kaper, 1998).

ETEC produce two main types of virulence factors, *viz.*, adhesins and enterotoxins. When an ETEC bacterium encounters an intestinal epithelial cell, it expresses adhesins that are often encoded on plasmids, *e.g.*, EtpA in some strains, which mediates initial attachment to the host. This is followed by the expression of other adhesins, sometimes encoded on the chromosome, which effect a more permanent attachment (Roy *et al.*, 2008; Roy *et al.*, 2009, Fleckenstein *et al.*, 2010). These events are followed by the production and secretion of enterotoxins that are delivered to the host cell by direct delivery (Dorsey *et al.*, 2006) or through encasement in outer membrane vesicles (Kesty *et al.*, 2004). In either case, the enterotoxins are the main direct cause of net fluid loss from the intestine (Dubreuil, 2012; Fleckenstein *et al.*, 2013).

There are more than 20 antigenically diverse, proteinaceous fimbrial colonization factors (CF) that are important in the colonization of the host intestine by ETEC (Nataro & Kaper, 1998). Among ETEC that colonize humans, CF include colonization factor antigen (CFA) and coli surface antigen (CS), the latter also known as putative colonization factor (PCF). CFA/I, CFA/II, and CFA/IV51 are expressed by approximately 75% of human ETEC isolates (Blanco *et al.*, 1993; Wolf, 1997). Colonization by ETEC is mitigated by anti-CF antibodies that are produced as a result of the host adaptive immune response (Gaastra & De Graaf, 1982; Sun *et al.*, 2000).

Expression of colonization factors unique to animal ETEC isolates is an important step in the progression of diarrheal disease in animals (Fleckenstein *et al.*, 2013). The most common fimbrial intestinal CF expressed by animal isolates, particularly those of porcine origin, are F4 (K88) and F5 (K99), which have not been found in human ETEC strains (Mol & Oudega, 1996; Jin & Zhao, 2000). Animals that are receptor positive for these CF, such as those with the F4 receptor, intestinal mucin-type sialoglycoprotein (IMTGP), are particularly susceptible to disease upon infection with the respective adhesin (Erickson *et al.*, 1992; Grange *et al.*, 1998; Francis *et al.*, 1998).

Enterotoxins produced by ETEC can be classified based on their thermal stability. There are two main types of enterotoxins, heat-labile (LT) and heat-stable (ST). Since the genes are usually carried on plasmids, it is not uncommon for a particular isolate to produce more than one enterotoxin (Kaper *et al.*, 2004; Croxen & Finlay, 2010).

Enterotoxin plasmids of ETEC

Ent plasmids carrying the genes for LT and ST were first identified in 1972 in the human isolate H10407 (Skerman *et al.*, 1972). These large plasmids are present in both human and animal isolates, and vary from 94-117 kb. These plasmids encode for approximately 100 bacterial proteins that average about 235 amino acids in length; the plasmids are conjugatable and efficiently self-transmitted to compatible *E. coli* strains (Gyles *et al.*, 1974; Dallas *et al.*, 1979; Mazaitis *et al.*, 1981). In addition to the enterotoxin genes, Ent plasmids typically carry genes for conjugation and resistance to antimicrobics such as tetracycline, streptomycin, sulfonamides and mercury, among others (Gyles *et al.*, 1978; Mazaitis *et al.*, 1981). It has been hypothesized that these

plasmids may have arisen by either recombination between plasmids of incompatibility group I (IncFI) carrying the enterotoxin genes, and an R plasmid of incompatibility group FII (IncFII) carrying drug resistance genes, or by insertion into the IncFI by two transpositions mediated by *tet* and R-determinant transposons (Gyles *et al.*, 1978; Mazaitis *et al.*, 1981). The presence of drug resistance genes in stem loop structures of the plasmids, and the flanking of both LT and ST genes by repeated, inverted DNA sequences give credence to this hypothesis (Yamamoto & Yokota, 1981). The association between the carriage of genes for drug resistance, enterotoxins, and other virulence factors (e.g., fimbria) in R plasmids may have been exacerbated by the overuse of antibiotics (Gyles *et al.*, 1978).

Ent plasmids can be categorized based on the different types and combinations of enterotoxin genes they carry. They may have genes for LT (*eltAB*), STa (*estA*), STb (*estB*), or enteroaggregative *E. coli* heat-stable toxin-1 (EAST1; *astA*), or any combination thereto. ETEC isolates from humans that produce STa make a variant designated STaH, whereas, isolates from pigs make a variant called STaP (Nair & Takeda, 1968). These toxins vary slightly in their amino acid sequences. STb-encoding plasmids are primarily found in porcine strains, as this toxin does not have an effect on human intestinal epithelium (Gyles *et al.*, 1974; Dubreuil, 2008). Human isolates often carry plasmids that encode LT and STaH, while plasmids encoding STb usually also encode LT, as the two genes are linked in isolates of porcine origin (Gyles *et al.*, 1974; Smith, 1984). Ent plasmids usually carry only one copy of their respective enterotoxin genes; however, there have been reports of multiple gene copies for an enterotoxin, typically LT, being carried in the same plasmid (Murphy & Dallas, 1991).

Heat-labile enterotoxin (LT)

LT closely resembles cholera toxin (CT) produced by *Vibrio cholerae* (Spangler, 1992). These two toxins are multimeric proteins belonging to the AB_5 family of toxins. They evoke a diarrheal response from the host by interfering with electrolyte and water absorption and secretion mechanisms of intestinal cells. Furthermore, the antigenic responses generated by CT subunit vaccines have been shown to be protective against ETEC (Zhang & Sack, 2012). The existence of LT was first demonstrated in ligated pig intestines (Gyles & Barnum, 1967). It was then shown that the component causing fluid accumulation in ligated procine intestinal loops and induction of diarrhea in pigs in cases of disease was an enterotoxin (Smith & Halls, 1968). Furthermore, the diarrheagenic activity was shown to be inactivated by heating at 60°C for 30 min (Gyles & Barnum, 1969). LT is found in two antigenic forms, LT-I and LT-II. The one found predominantly in human and animal isolates is LT-I, while LT-II is found in a few animal ETEC isolates (Mudrak & Kuehn, 2010).

Structure of LT

LT and CT are homologous proteins, with nucleotide and amino acid sequence identity of 77.9% and 78.8%, respectively (Yamamoto *et al.*, 1984). Both LT and CT holotoxins are heterohexamers with an AB_5 toxin configuration. This configuration consists of a single catalytically active A subunit, and a ring-shaped binding region comprised of five identical B subunits (Dallas & Falkow, 1979; Spicer & Noble, 1982; Mekalanos *et al.*, 1983). Although the B subunits are identical for a given holotoxin molecule, the molecular weights of the subunits for different LT holotoxin molecules,

especially those of different sources, can vary, as evidenced by the observation of different migration rates by SDS-PAGE (Gill *et al.*, 1981). The monomeric B subunits of LT from human (LTh) and porcine (LTp) isolates have masses of approximately 12,700 and 11,500 Da, respectively. In contrast, the A subunits for both LTh and LTp have masses of approximately 28,000 Da (Geary *et al.*, 1982). The final assembled holotoxins for LTh and LTp are approximately 85,000 Da (Spangler, 1992). The A subunit consists of two peptides, A1 and A2, which are synthesized as a single polypeptide, but later proteolytically cleaved into two segments. The two peptides are connected by a single disulfide bond between two cysteine residues. The A1 peptide is the enzymatically active portion of the A subunit, while A2 anchors the A1 to the B pentamer and is buried in it (Clements & Finkelstein, 1979). When the toxin is internalized by the host cell, the disulfide bond is reduced, releasing the A1 peptide. Binding of LT to the host cell occurs via the ring-shaped B subunit pentamer (Lencer *et al.*, 1999). The B subunits bind to GM1 gangliosides on the apical cell membranes of enterocytes, which serve as receptors for the toxin (Gyles, 1992).

Function and mechanisms of LT

Although LT, via the B subunits, binds to the GM1 ganglioside as the main host cell receptor, it may also bind very weakly to ganglioside GD1b (Nataro & Kaper, 1998). At the molecular level, these binding events are mediated by the terminal galactose units in the receptor and the Cys residues of the B subunits (Horstman & Kuehn, 2002). Binding between the GM1 and the B subunits in particular is very tight, with a dissociation constant in the range of 10^{-9}. The A subunit is proteolytically degraded and

the resulting A1 and A2 peptides are released once the holotoxin touches the cell surface, a process which is relatively slow and temperature-dependent (Field, 1979; Rao & Field, 1984). Subsequent to these processes the whole receptor/toxin complex is internalized and the A1 peptide is trafficked in the cytosol in a retrograde manner using the trans-Golgi apparatus (Spangler, 1992; Horstman & Kuehn, 2002).

Once the A1 peptide reaches the basolateral membrane of the intestinal cells, it activates adenylate cyclase (Field, 1979). A1 peptide has ADP-ribosyl transferase activity, which enables the transfer of an ADP-ribosyl moiety from NAD to the α-subunit of the stimulatory G proteins (G_s; Moss & Richardson, 1978). This causes constitutive activation of adenyl cyclase complex in the intoxicated cell with a resultant increased intracellular concentration of cAMP. The elevated cAMP level causes constitutive activation of cAMP-dependent protein kinase A (PKA), which phosphorylates chloride channels in the apical cell membranes of the intestinal epithelium (Sears & Kaper, 1996).

The major chloride channel in affected epithelial cells is the cystic fibrosis transmembrane conductance regulator (CTFR; Nataro & Kaper, 1998). The net result of constitutive CFTR phosphorylation resulting from A subunit activity is increased Cl^- secretion from secretory crypt cells and reduced absorption of Na^+ from absorptive cells on the villi. This results in increased Na^+ concentration and osmotic pressure in the intestinal lumen, and diarrhea caused by paracellular water flow (Sears & Kaper, 1996).

The A subunit is also capable of producing secretagogues via stimulation of prostaglandin synthesis. An alteration in arachidonic acid metabolism results in production of PGE_2 which stimulates secretion of water and electrolytes from the intestinal cells (Nataro & Kaper, 1998).

Similar to CT, LT is a potent immunogen, and has been used as an adjuvant in several ETEC vaccines (Pizza *et al.*, 1991). When incorporated alone or co-administered with another antigen, it has been shown to be capable of generating substantial systemic and intestinal antibody responses (Boedeker, 2005).

Factors affecting the production of LT

Many factors affect the production of LT, specifically, temperature, pH, salt concentration, and availability of oxygen, short chain fatty acids, and other nutrients. LT is produced maximally at 37°C. At 40°C, LT is still produced in significant amounts, but at 42°C the enterotoxin level is reduced approximately ten-fold from its optimum. At temperatures below the optimum (33°C), there is a significant amount of LT production, but it becomes barely detectable below 26°C (Kunkel & Robertson, 1979; Mundell *et al.*, 1976). The pH of the environment has a significant effect on LT production. LT is produced maximally in neutral or alkaline conditions in the 7.0-9.2 pH range (Gilligan & Robertson, 1979; Gonzales *et al.*, 2013; Hegde *et al.*, 2009). In acidic conditions, LT production is abrogated (Gonzales *et al.*, 2013; Hegde *et al.*, 2009). LT production is limited to a narrow range of osmotic conditions equivalent to 0.1-0.3 *M* NaCl, and is optimal at 171-200 m*M* NaCl (Trachman & Yasmin, 2004; Hegde *et al.*, 2009). Another factor that increases LT production is the oxygen concentration, which is optimal at very low (microaerobic) amounts (Trachman & Yasmin, 2004).

The production of LT and ST are subject to catabolite repression (Alderete & Robertson, 1977; Gilligan & Robertson, 1979). Carbon sources shown to repress ST production have the opposite effect on LT. L-arabinose, lactose, glucose, galactose,

glycerol, pyruvate and sucrose all increase the production of LT (Gilligan & Robertson, 1979). However, among all the carbon sources tested, glucose has the greatest effect on LT production. Maximal LT production occurs when the concentration of glucose in the medium at the initiation of batch culture is 2.5 g/L (0.25%; Hegde *et al.*, 2009; Wijemanne & Moxley, 2014).

Short chain fatty acids (SCFA) reduce the level of LT produced by ETEC. This effect is not thought to be due to destruction of LT by SCFA or the inhibition of LT produced from the ETEC. SCFA with a carbon chain length from C2 to C7 inhibit biosynthesis of LT, and the longer the chain length the greater the inhibitory effect (Takashi *et al.*, 1989; Binder 2010).

LT Secretion

The two subunits of LT, LT-A and LT-B, are transcribed from the *eltAB* operon and, after translation, the subunit proteins are translocated separately to the periplasmic space. Each subunit contains a signal sequence which guides its localization into the periplasm via the Sec transport system (Hirst, 1991). Both signal sequences for LT-A and LT-B, which are 18 and 21 amino acids in length, respectively, have the typical arrangement of amino acids needed for Sec-dependent transport. This arrangement consists of positive charges at the start followed by hydrophobic residues and tyrosine or histidine at the cleavage site (Spicer & Noble, 1982; Yamamoto *et al.*, 1982).

Early workers on LT concluded that *E. coli* did not have the capacity to secrete this toxin (DiRita *et al.*, 1991). This conclusion was based on the finding that LT was sequestered in the periplasmic space, and not found in the culture supernatant until cell

lysis had occurred. This finding was further suggested by the fact that ETEC containing plasmid-borne CT was also found only in the periplasm, while *V. cholerae* strains expressing the plasmid-borne LT were found to secrete the toxin (Gennaro *et al.*, 1982; Hirst *et al.*, 1984; Neill *et al.*, 1983). That being said, Tauschek *et al.* (2002) demonstrated the presence of a functional type II secretion system (T2SS) in the prototypic human ETEC strain H10407. Not only did this T2SS share a high degree of amino acid sequence identity with a similar system used in *V. cholerae* to secrete CT, it also had the same functionally analogous components, *i.e.*, it was homologous. The discovery of a T2SS essential for the secretion of LT, together with the evidence that laboratory strains used in previous studies lack a functional T2SS, corrected the misconception that ETEC strains do not have the potential to secrete LT (Tauschek *et al.*, 2002; Mudrak & Kuehn, 2010). Expression of this general secretory pathway T2SS operon in the commonly used laboratory K-12 strain MC4100 resulted in LT secretion when it was transformed with an LT-encoding plasmid (Horstman & Kuehn, 2002). ETEC carrying this T2SS was also shown to secrete CT in the same manner that LT is secreted by *V. cholerae* (Mudrak & Kuehn, 2010). Regulation of the T2SS by H-NS, a histone-like protein known to influence transcription of the LT operon, implies that the secretory apparatus is upregulated in conditions that favor LT production (Yang *et al.*, 2007; Francetic *et al.*, 2000).

After they are transported singly, the assembly of A and B subunits into the AB_5 holotoxin conformation occurs spontaneously and rapidly in the periplasm. Most of the LT-B transported to the periplasm is assembled into pentamers within one minute, and about half of these assembled pentamers are associated with the LT-A subunit (Hofstra &

Witholt, 1985). This assemblage is further enhanced in the presence of LT-A, as is evidenced by the presence of a high concentration of LT-B pentamers when co-expressed with LT-A (Hardy *et al.*, 1988), and slow formation of pentamers when the terminal 14 amino acid residues are deleted (Streatfield *et al.*, 1992). The first 10 amino acids at the N-terminus are also essential for the self-assembly of LT-B (Chung *et al.*, 2006). Pentamers are dissociated when exposed to strong acidic conditions, but can reassemble spontaneously when the pH again is neutral (Hardy *et al.*, 1988). Although > 90% of LT-A in the periplasm is associated with the LT-B pentamer, only about 50% of newly translocated LT-A is associated with the holotoxin. Therefore, LT-A is thought to be easily degradable in the periplasm, which may explain why 1.5 LT-A subunits are found for every LT-B pentamer in the periplasm (Hofstra & Witholt, 1985). The last four amino acid residues of the LT-A are essential for the anchoring of the subunit to LT-B pentamer, as deletion of these results in pentamers not being associated with LT-A (Streatfield *et al.*, 1992). Unlike the pentamer, once the holotoxin is assembled, it is able to withstand large pH fluctuations (Chung *et al.*, 2006). The only factor needed for the LT holotoxin formation is the periplasmic disulfide oxidoreductase, DsbA, which is needed for the disulfide linkage between Cys-9 and Cys-86 of LT-B, and for the disulfide bridge between the Cys-187 and Cys-199 residues of the LT-A (Hardy *et al.*, 1988; Yu *et al.*, 1992).

Release of LT from the periplasm is not dependent on other factors in the periplasmic space. The assembled holotoxin is released to the outside milieu via the porin structure formed by the polymerization of the GspD protein of the T2SS. In addition to the holotoxin, the pentameric LT-B subunit is also secreted to the outside by ETEC.

However, neither the LT-A subunit nor the individual LT-B monomers are secreted (Hirst, 1991). Deletion of *eltA* did not result in LT-B pentamer assembly or secretion, whereas deletion of *eltB* resulted in no detection of LT-A in the medium, leading to the conclusion that LT secretion is dependent on the presence of LT-B pentamer while LT-A does not influence the secretion of the holotoxin (Hirst *et al.*, 1984; Hirst, 1991).

LT secretion is also enhanced by factors other than T2SS. A pathogenicity island in ETEC strain H10407 containing the adhesin Tia, was also demonstrated to be carrying genes associated with bacterial secretion apparatuses. One of the key genes in this pathogenicity island, the deletion of which negatively influenced LT secretion, is *leoA* (Fleckenstein *et al.*, 2000). Although LeoA is not found in the vast majority of ETEC isolates, it seems to have a profound effect on the pathogenicity of strain H10407, affecting the motility as well as enterotoxin secretion in Δ*leoA* mutants (Brown & Hardwidge, 2007). Analysis of the LeoA protein revealed that it contains a potential ATP/GTP binding motif, as well as being homologous to EpsE of *V. cholera*e and a number of ATP-binding cassette transporters. *In vitro* assays performed with LeoA demonstrated that it acts as a functional GTPase (Brown & Hardwidge, 2007). While LeoA is not essential for the LT secretion in H10407, its presence seems to enhance the secretion process, producing maximal levels compared to Δ*leoA* clones. This led to the conclusion that the presence of GTPase, in addition to the ATPase associated with the T2SS, may provide additional boost to the secretory apparatus (Brown & Hardwidge, 2007). An absence of LeoA also affects the formation of outer membrane vesicles (OMV) carrying LT molecules. The total number of OMV, total protein content and LT concentration of OMV are all decreased in the absence of LeoA. It was determined that

the GTP-binding domain of the protein was essential to its functions related to OMV (Brown & Hardwidge, 2007).

All Gram-negative bacteria studied to date produce OMV as a general stress response. It is an independent process whose purpose is thought to be the expulsion of misfolded proteins which accumulate under stress conditions (McBroom & Kuehn, 2007). OMV are spheroid particles, which can range from 10-300 nm in diameter (Bonnington & Kuehn, 2013). Since OMV are formed when the bacterial outer membrane encloses periplasmic contents and buds off, they are mainly composed of protein and lipid. Approximately 1% of outer membrane material and 0.5% of periplasmic contents are packaged into OMV in *E. coli*, while other species like *Neisseria* may divert 8-12% of total protein into OMV (Hoekstra *et al.*, 1976; Devoe & Gilchrist, 1973).

In ETEC strains, release of OMV is coordinated with the secretion of LT (Wai *et al.*, 1995). A ten-fold increase in OMV production by pathogenic ETEC strains containing LT, compared to non-pathogenic *E. coli*, suggests that ETEC utilizes the OMV as another pathway to release LT. ETEC OMV, in addition to being packed with LT, are also covered on the outside with LT molecules anchored to their membrane (Horstman & Kuehn, 2000). In certain isolates OMV have been shown to carry the largest amount of secreted LT, but studies done with clinically isolated strains have shown that freely secreted, non-OMV associated LT consists of the majority of secreted enterotoxin (Lasaro *et al.*, 2006). The advantage of OMV to ETEC cells may be in their ability target intestinal cells and deliver a large dose of enterotoxins compared to free, non-OMV bound LT. Two pathways have been suggested for the OMV mediated toxin

delivery: one proposes that LT is delivered to the intestinal cells via vesicles but is dissociated from the vesicle before entering the cell; the second suggests that the vesicle is internalized with LT still bound, delivering the enterotoxin directly into the cell. Surface adhesins, as well as LT coating OMVs are responsible for the attachment of vesicles to host cells (Kesty *et al.*, 2004).

In addition to the secretion of LT as free or membrane-bound, ETEC also may deliver LT directly to the host cells. Once ETEC is in contact with the apical surface of the intestinal epithelial cell, LT and the T2SS are assembled exclusively at the pole of the bacterium that is in contact with the cell. This directed delivery of LT is thought to benefit ETEC by circumventing exposure of the toxin to anti-LT antibodies (Dorsey *et al.*, 2006).

LT gene transcription and translation

The genes for the two subunits of LT, *eltA* and *eltB*, are arranged in a 1.2-kb operon and transcribed into one dicistronic mRNA (Yamamoto *et al.*, 1982). Both *eltA* and *eltB* have been cloned separately to analyze their DNA sequences using minicells and deletion mutants (Dallas & Falkow, 1979). LT and CT share nucleotide sequence identities of 75% and 77%, respectively, in their A and B subunit toxin genes, with corresponding amino acid sequence identities of 76 % and 78% (Mekalanos *et al.*, 1983). The G+C content of *eltA* and *eltB* is 38% and 37%, respectively, which is relatively low and more similar to *V. cholerae* than *E. coli*.

The highest degree of amino acid sequence identity (91%) between the LT A subunit (LT-A) and the CT A subunit (CT-A) occurs at the amino-terminus, while the

lowest (52%) is observed in the carboxyl-terminus of the protein (Spicer & Noble, 1982). The sequence for *eltA* of human and porcine isolates encode for a precursor polypeptide of 254-258 amino acids (29,673-29,873 Da) consisting of a highly conserved, 18-amino acid leader sequence at the amino-terminus, which is cleaved to produce the final LT-A (27,588 Da) product (Spicer & Noble, 1982; Yamamoto *et al.*, 1984).

LT-A is post-translationally processed to into peptides LT-A_1 and LT-A_2, which are linked by a disulfide bond and proteolytically cleaved into two regions inside the host cell. The cleavage occurs between Arg-192 and Thr-195, and results in polypeptides containing 192- (LT-A_1) and 46- amino acid (LT-A_2) residues (Yamamoto *et al.*, 1984). Nine nucleotides upstream from the *eltA* initiation sequence, ATG, the four-base pair sequence, TAAG, has been identified as the ribosome binding site with -5.2 kcal binding energy. In the 200 base pairs upstream of the initiation sequence, surrounded by a highly A/T rich region, lies the -35 and -10 regions, homologous to RNA polymerase recognition and binding sites, which act as the promoter for *eltAB* operon (Yamamoto *et al.*, 1984).

The *eltB* sequence of 375 bp, with low G+C content (37%), encodes for a precursor polypeptide of 124 amino acids, consisting of a 21-amino acid leader sequence at the amino-terminus. The precursor LT-B is processed into the final LT-B (11,780 Da) product by the cleavage at Gly-21 residue (Dallas & Falkow, 1980; Yamamoto & Yokota, 1983). The proximal 180 nucleotides of *eltB* do not contain a Pribnow box or similar structure, capable of binding RNA polymerase. However, three nucleotides upstream from the initiation codon of *eltB*, a Shine-Dalgarno sequence is present. The region upstream of the *eltB* initiation codon, including the Shine-Dalgarno sequence

(GGAAUGA), is considered to be highly favorable for ribosome binding with a binding energy of -7.2 kcal. The presence of nucleotides complementary to the 3′ end of the 16S rRNA, and an adenosine adjacent to the *eltB* initiation codon increases the efficiency of ribosome binding (Dallas & Falkow, 1980).

The two nucleotide sequences for *eltA* and *eltB* exist as overlapping genes in the *eltAB* operon, sharing four nucleotides. The distal end of *eltA* (5′-TTATGA) overlaps with the proximal end of *eltB* (5′-ATGAAT). The first adenosine in the shared four nucleotides is used in a TTA reading frame for the carboxy-terminal leucine of LT-A, while simultaneously being used as the ATG reading frame for amino-terminal methionine of LT-B (Yamamoto *et al.*, 1982).

The *eltAB* operon was originally hypothesized to be transcribed as one polycistronic (or more accurately, dicistronic) mRNA by Dallas *et al.* (1979); this hypothesis was subsequently supported by the work of other authors (Yamamoto & Yokota, 1981). Both *eltA* and *eltB* are transcribed from the same promoter region upstream of *eltA*. The -10 region (AGTGGTTATCTTT), binding RNA polymerase, as well as the -35 region (TGTTGCATATAG), which recognizes *E. coli* RNA polymerase, of the *eltAB* operon are highly similar to the consensus sequences of -10 (TGTTGACAATTT) and -35 (ATTTGTTATAATG) regions of *E. coli* promoters (Yamamoto & Yokota, 1981; Spicer & Noble, 1982). The mRNA of *eltA* contains a 34-nucleotide long leader region with two AUG codons. The first AUG codon, 21 nucleotides upstream, without an accompanying ribosome binding site, is considered the translation initiation site, while the second AUG site with a four nucleotide ribosome

binding site 5'-UAAG-3', nine nucleotides upstream is considered the starting codon with the amino terminal sequence of Met-Lys-Asn-Ile-N- (Spicer & Noble, 1982).

Since *eltA* and *eltB* are transcribed into one dicistronic mRNA but the LT holotoxin consists of one LT-A and five LT-B subunits, the translation rates for the two coding sequences was hypothesized to differ. This hypothesis was in fact observed in minicells containing the LT+ plasmid EWD299, which produced higher levels of LT-B compared to LT-A. The production of LT-B by ETEC strains of human origin is on average more efficient than that of LT-A, at 2.2 mol to 1.4 mol (Yamamoto & Yokota, 1981; Yamamoto *et al.*, 1982). In some cases, the LT-B subunit has been shown to be >5 mol compared to LT-A (Dallas & Falkow, 1979). The higher rate of LT-B to LT-A synthesis, even though *eltA* is closer to the promoter region, results from the *eltB* mRNA ribosome binding site having a higher binding energy (-7.2 kcal) than that for *eltA* (-5.2 kcal; Yamamoto *et al.*, 1984). Secondary structures of *eltB* mRNA, particularly the stem loops formed by sequences of dyad symmetry, enhance translation. Stable stem loops formed by codons from 21-30 and 38-46, among other secondary structures, are considered to be of importance in this regard (Dallas & Falkow, 1980; Yamamoto & Yokota, 1983).

Although it was at first thought that no typical mRNA transcription termination signals were present distal to *eltB*, later it was shown that the stop codon TGA is present (Dallas & Falkow, 1980; Yamamoto & Yokota, 1983). Two regions of dyad symmetry are considered to enhance the transcription termination, one at nucleotides 393-416 near the *eltB* stop codon, and the other at nucleotides 515-548 making a very stable prominent stem loop structure. Beyond the transcription stop codon lies a G/C rich region (535-

542) followed by T-rich region (543- 546), typical of *rho*-independent transcription termination.

Translation of the polycistronic mRNA was different from the typical codon usage observed in *E. coli* genes, but was similar to the codon usage seen in CT-A and CT-B translation. Usage of codons that initiated or terminated with A- or U- over those with G- or C- was preferred with Arg, Leu, Ser, Thr, Pro, Ala, Gly, Val, Lys, Asn, Glu, Tyr, Cys, Phe, and Ile, while codons initiated with A- or U- was preferred for Arg and Leu. Other deviations from typical *E. coli* protein translation were the use of AGG for Arg, which is rarely used by *E. coli*, and none of the Cys codons consisted of UGC. Together with the fact that *eltAB* is flanked by transposable insertion sequences, this supports the hypothesis that LT originated in a species with low G+C content, such as *V. cholerae*, rather than that of *E. coli* (Yamamoto *et al.*, 1984).

Thermoregulation of eltAB

The *eltAB* operon is regulated at the transcriptional level, in part, by temperature (Trachman & Maas, 1998). The highest levels of production of LT mRNA and protein are observed at 37°C, and transcription steadily decreases as the temperature is lowered to <18°C. Thermoregulation of LT operon involves the histone-like nucleoid structuring (H-NS) protein, a global regulator belonging to the cold shock regulon (Trachman & Yasmin, 2004). HN-S regulates transcription of a large number of genes involved in cellular processes as well as that of virulence genes (La Teana *et al.*, 1991). H-NS represses *eltAB* transcription at low temperatures (Trachman & Maas, 1998). H-NS is thought to interact with a downstream regulatory element (DRE) in the N-terminal region

of *eltA* rather than the upstream control region (UCR) of the promoter since deletion of the UCR does not affect the temperature-dependent effects of H-NS on LT transcription (Trachman & Maas, 1998). The DRE binding region of H-NS consists of two distinct silencer regions separated by a stretch of 350 bp. One silencer region is located between +31 and +110, while the other is found between +460 and +556 from the transcription start site (Yang *et al.*, 2005). Deletion of this region produces elevated levels of LT mRNA, even at low temperatures (Trachman & Maas, 1998). However, experiments performed with Δ*hns* mutants have shown that the absence of H-NS does not interfere with normal LT synthesis, which led to the conclusion that this interaction is needed only for LT repression and not for activation of *eltAB* (Trachman & Maas, 1998).

Osmoregulation of eltAB

Osmolytes in the cultivation medium influence the production of enterotoxins, especially LT (Hegde *et al.*, 2009). This regulation is limited to a range of osmotic concentrations and is dose-dependent (Trachman & Yasmin, 2004; Hegde *et al.*, 2009). H-NS plays a role in osmoregulation of LT, but this effect does not involve binding to the DRE, which as noted above is responsible for thermoregulatory effects. The lack of DRE involvement in osmoregulation was observed in strains containing plasmids carrying a copy of *eltAB* with the DRE deleted. In this study, LT production was highest when the NaCl concentration of the media was <0.171 *M* (Trachman & Yasmin, 2004). In addition, LT production was not affected under varying osmotic conditions in experiments performed with Δ*hns* mutants. However, the H-NS mediated osmoregulation requires acting in concert with the glucose mediated regulation to exert control over *eltAB*

(Haycocks *et al.*, 2015). The osmoregulation does not seem to be dependent on any particular osmolytes or whether the osmolytes are ionic or non-ionic, as sucrose and lithium chloride used to influence the osmotic stress in the medium seem to affect the LT production in the same manner as NaCl. However, the levels affected by different osmolytes vary (Trachman & Yasmin, 2004). The osmoregulation of *eltAB* is under the influence of the ambient temperature and oxygen concentration. The highest levels of LT are observed at 37°C and under microaerobic conditions, irrespective the osmolytes used in the media (Trachman & Yasmin, 2004). It has been suggested that osmolytes with higher hydration than NaCl, such as sucrose or lithium chloride, may have a more stimulatory effect on the transcription of LT (Trachman & Yasmin, 2004).

Catabolite regulation of eltAB

Carbon sources in the medium have the ability to exert catabolite repression on ST synthesis; however, the same carbon sources have been shown to have the opposite effect on LT (Alderete & Robertson, 1977). This effect was observed independent of other potential metabolites, such as amino acids, capable of exerting repression (Gilligan & Robertson, 1979). The most profound effect on LT synthesis was observed with glucose (Mundell *et al.*, 1976; Gilligan & Robertson, 1979). The maximum level of LT produced occurred when glucose was present in the medium at a starting concentration of 0.25 % (2.5 g/L; Hegde *et al.*, 2009; Wijemanne & Moxley, 2015). It was determined that this effect of glucose on LT regulation was under the control of cAMP. Specifically the complex formed by the interaction of cAMP and the cAMP receptor protein (CRP) is responsible for the positive modulation of *eltAB* transcription (Gibert *et al.*, 1990).

Bodero and Munson (2009) were the first to elaborate a detailed molecular mechanism to explain the cAMP-CRP complex-mediated enterotoxin gene regulation. Three binding sites for CRP within and adjacent to the LT promoter region were identified by DNase I footprinting, and designated *eltA1o*, *eltA2o*, and *eltA3o*. The nucleotide identities of *eltA1o*, *eltA2o*, and *eltA3o* in relation to the consensus CRP binding site, AAATGTGANNNNNNTCACATTT were 69%, 75% and 63% respectively. Of the three binding sites, *eltA1o*, had the highest affinity for CRP while *eltA2o* the lowest. The discrepancy among the CRP affinity to binding sites may be attributed to the arrangement of those sites. Only *eltA1o*, located -31.5 upstream from the promoter start site, has the 6-bp spacing region separating the inverted repeats typical of the CRP binding sites. Both *eltA2o* and *eltA3o*, located around -132 and -261 upstream from the promoter start site, respectively, have 7-bp spacers separating the inverted repeat sequences (Bodero & Munson, 2009). Furthermore, since *eltA1o* straddles the -35 hexamer, which recognizes the RNA polymerase, its occupancy by CRP has a much more profound effect on the LT transcription than the CRP occupancy of the other two sites. For CRP to occupy its binding sites, it needs to be in a complex with cAMP. The levels of cAMP are highly dependent on the glucose concentration. Higher glucose concentration suppresses cAMP synthesis leading to a decrease in the cytotolic concentration of cAMP-CRP complex. This reduced concentration reduces the cAMP-CRP complex binding to the LT-promoter, allowing RNA polymerase to bind and transcribe *eltAB*. Conversely, at low glucose concentrations, the resulting high cAMP levels promote cAMP-CRP complex formation, which increases complex binding to the LT promoter, repressing transcription. Therefore, the effect of glucose on LT

transcription is de-repression (Bodero & Munson, 2009). To observe the effects of LT repression, it is only necessary to occupy the *eltA1o* site, while deletion of *eltA2o* and *eltA3o* slightly alter repression. It has been hypothesized that the two low affinity sites, mainly *eltA3o*, may not be present in all ETEC strains, but when present they are essential for the glucose mediated de-repression of the LT gene (Bodero & Munson, 2009).

The model proposed by Bodero and Munson (2009) for direct repression or de-repression of *eltAB* by CRP was recently disputed (Haycocks *et al.*, 2015). In this study, CRP binding sites for the whole ETEC genome were identified by chromatin immunoprecipitation (ChIP) coupled with next-generation DNA sequencing (ChIP-seq) methods. Even though 111 binding sites with high affinity for CRP were found, none were identified in the *eltAB* promoter region as reported by Bodero and Munson (2009).This was in contrast to the presence of such sites in the ST promoter regions from the same study. Further experiments with electrophoretic mobility shift assays (EMSA) failed to yield the suspected CRP binding sites previously reported, although non-specific CRP binding to the promoter region was observed at high concentrations of CRP. The transcription of *eltAB* was not affected when these regions were removed. These data led to the hypothesis that although LT repression occurs in the presence of CRP, this process occurs indirectly, rather than by the direct manner previously elaborated (Haycocks *et al.*, 2015).

Interaction between H-NS and CRP in eltAB regulation

The possibility that H-NS and CRP, which have been implicated in the regulation of LT via different mechanisms may be involved in a broader coordinated regulation of *eltAB* was previously suggested (Trachman & Yasmin, 2004). This hypothesis was supported by the aforementioned recently published study, which elaborated a series of complex, coordinated mechanisms that are responsible for LT and ST gene regulation in response to salt and glucose concentrations (Haycocks *et al.*, 2015). Five different mechanisms were suggested in this model: (a) LT is directly repressed by H-NS; (b) ST is directly repressed by H-NS; (c) ST transcription is directly activated by CRP; (d) H-NS competes for CRP binding sites of ST to represse transcription; (e) LT transcription is repressed by CRP via an indirect manner, and is influenced by H-NS.

H-NS mediated repression of ST can be abrogated by increasing the osmotic concentration of the medium. When both H-NS and CRP act on the same operon, as is the case with *eltAB* in this model, the effects are considered epistatic with respect to the glucose and osmotic concentration (Haycocks *et al.*, 2015). This occurs because of the propensity of H-NS to indiscriminately bind high-affinity CRP binding sites when they are not occupied, as well as to exclude CRP from binding to enterotoxin promoters (Haycocks *et al.*, 2015).

Heat-Stable Enterotoxin (ST)

An enterotoxin able to retain its activity after 30 min at 100°C, but lose its activity after 30 min at 121°C, was first described in 1970 and named heat-stable enterotoxin (ST; Smith & Gyles, 1970). Those toxins falling within the designation, ST, are small and

consist of only single peptides. These toxins include two unrelated classes, STa and STb, which differ both in structure and mechanism of action. The host cell receptor for STa is the extracellular domain of guanylyl cyclase C (GC-C), whereas that for STb is sulfatide. Only toxins of the STa class have been associated with human disease (Nataro & Kaper, 1998).

Two types of STa are produced by ETEC: STaP and STaH, which are found in animal (*viz.*, P for porcine) and human isolates, respectively (Picken *et al.* 1983; Okamoto *et al.*, 1995; Yamanka *et al.*, 1997). Both STaP and STaH are synthesized from a 72-amino acid precursor molecule consisting of a pre-, pro- and mature ST- region (Yamanaka *et al.*, 1994; Okamoto *et al.*, 1995). The pre-regions act as leader sequences, which are identified by the Sec-pathway, and as a result are translocated into the periplasm (Yamanaka & Oakamoto, 1996). In the periplasm, the pre-form is modified into the mature form that is capable of being translocated across the outer membrane by being associated with the TolC receptor (Yamanaka *et al.*, 1998). For this process, DsbA, a protein present in the periplasm is essential. DsbA catalyzes the formation of three intramolecular disulfide bonds, between Cys-5 and Cys-10, Cys-6 and Cys-14, and Cys-9 and Cys-11 (Yamanaka *et al.*, 1994, 1997). The final product from the processing of this precursor is an 18-amino acid STaP and a 19-amino acid STaH (Aimoto *et al.*, 1982; Takao *et al.*, 1983).

STa enterotoxins bind GC-C, a large protein expressed at the apical plasma membrane of intestinal epithelial cells (Weiglmeier *et al.*, 2010). In addition, STa has been shown to bind receptors on renal cells which exhibit the same extracellular domain (Lima & Fonteles, 2014). The N-terminal 14 residues are the essential component for this

action (Yoshimura *et al.*, 1985). Binding of STa to GC-C imparts a conformational change and initiates a cascade of events that result in fluid secretion from the cell. The catalytic domain of activated GC-C generates excessive amounts of cGMP from GTP, leading to the activation of cGMP-dependent protein kinase II (PKGII) (Katwa *et al.*, 1992; Lima & Fonteles, 2014).

CFTR at the apical membrane is phosphorylated by PKGII, which opens up to release Cl^- into the intestinal lumen. Increased cGMP leads to increased cAMP by its inhibitory action on cAMP hydrolyzing phosphodiesterase-3. High cAMP levels activate PKA, leading to further phosphorylation of CFTR. Furthermore, cGMP inhibits the Na^+/H^+ exchanger (NHE) channels that absorb Na^+ from the intestine (Laohachai *et al.*, 2003; Lima & Fonteles, 2014). Ultimately, this results in increased Cl^- and Na^+ in the intestinal lumen leading to massive fluid secretion from the cells.

STa has been suggested to act as a protective agent against colorectal carcinoma, based on the observation of an inverse relationship between the incidence of STa-mediated diarrhea and colon cancer (Pitari *et al.*, 2003; Parkin *et al.*, 2005). It has been shown that signal cascades initiated by the GC-C receptor are essential for arresting the events that lead to cancer formation in colon cells. In colorectal carcinoma, the usual ligands that bind GC-C, *e.g.*, guanylin and uroguanylin are not expressed (Pitari *et al.*, 2003). However, the presence of STa may induce GC-C mediated signal pathways and provide the protective effect from cancer (Weiglmeier, 2010; Lima & Fonteles, 2014).

Production of STb by ETEC isolates is associated with clinical disease in pigs (Dubreuil, 2008; Taillon *et al.*, 2008). STb is a 48-amino-acid peptide containing two disulfide bonds, which is initially synthesized as a 71-amino acid precursor (Picken *et al.*,

1983; Dubreuil, 1997). The formation of disulfide bonds is mediated by DsbA in the periplasm. The amino acid residues from positions 19-36 in the final processed product are important for translocation of the toxin across the outer membrane (Okamoto *et al.*, 1995; Okamoto *et al.*, 2001). Unlike STa, STb has been implicated in the causation of morphological damage to the intestinal cells, with resultant enterocyte sloughing and villus atrophy (Whipp *et al.*, 1985; Whipp *et al.*, 1986; Rose *et al.*, 1987).

The binding site for STb on enterocytes is sulfatide, an acidic glycosphingolipid (Rose *et al.*, 1998). The pathogenesis of net fluid secretion in enterocytes attributable to STb involves several mechanisms, none of which involves adenylate or guanylate cyclase (Hitotsubashi *et al.*, 1992). Once the toxin is internalized it activates a GTP-binding protein, which opens up calcium channels. The resulting increased intracellular Ca^{2+} concentration activates a calcium-dependent protein kinase, which ultimately opens up several intestinal ion channels, including the CFTR (Hitotsubashi *et al.*, 1992; Dreyfus *et al.*, 1993; Fujii *et al.*, 1997). Opening of the CFTR results in constitutive Cl^- and HCO_3^- secretion with resultant effects resembling that of LT intoxication (Laohachai *et al.*, 2003; Sellers *et al.*, 2004). High intracellular Ca^{2+} concentration also induces the production of elevated intracellular concentrations of prostaglandin E2 (PGE_2) and serotonin (5-hydroxytryptamine), additionally leading to water and electrolyte transport out of the cells. These net water and electrolyte losses are also exacerbated by STb stimulation of the enteric nervous system (Harville & Dreyfus, 1995; Peterson & Whipp, 1995). Th-e cumulative effect of these actions, depending on the extent of infection, is severe fluid loss from the intestine.

Fimbrial adhesins in ETEC pathogenesis

Fimbriae are the main adhesins produced by ETEC. Fimbriae are peritrichously arranged protein heteropolymers expressed on the cell surface which facilitate adherence to epithelial cells of the host. They can be assembled into structures measuring approximately 2 μm in length, and vary in diameter from 2-10 nm (Mol & Oudega, 1996). The first classification of fimbriae was based on their morphology as evidenced by electron microscopy. Based on these observations, fimbriae were grouped into two types (Brinton, 1965). The first type was characterized by a structure that included a right-handed helix containing 3.2 subunits per turn. Fimbriae of this type also were 7 nm in diameter with a 1.5 nm diameter central axial hole (Gong & Makowski, 1992). The second type consisted of a structure that lacked a central axial hole and were thin, flexible, and typically had a diameter of 2-4 nm (Bullitt & Makowski, 1995). Subsequent studies indicated this method of classification was unreliable as the visualization of the two types could alter depending on the technique used, and some of the features upon which the classification was based may have been artifacts (Mol & Oudega, 1996).

A better method of classification of fimbriae was discovered, which was based on their adhesiveness to red blood cells of different animal species (Gaastra & De Graaf, 1982). Based on hemagglutination, fimbriae have been categorized as either mannose-sensitive or mannose-resistant. When D-mannose can block the reaction, they are called mannose-sensitive, while fimbriae whose attachment is not blocked by D-mannose are called mannose-resistant (Mol & Oudega, 1996). However, not all fimbriae agglutinate red blood cells, and in this case cannot be categorized under this method (Gaastra & De Graaf, 1982).

The specificity of fimbria-receptor binding restricts host susceptibility to certain fimbrial types. For example, porcine and human ETEC strains express distinctly different kinds of fimbrial adhesins, and these attach only to specific receptors found on enterocytes of respective host species (Erickson *et al.*, 1992; Nataro & Kaper, 1998; Jin & Zhao, 2000).

Fimbrial adhesins of human isolates

In human ETEC isolates, the fimbriae are designated by antigen type, termed colonization factor antigen (CFA) or coli surface antigen (CS). In addition, human ETEC isolates express other fimbrial structures such as, type 1 fimbriae (T1F) and *E. coli* common pilus (ECP). Altogether, 25 different kinds of colonization factors, both fimbrial and non-fimbrial have been identified in human isolates. When a host is infected by a strain expressing a particular colonization factor, this event provides protection against infection of other strains carrying the same fimbrial antigen.

Thus far there are four different kinds of CFA associated with human ETEC isolates: CFA/I, CFA/II, CFA/III, and CFA/IV (Wolf, 1997). The subunit structures of the CFA are similar in nature, and each with a mass of approximately 12,000-13,000 Da. CFA are additionally divided on the basis of morphological characteristics into three subgroups: rigid rods (CFA/I), bundle-forming flexible rods (CFA/III), and thin flexible wiry structures (CFA/II, CFA/IV; Gaastra & De Graaf, 1982).

Genes for fimbrial synthesis are carried on plasmids that usually also contain genes for enterotoxins, *e.g.*, LT and ST. The typical arrangement of the CFA operon consists of genes for a major subunit and accessory proteins needed for fimbrial

arrangement (Fleckenstein *et al.*, 2010). CFA/I was first identified in the prototype human ETEC isolate, H101407 (Evans *et al.*, 1975). Genes for CFA/I are carried on a 94.8-kb plasmid. The operon encodes for the major fimbrial subunit (CfaB), minor subunit (CfaE), periplasmic chaperone (CfaA) and outer membrane chaperone (CfaC). The final assembled structure consists of roughly 1,000 CfaB subunits, and can be as long as 1 μm, with the minor subunit restricted to the tip of each fimbria (Jansson *et al.*, 2006). The receptors for these adhesins have not been identified with any certainty.

CFA/I and CFA/II are thought to bind to GM2 and GM1, respectively. The likely receptors for CFA/III and CFA/IV are unknown, although they are believed to bind to both acidic and non-acidic glycosphingolipids (Croxen & Finlay, 2010). Among the strains tested so far, CFA/I, CFA/II and CFA/IV account for the majority of fimbrial adhesins (Wolf, 1997). CFA/I was found in 23 different O serogroups, with 90% of these isolates belonging to O78, O153, O128, O126, O127 and O63 (Wolf, 1997). CFA/I is associated with either ST or both LT and ST, but not LT alone. CFA/II is associated with any of 21 different O serogroups, but in >two-thirds of the isolates it is associated with O6 and O8 (Wolf, 1997). Over 85% of isolates with CFA/II also carry both LT and ST. CFA/IV is distributed widely among a variety of O serogroups, with no particular group dominating. However, in >80% of isolates with CFA/IV, ST alone is found (Wolf, 1997).

CS adhesins consist of fibrillar antigens and are found in association with a particular CFA (Wolf, 1997). CS5 and CS6 are commonly found in isolates from around the world (Qadri *et al.*, 2005). The operon for CS usually consists of genes for a major subunit, a minor subunit, an outer membrane usher, chaperone(s) and pilus length regulatory protein (Nicklasson *et al.*, 2012); however, some CS, *e.g.*, CS6, are afimbrial

in nature and consist of two subunits. These subunits are composed of proteins transcribed by four genes in an operon that also contains genes for a chaperone and an usher (Wolf *et al.*, 1997).

T1F, also called somatic or common fimbriae, are expressed by most *E. coli*, including ETEC. More than 70% of wild-type ETEC strains carry these fimbriae. These are threadlike structures, composed of about 1,000 subunits of FimA, encoded by a chromosomal operon. T1F bind to D-mannose on the host cell membrane via adhesive protein FimH, suggesting that they may prefer glycoproteins with *N*-linked oligomannose as the natural receptor (Krogfelt *et al.*, 1990).

T1F expression is regulated by the proteins FimB and FimE, which bind upstream of *fimA* to allow or block transcription, respectively, through a phase variation mechanism (Klemm, 1986). Type I fimbriae are important virulence factors in the pathogenesis of UPEC infection, in contrast to that of ETEC; however, when ETEC are adherent to host cells, the expression of T1F is typically also upregulated (Fleckenstein *et al.*, 2014).

First identified in NMEC and EHEC strains, ECP is yet another fimbrial adhesin found in both pathogenic and non-pathogenic *E. coli* (Blackburn *et al.*, 2009; Fleckenstein *et al.*, 2014). Despite the fact that >80% of strains carry *ecp*, this adhesin has not yet been implicated in the pathogenesis of ETEC infections. The organization of the *ecp* operon and its products are highly similar to other fimbrial adhesins.

Fimbrial adhesins of porcine isolates

As noted earlier, ETEC isolates from pigs and humans express different fimbrial adhesins. Some of the main fimbrial adhesins in porcine isolates include F4 (K88), F5 (K99), and F6 (987P; Gaastra & De Graaf, 1982; Kaper *et al.*, 2004). These adhesins were first detected and named according to their identifying antigens by Orskov *et al.* (1961), with the first such adhesin identified being K88; however, they mistakenly identified these as capsular antigens, hence the designation with the letter K for *kapsel* (Danish for capsule; Mol & Oudega, 1996).

Ten genes arranged in an operon encode for the synthetic, structural and regulatory proteins of F4, and are carried on a large plasmid. These genes include *faeABCDEFGHIJ* (Mol & Oudega, 1996), and are negatively regulated by the binding of leucine-responsive regulatory protein (Lrp) to the *faeA* promoter (Huisman *et al.*, 1994; Huisman & De Graaf, 1995). Three different antigenic subtypes of F4 have been identified: F4ab, F4ac and F4ac (Jin & Zhao, 2000). The differences between F4ab and F4ac are in the FaeG subunit. Of these three, F4ac is the most highly prevalent world-wide (Mol & Oudega, 1996). The receptor for F4ac in the porcine intestinal brush border is IMTGP (Grange *et al.*, 1998).

The F5 adhesin is found in ETEC strains that cause diarrhea in neonatal pigs. It is composed of two components, the major subunit (22,500 Da) and the minor subunit (29,500 Da), present at a ratio of 5:1 (Gaastra & De Graaf, 1982). Genes responsible for the synthesis, assembly and regulation of F5 are encoded by an operon with eight genes, located on a large conjugative plasmid (Mol & Oudega, 1996). The major subunit, FanC, interacts with the receptor for the binding. A brush border ganglioside, Neu5Gc-α(2-

3)Galp-β(1-4)Glcp-β(1-1)-Ceramide, has been identified as the probable receptor (Smit *et al.*, 1984).

F6 antigens are present in ETEC strains that cause diarrhea in piglets (Mol & Oudega, 1996). Genes for F6 are transcribed by the *fasABCDEFGH* operon located on a 35-MDa plasmid, and are responsible for transcribing subunits as well as the chaperones needed for maturation of F6 (Edwards & Schifferli, 1997). These genes are located very close to STa genes in the plasmid (Schifferli *et al.*, 1991). The F6 adhesin is composed of a major subunit FasA, and two minor subunits FasF and FasG. FasG is responsible for the adhesive property of F6 (Edwards & Schifferli, 1997).

Non-fimbrial adhesins in ETEC pathogenesis

In addition to fimbrial adhesins, non-fimbrial adhesins are also implicated in ETEC pathogenesis. Some of the proteins important in this regard are Tia, TibA, EtpA, EatA, and EaeH. Tia and TibA are expressed by invasion loci located in ETEC chromosomes (Fleckenstein *et al.*, 2010). Tia is an outer membrane protein that binds to an epithelial cell surface glycoprotein to enable bacterial adherence. TibA is an autotransporter protein that allows ETEC to aggregate on the surface epithelial cells, thereby promoting adherence. TibA is positively regulated by the cAMP-CRP complex binding to the promoter of the *tibDBCA* operon which activates transcription (Espert *et al.*, 2011).

EtpA is a conserved, secreted adhesin that belongs to the two-partner secretion locus family of proteins. This locus contains genes for three proteins: the glycoprotein EtpA which adheres to the flagellum; EtpB which forms the transport pore; and EtpC, a

glycosyltransferase needed for secretion of EtpA (Fleckenstein *et al.*, 2010). EtpA acts by binding to the tip of the flagellum and acting as a bridge between the epithelial cell and the ETEC, allowing the longer flagella to make the initial contact from a distance (Roy *et al.*, 2009). EatA, another secreted autotransporter, is involved in this process. This serine protease can degrade EtpA and affect the adherence process. In addition, it can degrade the intestinal mucin and increase the likelihood of ETEC contact with the host cell (Kumar *et al.*, 2014).

The surface expressed protein EaeH has been shown to increase ETEC adherence to epithelial cells, and intestinal colonization of animal models by this organism (Sheikh *et al.*, 2014). EaeH contains domains similar to adhesion domains found on eukaryotic cells (Fleckenstein *et al.*, 2014). The gene for EaeH is upregulated when ETEC contacts the host cell. Therefore, the role of EaeH in adhesion may be at a later step in the pathogenesis than that of other adhesins. In addition to enhancing adhesion, EaeH has been shown increase LT uptake by the host cell (Sheikh *et al.*, 2014).

Porcine ETEC pathogenesis

Enteric colibacillosis is an economically important disease of swine, caused by ETEC and rarely other pathovars (Fairbrother, 1999). In swine herds affected with enteric colibacillosis, diarrhea is a predominating clinical sign that leads to weight loss, reduced growth, and high mortality rates (Fairbrother, 1999). Over 10 million pigs worldwide are affected with enteric colibacillosis, with death losses in some cases amounting to >50% of the weaned pigs in a herd (Gyles, 1992; Bertscheinger, 1999). Enteric colibacillosis can be categorized into two types, depending on the age of the piglet and the weaning

status: neonatal (<7-day-old) and post-weaning diarrhea. Pigs >8 weeks of age are seldom clinically ill as a result of ETEC infection (Linton & Hinton, 1988; Verdonck *et al.*, 2002; Francis, 2002; Do *et al.*, 2006).

Pathogenesis of ETEC-mediated neonatal diarrhea (ND) in pigs

Strains of ETEC that cause ND are characterized by the presence of LT and STa or STb, and usually express F4 (K88), F5 (K99), or F6 (987P) adhesins. These strains typically belong to serogroups O8, O9, O20, O101, O141, O147, or O157. Less often, strains expressing adhesins F41, F42, or F165 are implicated in ND (Fairbrother *et al.*, 1988; Sperandio & Silvera, 1993; Vazquez *et al.*, 1996). Of the strains that cause ND, those expressing F4 are the most common, and account for 40-60% of the cases. All three known variants of F4 adhesins, *viz.*, F4ab, F4ac and F4ad, are implicated in the pathogenesis of ND. Non-F4 strains account for 20-30% of the ND cases. Diarrhea caused by $F4^+$ strains is often more severe and frequently occurs at 1-4 days of age. Non-F4 strain mediated ND is mild and usually occurs at 4-14 days of age (Nagy & Fekete, 1999).

The pathogenesis of ND relies heavily on the presence of receptors for the fimbrial adhesins in the pig intestinal epithelium. Resistance to fimbrial adherence attributable to a lack of receptors is inherited as a homozygous recessive trait (Bijlsma & Bouw, 1987). Receptor function is dependent on the b and c components and in genetically resistant piglets the receptors are usually absent for both of these components (Jin & Zhao, 2000). *In vitro* adhesion tests have revealed a polymorphism of intestinal receptors for F4 and indicate the presence of five to six adhesion patterns for F4ab, F4ac

and F4ad in piglets (Bijlsma *et al.*, 1982; Baker *et al.*, 1997; Erickson *et al.*, 1997). Francis *et al.* (1998) identified IMTGP as the relevant receptor for F4ab and F4ac. The production of receptors also influences age-related resistance to the disease. F4 receptors are abundant in newborn pigs and decrease with age, but remain relatively stable in their presence throughout the ages at which pigs are weaned and into the post-weaning period (Erickson *et al.*, 1992). Receptors for F5 decrease gradually with age, while receptors for F6 increase with age (Francis, 2002). In the case of F6, the receptors, which are glycolipids, are also present in goblet cell membranes shed in the mucus. As pigs age, these receptors are predominantly found in the mucus, which is thought to bind to $F6^+$ ETEC and prevent their adhesion to intestinal epithelial cells (Dean-Nystrom & Samuel, 1994). The relationship between piglet age and expression of F41 receptors is unknown, but data suggests that they may continue to be produced until the typical age of weaning (Gaastra & De Graaf, 1982).

Pathogenesis of ETEC mediated post-weaning diarrhea (PWD) in pigs

Post-weaning diarrhea (PWD) is usually the most constant disease problem on large swine farms, especially of those that wean around 3-4 weeks of age. PWD is usually seen within a few days after weaning. During this time period, the passive immunity acquired from the milk of the sow is lost, newly weaned pigs from different litters are commingled in new pens, and pigs are switched to solid feed (Nagy & Fekete, 1999). The pathogenesis of PWD is complex, but usually involves specific ETEC serotypes and pathotypes. It is frequently seen in almost all large-scale farms, which

suffer enormous economic losses due to weight loss and reduced growth after episodes of diarrhea in weaned pigs (Melkebeek *et al.*, 2013).

ETEC isolates that produce LT, STa or STb, belonging to O serogroups O8, O138, O141, O147, O149 and O157, are most frequently associated with PWD (Nagy & Fekete, 1999; Van Beers-Schreurs *et al.*, 2015). Most strains associated with PWD produce α-hemolysin, although there is no evidence this toxin plays a significant part in the disease process (Moxley *et al.*, 1998). The main adhesin implicated in PDW is F4, although F5, F6, F18 and F41 have also been reported in certain cases. Some strains produce more than one type of adhesin, but whether this imparts a particular advantage over production of only one type of adhesin has not been established. As noted above, production of F4 receptors occurs from birth through post-weaning periods (Erickson *et al.*, 1992); however, production of these receptors decreases with age (Francis, 2002). F18 receptors on the other hand are produced in an increasing number up to 10-21 days of age. Ileal Peyer's patches are particularly rich in certain variants of the F18 receptor. Newborn pigs lack the receptors for certain F18 adhesin variants like F18ab and F18ac, which may explain why enteric colibacillosis occurs as PWD and not ND with F18 fimbrial types (Nagy & Fekete, 1999; Melkebeek *et al.*, 2013; Van Beers-Schreurs *et al.*, 2015).

ETEC vaccines for pigs

Vaccination of pigs to prevent ETEC infections has become increasingly important, since the use of antibiotics in farm animals to prevent diseases and the subsequent rise of antibiotic-resistant strains has become of increasing concern

(Genovese *et al.*, 2000). Most neonatal infections are prevented to some extent by colostral antibodies obtained from sows immunized with parenterally administered ETEC vaccines (Rutter & Jones, 1973). However, since ETEC infection is essentially limited to the intestinal mucosal surface, local active mucosal immunity, rather than systemic immunity acquired through the colostrum is a much more important factor in the prevention of disease (Melkebeek *et al.*, 2013).

Commercial vaccines that are currently available contain either the inactivated bacteria with the adhesin or purified adhesin with or without LT, and are administered parenterally, *e.g.*, by subcutaneous injection. This results in induction of systemic, but not mucosal immunity. Vaccination of sows with these parenteral vaccines provides passive immunity to suckling pigs that is available immediately at birth through the colostrum, but is not available in the milk, within a few days of lactation. Hence, piglets born in herds with endemic ETEC infections suckling sows vaccinated with parenterally administered ETEC vaccines typically become susceptible to ETEC within the first week of life. Protective immunity requires an active mucosal immune response from the intestine, where antigen-specific secretory IgA is produced locally and secreted into the intestinal lumen (McGhee *et al.*, 1992).

ETEC vaccinations against ND

As noted above, vaccines against ND are typically administered to sows with the intent of providing an immediate source of protection to piglets at birth through the colostrum. Typical components used in vaccines against ND include the most prevalent adhesins, *viz.*, F4, F5 and F6, and also LT, since it is immunogenic (Moon & Bunn,

1993). As noted earlier, these vaccines are typically given parenterally to the sow before farrowing, which provides protective immunity for the piglet through colostral antibodies for up to one week. For this to be effective, piglets must consume adequate volumes of colostrum within the first 12 h of life. To improve the efficacy of maternal parenteral vaccines, immune-stimulating complex (ISCOM) coupled with protective antigens has been used. ISCOM consists of saponin adjuvant Quil-A and lipids in equal ratios encased in a matrix that have antigens attached to it by hydrophobic reactions to enable much more effective vaccine delivery (Morein *et al.*, 1984; Nagy *et al.*, 1990).

ETEC vaccinations against PWD

Vaccinations against PWD have involved different strategies. These include oral subunit and live vaccines, encapsulated subunit and live vaccines, and parenteral vaccines. Since most cases of PWD are caused by $F4^+$ and $F18^+$ strains, strategies have involved the generation of F4- and F18-specific secretory IgA antibodies to prevent the colonization of the small intestine by ETEC (Melkebeek, 2013).

The first oral subunit vaccination developed involved the use of purified F4ac fimbrial antigen (Van den Broeck *et al.*, 1999). F4-specific antibodies were detectable in samples from Peyer's patches, intestinal lymph nodes and blood within days of vaccination. The IgA responses generated were similar to those obtained by oral ETEC challenge (Van den Broeck *et al.*, 1999). The presence of the F4 receptor on small intestinal cells is important in the induction of a protective mucosal response. To deliver FaeG, the major fimbrial subunit of F4, alfalfa plants have been used: however, oral immunization by this method provided only a limited and weak immune response. The

main advantage of this method is the ability to produce large quantities of stable, plant-synthesized FaeG (Joensuu *et al.*, 2006). Oral immunization with purified F18 did not produce as a strong protective response as that observed with the F4 subunit vaccine. This held true even when the doses were increased far higher than that for the F4 vaccine (Verdonck *et al.*, 2007). The difference in immunogenic responses may be attributed to the structural differences between the two adhesins. The interactions between the major and minor subunits of F18 are not as stable as those of F4. This renders the F18 subunit vaccine less stable in the gastrointestinal tract (Tiels *et al.*, 2007). In order to overcome this problem, FedF, the F18 subunit, was coupled to the F4 adhesin covalently. The resulting vaccination produced lower shedding of F18 ETEC after challenge (Tiels *et al.*, 2008).

Live oral vaccines consisting of live attenuated wild-type *E .coli* are used to stimulate mucosal immunity in the intestines, which is especially important to protect against PWD. Coliprotec (Prevtech microbia, inc., Montreal, Canada), a live vaccine against F4-mediated PWD in pigs, which is delivered to pigs in the drinking water, has been licensed for used in Canada, Brazil, USA and Mexico. The key antigenic component in this vaccine is a non-virulent *E. coli* strain that expresses F4 fimbriae, but no enterotoxins. Immunization reduces colonization of the intestines by $F4^+$ ETEC, as well as reduces the duration and severity of such an infection. Fluid accumulation in the intestine after an ETEC challenge in Coliprotec-vaccinated pigs was shown to be significantly lower than that in non-vaccinated pigs (Melkebeek *et al.*, 2013).

Live vaccines containing $F18ac^+$ *E. coli* have also been used to take advantage of the fact that the vaccine strain is better able to bind to M cells when complexed with

specific secretory maternal IgA antibodies. Such pathogen-anibody complexes have been used to target the M cells into taking them up and delivering to the underlying immune cells in experimental animal studies (Weltzin *et al.*, 1989). However, the contribution of these vaccines to the level of total protective immunity has come under question because the initial level of maternal-origin IgA in sow's milk which is not complexed to the vaccine has not been determined (Bertschinger *et al.*, 2000).

Encapsulated vaccines are used to protect the live oral and subunit vaccines' passage through the gastrointestinal tract. They also help protect the vaccines against antibodies and receptor analogues that may neutralize the vaccine before it reaches the target (Atroshi *et al.*, 1983). Felder *et al.* (2000) first encapsulated $F18^+$ *E. coli* and F18 adhesin in PLGA (poly lactide-co-glycolide) and used it to orally inoculate weaned pigs. These vaccines failed to produce the expected immunity in PWD pigs, but did produce immunity in suckling pigs. The most probable reasons for this may be that the encapsulated vaccines may have altered the normal immune induction mechanisms (Felder *et al.*, 2000).

In addition to vaccination of pregnant sows, parenteral vaccines are also used in suckling pigs to circumvent the presence of maternal antibodies acquired through the milk. Parenteral vaccination has the potential to boost mucosal immunity that has been acquired already through oral immunization with an attenuated vaccine strain or previous infection. In intramuscular injections, the dose plays a vital role in the immune induction. Larger doses induce a lower immunogenic response than a lower dose of $F4^+$ ETEC *E. coli*. Immunogenic responses may be further improved when a fusion protein containing FaeG, FedF and LT are given intramuscularly. IgA antibodies were observed in serum,

feces, and intestines after inoculation with the triple-fusion protein (Ruan *et al.*, 2011). To induce a systemic secretory IgA response, 1α-25 dihydroxyvitamin D_3 (vitamin D_3) was co-administered with vaccines (Van der Stede *et al.*, 2001; Daynes *et al.*, 2003; Enioutina *et al.*, 2000). When carried out in pigs, it resulted in an increase in the numbers of IgA antibody-secreting cells in the Peyer's patches (Enioutina *et al.*, 1999).

Type II secretion system (T2SS)

Bacteria possess several mechanisms by which they export proteins destined for the extracellular milieu. In Gram-negative bacteria, the secretion of these extracellular proteins poses a particular obstacle as they have to cross two membranes and the periplasmic space to exit the cell. One mechanism is utilized by Gram-negative bacteria for this purpose is the type II secretion system (T2SS). The T2SS is also known as the general secretory pathway (GSP), Sec-dependent secretion system, or simply the classical pathway (Pugsley, 1988). In general, its main function is the translocation of folded proteins from the periplasm to the outside environment. The first such T2SS discovered was in *Klebsiella oxytoca* in 1980 (Nivaskumar & Francetic, 2014). Since 1980, T2SS have been found in hundreds of bacterial species in all classes of *Proteobacteria* which occupy many different environmental niches such as pathogenic, commensal, free living and mutualistic bacteria (Tseng, 2009; Korotkov *et al.*, 2012; Cianciotto 2005; Sandkvist, 2001a). T2SS can also be found in other bacterial species, like chlamydia, spirochetes and cyanobacteria (Nivaskumar & Francetic, 2014).

Human pathogens that contain one or more T2SS include *Vibrio cholerae, Klebsiella oxytoca, Klebsiella pneumoniae, Pseudomonas aeruginosa, Burkholderia*

mallei, Burkholderia pseudomallei, Burkholderia cepacia, Aeromonas hydrophila, Legionella pneumophila, Yersinia enterocolitica, Yersinia pestis, Yersinia pseudotuberculosis and different pathovars of *Escherichia coli*. Plant pathogens with a T2SS include *Erwinia chrysanthemi, Erwinia carotovora, Ralstonia solanacearum Xanthomonas campestris, Xanthomonas axonopodia, Pseudomonas syringae, and Xylella fastidiosa*. Non-pathogenic bacteria that contain a T2SS include *Shewanella oneidensis, Acinetobacter calcoaceticus, Idiomarina loihiensis, Methylococcus capsulatus, Photobacterium profundum, Pseudomonas alcaligenes, Pseudomonas fluorescens, Pseudomonas putida, Bradyrhizobium japonicum, Caulobacter crescentus, Gluconacetobacter diazotrophicus, Mesorhizobium loti, Chromobacterium violaceium, Bdellovibrio bacteriovorus, Geobacter sulfurreducens* and certain free-living *Escherichia coli* (Tseng, 2009; Korotkov et al., 2012; Cianciotto 2005; Sandkvist, 2001b; Campos *et al.*, 2013; Saier, 2006; China & Goffaux, 1999; Nivaskumar & Francetic, 2014).

Substrates secreted by T2SS

The T2SS is typically found in bacteria that tend to colonize host surfaces or form a biofilm, but not invade the host cell. As a result, they use the T2SS to secrete a multitude of different proteins (Sandkvist, 2001b). Proteins targeted for secretion by the T2SS contain an N-terminal signal sequence. These signal sequences are recognized by the Sec pathway, and the peptide sequences are sequestered in the periplasm. The main function of the T2SS is to translocate mature, folded proteins, which enter the apparatus as unfolded peptides but are later packaged into the final product by the chaperones

associated with that particular T2SS or in the periplasm (Sandkvist, 2001a; Cianciotto, 2005; Rondelet & Condemine, 2013).

With few exceptions, the different types of proteins secreted through the T2SS do not seem to share many biochemical, structural, or functional similarities. T2SS can transport either monomeric proteins such as elastase or multimeric proteins with complex structures such as CT, LT, and aerolysin (Sandkvist, 2001a; Cianciotto 2005). That being said, one feature in common among most of these secreted proteins is that their three dimensional structure is replete with β-sheet conformations (Sandkvist, 2001a). In some cases these β-sheets are arranged exclusively in an antiparallel manner, as is the case with exotoxin A secreted by *P. aeruginosa* (Allured *et al.*, 1986). This may be attributed the fact that the putative signal sequence recognized by the T2SS for substances that are targeted for secretion has been found to be rich in β-sheet conformations. However, removal of such structures from the protein has still resulted in the translocation of that protein across the cell envelope in certain cases (McVay & Hamood, 1995). Therefore, it may not be the only requirement for a secretion signal.

Another hypothesis as to what determines secretion of a protein through the T2SS is the overall structure or a particular structural motif, as opposed to a sequence of amino acids located near its N- or C- terminus. For example, LT-IIb, a variant of heat-labile enterotoxin, is secreted in a fashion similar to CT using a T2SS (Connell *et al.*, 1995). This is despite the fact that LT-IIb shares only 11% amino acid sequence identity with CT, unlike LT-I which is virtually identical to it. However, the final arrangement of the pentameric B subunit structure between both CT and LT-IIb is very similar. Therefore, it is likely that the T2SS recognizes the final structure of the folded protein for secretion,

which may explain why bacteria that secrete similarly shaped proteins carry a complete set of genes for a T2SS (Tseng *et al.*, 2009; Sandkvist, 2001a; Sandkvist, 2001b). The large outer membrane pore (~6 nm in diameter), the lack of discrimination in what it secretes, and the apparent high stability of the structure under extremes of pressure, temperature and salt has rendered the T2SS as the best secretion system for transport of a large number of proteins outside the bacterium, as well as for the translocation of structural proteins to the cell surface (Nivaskumar & Francetic, 2014). This promiscuity is observed in several species, which secrete many different proteins through the T2SS: *L. pneumophila*, >25; *V. cholerae*, >21; *Aeromonas* spp., >20; *Dickeya dadantii*, >12. In contrast, *K. oxytoca* secretes only one protein through its T2SS (Rondelet & Condemine, 2013; Douzi et al., 2015).

In addition to secreting toxins such as aerolysin (*Aeronmonas salmonicida*), hemolysin (*Burkholderia vietnamiensis*), CT, LT and exotoxin A, the T2SS is responsible for secreting a wide variety of virulence factors, enzymes, lipoproteins, and surface associated proteins. Most of the enzymes secreted by bacteria via the T2SS are used to provide nutrients for the bacteria, such as lipases, phospholipases, proteases, cholesterol acetytransferases, aminopeptidases, amylases, cellulases, and pullulanases (Cianciotto, 2005; Nivaskumar & Francetic, 2014). Plant pathogens, *e.g.*, *Dickeya dadantii, Pectobacterium carotovorum* and *Xanthomonas campestris*, secrete a wide variety of proteins that degrade cell walls, such as chitinases, cellulases, pectate lysases, pectin methylesterases, and polygalaturonases, (Tseng, 2009; Sandkvist, 2001b). In the case of *L. pneumophila* and *V. cholerae*, the chitinases secreted are used for attachment to the host or to be released from it. Therefore, T2SS is instrumental in determining whether

certain bacteria remain in association with a host or in free-living planktonic form (Nivaskumar & Francetic, 2013).

Another class of proteins that utilizes the T2SS for transport is the outer membrane (OM) proteins. These are typically lipoproteins that depend on the T2SS for their proper localization. A wide variety of organisms including *K. oxytoca, E. coli, S. oneidensis, D. dadantii*, utilize this pathway for surface protein localization (Tseng *et al.*, 2009; McLaughlin *et al.*, 2012; Rondelet & Condemine, 2013). In *K. oxytoca*, PulA, an OM protein that forms large complexes whose function is to remove branches from starch molecules, is located to the surface by T2SS. This protein can be either bound to the OM or released and exist as a free form. In the absence of the *pul* operon for the T2SS, PulA is tethered to the inner membrane (IM; Pugsley *et al.*, 1991).

In certain *E. coli* pathovars, such as EPEC, EAEC, EIEC and extraintestinal *E. coli*, a T2SS is used to translocate SslE protein to the OM. This protein is suspected to act as a protective antigen in these pathovars. Moreover, mutation of *sslE* or the absence of a complete T2SS in certain *E. coli* results in defective biofilm formation, cell attachment, and cells that are generally less virulent than their counterparts with both the functional gene and a complete T2SS (Baldi *et al.*, 2012; Rondelet & Condemine, 2013).

Some of the most unusual and remarkable lipoproteins translocated by T2SS are the c-type cytochromes that consist of multiple heme units. These multimeric electron transporters and their associated T2SS are found in *Shewanella oneidensis* and certain *Geobacter* spp. They allow these bacteria to utilize >20 different molecules, including insoluble Fe (III), Mn (IV) oxides, uranium or dimethyl sulfoxide (DMSO), as terminal electron acceptors for respiration in the absence of oxygen. Several such proteins that

render such a feat have been identified include: CymA, an IM protein that oxidizes quinol; MtrB, a trans-OM porin-like protein that transfers electrons across the OM to cytochromes; MtrC and OmcA, OM proteins that act as terminal reductases; and DmsA and DmsB, OM proteins that make up the DMSO reductase (Rondelet & Condemine, 2013; Nivaskumar & Francetic, 2014). In all these cases, expression of the T2SS along with the electron transport protein is essential for their function (Rondelet & Condemine, 2013).

Nomenclature for transport systems

The T2SS have been previously referred to as the main terminal branch (MTB) of the GSP, a two-step secretory pathway consisting of a general export pathway (GEP) to target relevant proteins from the cytosol to the periplasm, which are then folded and, utilizing energy in the form of ATP are transported, translocated or secreted using the OM pore (Desvaux *et al.*, 2004; Saier, 2006; Nivaskumar & Francetic, 2014). However, the indiscriminate use of the acronyms GSP and T2SS has prompted an effort for their clarification. The GEP is now referred to more precisely as either Tat (twin-arginine translocation) or the Sec pathway, which is responsible for sequestering the cytosolic proteins carrying the appropriate N-terminal signal sequence. Instead of using the MTB, the protein complexes that secrete the final mature product are referred to as type II, type IV or type V secretion pathways. Finally, GSP is only referred to when describing translocation of proteins utilizing the Sec pathway (Desvaux *et al.*, 2004). T2SS is usually referred to as the Sec-dependent pathway, owing to the fact that proteins that are

secreted by T2SS are first translocated to the periplasm via the Sec machinery and its associated chaperones (Tseng *et al.*, 2009).

Structure of T2SS genes

Proteins associated with the assembly of the T2SS are generally referred to as GSP proteins (Gsp). These are encoded by 12-16 genes and include ≥12 different proteins that interact to assemble three substructures: the pseudopilus, IM platform and the OM platform (McLaughlin *et al.*, 2012). Usually the genes that encode Gsp are arranged in one large, highly conserved operon designated as *gspCDEFGHIJKLMNO* (Sandkvist, 2001b, Nivaskumar & Francetic, 2014; Douzi *et al.*, 2015; Korotkov *et al.*, 2012). The *gspC-O* operon represents the core genes for the T2SS apparatus (Douzi et al., 2015). In some bacterial species, extra genes that contribute to the secretion mechanism are present in the same operon.

In *K. oxytoca* and *E. chrysanthemi* the extra genes are *gspS* and *gspB*, while in *V. cholerae, A. hydrophila* and *E. coli* K-12 species the extra genes are *gspA* and *gspB*, while in certain *E. coli yhgG* and *aspS* are closely associated with the T2SS operon. Most of these extra genes, when present, encode for proteins that help in the localization and assembly of GspD, which forms the OM channel of the T2SS. The genes, *gspA* and *gspB* encode for a peptidoglycan-binding complex that helps in the multimeric assembly of the GspD pore in the OM, while *gspS, aspS* and *yhgG* encode a protein called pilotin, which enables the targeting of GspD to the OM. However, some researchers have considered *gspN* as a non-core gene (Douzi *et al.*, 2015).

Although the T2SS operon is highly conserved among species, the level of amino acid sequence identity varies between the genes of different species. Among proteins encoded by *gspC, gspL* and *gspM*, the amino acid sequence identity varies from 25 to 40%, while among *gspE, gspF* and *gspG* it ranges from 60 to 80% (Sandkvist, 2001b; Filloux, 2004; Douzi *et al.*, 2015). In most cases, the T2SS genes are arranged in a single continuous operon and named by convention in alphabetical order. In several instances, this arrangement results in overlapping genes. However, variations from this structure, when present, almost always occur at either the 5′ or 3′ ends. This is observed in *K. oxytoca, A. hydrophila,* and *P. aeruginosa*, where *gspO, gspS* or *gspA* exist separate from the rest by a stretch DNA, but are still closely associated with it.

The *gspO* gene is usually the last gene in the T2SS gene cluster, and bears a striking resemblance to the gene for prepilin peptidase, which is essential in the processing of type IV pilus (T4P) subunits. When *gspO* is not associated with the other T2SS genes it is found among genes required for T4P, where it methylates the T4P subunits and proteins encoded by *gspG-K*. Transcription of the operons can also vary among the species. Typically the *gspC-O* genes are all transcribed in alphabetical order, from the same promoter. However, in the case of *P. aeruginosa* and *P. alcaligenes*, the operon is arranged into two distinct transcription regions. The *gspC* and *gspD* genes in this case are transcribed in the opposite direction from the *gspE-N*. It is more than likely that two different promoters are used in the transcription of such divergently arranged operons (Sandkvist, 2001b; Filloux, 2004).

T2SS are highly similar in composition and structure to systems that build archaeal pili and flagella, class a, class b and competence pili, filamentous bacteriophage,

and T4P assembly systems. The T2SS and T4P are indeed considered subclasses of cellular nanomachines used to assemble filamentous structures (McLaughlin *et al.*, 2012). All these systems share the ability to promote the assembly of helical filaments composed of plasma membrane-embedded pilin subunits. Even when there is no detectable amino acid identity between T2SS and T4P, both systems share functional and structural overlap. Together, with the ubiquitous nature of T2SS among all *Proteobacteria* suggests they all share an early common origin (McLaughlin *et al.*, 2012; Nivaskumar & Francetic, 2014).

Structure and assembly of T2SS proteins

The 12-16 major proteins encoded by the T2SS operon perform specific functions in the assembly of the secretory apparatus (Filloux, 2004; Nivaskumar & Francetic, 2014). GspC is an inner membrane protein that interacts with the secretin component in the outer membrane. GspD is an outer membrane protein that forms the secretin pore. GspE is the secretion ATPase that provides the energy for protein translocation. GspF is an inner membrane platform protein. GspG is the major pseudopilin. GspH, GspI, GspJ, GspK are minor pseudopilin proteins that form the tip complex to initiate pseudopilus formation. GspL and GspM are inner membrane platform proteins. GspN is an inner membrane protein that interacts with GspD secretion. GspO is a prepilin peptidase that processes the minor pseudopilin units prior to their assembly (Filloux, 2004, Camberg & Sandkvist, 2005; McLaughlin *et al.*, 2012; Korotkov *et al.*, 2012; Douzi *et al.*, 2015).

The entire T2SS assembly is likely to span the cell envelope and form a bridge between the IM and the OM; however, such a membrane-spanning structure appears to be

a transient state as no crystal structures have been isolated (Sandkvist, 2001a). The complete T2SS assembly can be broken down into four distinct parts: the pseudopilus assembly; the outer membrane complex or secretin assembly; the secretion ATPase assembly; and the inner membrane platform assembly (Korotkov *et al.*, 2012).

Pseudopilus assembly

All five major and minor pseudopilins show a wide diversity within their 3D structures. They share a long hydrophobic, N-terminal α-helix followed by a variable region, and a semi-conserved β-sheet conformation in most of their domains (Korotkov *et al.*, 2012). The four pseudopilins, GspH, GspI, GspJ and GspK are targeted to the IM via the signal recognition particle (SRP) pathway or the Sec machinery. Their correct insertion requires a positively-charged polypeptide, 6-8 residues long, which serves as an N-terminal signal anchor (McLaughlin *et al.*, 2012).

Once the pseudopilins are inserted, GspO, the prepilin peptidase, removes the signal sequence by cleaving at a conserved Gly residue to enable the subunits to assemble into helical structures (Filloux, 2004). The order of assembly of the minor pseudopilins may be determined by the differences in their variable regions. Of the four subunits, extensive variable regions are present among GspH and GspJ, while GspI and GspK differ in having a single β-sheet in their variable regions (Korotkov *et al.*, 2012).

Once assembled, the helical pseudopilus has a tip composed mostly of GspK, GspI and GspJ. Of these, it is assumed that GspK occupies most of the tip, since it covers up the other two subunits. GspH is present during the secretion phase to add more GspG

to the helical structure. In some cases, GspN is also a part of this structure (Campos *et al.*, 2013).

The major pseudopilins are shaped as an elongated lollipop, similar to the same structures observed in homologous T4P. The stem of the GspG is a 53-residue S-shaped structure, the N-terminus of which is embedded in the membrane while the C-terminus protrudes into the periplasm (Korotkov *et al.*, 2012; Nivaskumar & Francetic, 2013). Within GspG, a conserved C-terminal loop in a β-sheet formation contains a Ca^{2+}-binding site. Mutations in this binding site result in impaired function of T2SS, which can be attributed to the reduced interaction among the pseudopilin subunits and the rest of the T2SS machinery (Sandkvist, 2001a).

GspG is the only protein involved in the composition of the hyper-pseudopili, the extracellular portion of the T2SS pili. Under physiological conditions, the helical structure formation by GspG is restricted to the periplasm (Korotkov *et al.*, 2012). The periplasmic pseudopilus is a 400-kDa, right-handed helical complex structure comprised mostly of GspG and GspH, very similar in structure to gonococcal T4P (McLaughlin *et al.*, 2012; Campos *et al.*, 2013). Other minor pseudopilins GspI, GspJ and GspK are found in low abundance in the pseudopilus, mostly due to protein degradation in the periplasm.

Contacts made between the different helical components of the pseudopilin complex are essential for protein secretion under physiological conditions (Korotkov *et al.*, 2012). The energy needed for the pseudopilus assembly is provided by the ATPase, GspE (Douzi *et al.*, 2015). Assembly of the minor pseudopilins has been implicated in secretin (GspD) channel opening. In the proposed model, extraction of GspK from the IM

to form the pseudopilus acts as a switch to activate GspE, which initiates ATP hydrolysis. This signal is transmitted via GspL which connects both GspE and GspK. The initial ATP hydrolysis provides the energy required for the assembly of GspG. GspH binds to the tip of the rapidly assembling GspG helical structure through its globular domain. The assembled pseudopilins ensure the opening of the secretin for maximal protein secretion. Consistent with the estimated low levels of minor pseudopilin subunits under physiological conditions, one initiation event would allow continued pseudopilus elongation catalyzed by GspE. However, this model has not been experimentally demonstrated. The minor pseudopilins are capable of binding specific exoproteins by forming a stable quaternary complex specific to certain substrates (Sandkvist, 2001a; Korotkov *et al.*, 2012; Howard, 2013; Douzi *et al.*, 2015).

Assembly of secretion complex in the OM

The OM complex, the secretin pore of the T2SS, is formed by the multimerization of GspD protein. Secretins are large multimers of approximately 80 kDa, consisting of 12-15 subunits arranged in a circular manner to form the pore that extrudes the folded proteins from the periplasm (Howard, 2013; Nivaskumar & Francetic, 2014). In most species, this arrangement is in the shape of dodecamers. Secretins are highly stable since their proteins consist of trypsin-resistant N- and C-terminal domains. They have been shown to withstand denaturation due to extremes of heat and exposure to detergents, probably due to the large multimerization interfaces of the subunits (Korotkov *et al.*, 2012; Douzi *et al.*, 2015).

The embedded secretin structure is constricted in the middle by a continuous periplasmic gate, which divides it into a large, segmented periplasmic vestibule consisting of three concentric rings and an external chamber with a smooth surface (Korotkov *et al.*, 2011). The periplasmic vestibule has a wide opening of 70 Å that narrows down to a 55 Å constriction approximately two-thirds of the way into the channel. The periplasmic region consists mainly of large, N-terminal domains connected by flexible linkers, which undergo large conformational changes in order to organize the subunits into a hexamer of dimers (Korotkov *et al.*, 2011; Korotkov *et al.*, 2012).

The secretin channel is only open when secretion of a protein is imminent, otherwise it remains closed in the resting state. The specific signal for this opening is hypothesized to be transmitted when the pseudopilus is extended (Sandkvist, 2001b; Campos *et al.*, 2013; Douzi *et al.*, 2015). The extracellular chamber is closed from the top by an extracellular gate that has a small opening of 10 Å, which is constitutively open. This opening acts similar to a porin channel allowing passage of molecules smaller than 600 Da (Howard, 2013).

GspS, also referred to as pilotins, are used in the targeting and insertion of secretin to the OM. In addition to the targeting, pilotins also provide protection for GspD from proteolysis in the periplasm (Sandkvist, 2001b; Filloux, 2004; McLaughlin, 2012). In species lacking pilotin genes like *Aeromonas, Vibrio* and *E. coli*, this function is achieved by the GspA-GspB protein complex. However, deletion of these genes does not seem to affect protein secretion, leading to the conclusion that they support an accessory role and are present as functional redundancies (Cianciotto, 2005; Nivaskumar & Francetic, 2014). The recent discovery that YhgG and AspS in *E. coli* and *Vibrio*,

respectively, provides the same function bolsters this argument. Chaperones present in the periplasm, such as DegP, SurA or Skp, may also play a part in the secretin targeting to the OM (Howard, 2013; Campos *et al.*, 2013). It has been established that the pilotins are only needed for the targeting of the subunits to the proper cell membrane and are not needed for the multimeric assembly. This has been shown to occur in the absence of pilotin or any other chaperones responsible for targeting to the OM (Korotkov *et al.*, 2012).

Secretion ATPase assembly

The proton motive force required for the assembly of T2SS components and the subsequent secretion of molecules through it is provided by GspE, an ATPase, present in all functioning T2SS (Sandkvist, 2001b). It functions optimally in low salt conditions, at pH 8.5-9.5, and at temperatures ranging from 37°C-44°C (Camberg & Sandkvist, 2005). For catalytic activity to occur, it requires Mg^{2+} ions (Camberg & Sandkvist, 2005). Zinc is needed, not for catalytic activity, but for coordination between its domains.

Based on crystallization and electron microscopy studies, the functional form of GspE is considered to be hexameric (Camberg & Sandkvist, 2005: Douzi *et al.*, 2015). GspE functions are provided by the Walker A and B motifs as well as His and Asp boxes (Douzi *et al.*, 2015). Mutations in the Walker A motif severely impair secretion, but mutations in Walker B motif do not. The 'Asp Box' that exists between the Walker A and B motifs interacts with the Mg^{2+} ions and stabilizes the nucleotide binding. The six Arg residues near the ATP binding site are essential for ATP hydrolysis and communication

among the subunits of the hexamer. A domain found in the C-terminal region binds Zn^{2+} using four Cys residues.

The ATPase activity of GspE is greatly enhanced by the GspL cytoplasmic domains, through which it is tethered to the inner membrane assembly (Korotkov *et al.*, 2012; Nivaskumar & Francetic, 2014). This interaction is necessary because GspE is mostly composed of hydrophilic residues and lacks hydrophobic domains that can anchor it to the IM. The binding and hydrolysis of ATP leads to large conformational changes within the hexamer, which are then transmitted to the other T2SS components via GspL. The conformational changes incur binding of pseudopilin subunits, leading to the extension of the pseudopilus (Korotkov *et al.*, 2012).

Inner membrane platform assembly

The inner-membrane platform comprises at least four different membrane proteins, GspM, GspL, GspF and GspC associated with GspE (McLaughlin *et al.*, 2012). GspM has a short cytoplasmic sequence, a transmembrane helix (TMH), and a peri-plasmic domain consisting of a ferredoxin fold (Korotkov *et al.*, 2012). GspL consists of a cytosolic domain, a TMH, and a periplasmic domain with homology to the ATPase family of proteins; however, no ATP-binding site has been found in the structure. The periplasmic domain of GspL also has the same ferredoxin fold observed in the periplasmic domain of GspM. GspL binds the ATPase between the cleft observed among domain II and III (Korotkov *et al.*, 2012; Douzi *et al.*, 2015). GspF is a transmembrane protein consisting of three TMHs and two homologous cytoplasmic domains (Sandvkist, 2001a; Korotkov *et al.*, 2012). GspC consists of a short cytoplasmic sequence, a TMH,

and two periplasmic domains, the homology region domain and a PDZ domain, a common structural component of signaling proteins consisting of 80-90 aminio acids. The peptide-binding groove in the PDZ domain in GspC is wider than PDZ domains in other proteins. It is hypothesized that the PDZ domain might be involved in the regulation of secretion specificity (Korotkov *et al.*, 2011, 2012).

The inner membrane platform (IMP) has been shown to consist of equimolar amounts of GspL, GspM, GspC and GspE (McLaughlin, 2012). How many subunits of GspF take part in the assembly is unknown. The IMP structure is stabilized by the interaction between GspL and GspM. The final assembled IMP complex interacts with the other components of the T2SS. The IMP interacts with the pseudopilus through the GspL component. GspC is the main component which connects the outer membrane complex to the IMP (Nivaskumar & Francetic, 2014). This interaction with the GspD secretin is mediated via the periplasmic HR domain of GspC. GspN, when present in the T2SS, also takes part in the interaction between inner and outer membrane protein assemblies (Korotkov *et al.*, 2011; Nivaskumar & Francetic, 2014).

Secretion mechanisms of T2SS

Secretion of exoproteins via the T2SS is a two-step process (Desvaux *et al.*, 2004; Saier, 2006). The protein subunits or peptides to be secreted are first transported to the periplasmic space via the Sec-pathway (Mori & Ito, 2001). In the case of enterotoxins such as CT or LT, the individual peptides are targeted to the periplasm via a hydrophobic N-terminal signal sequence consisting of approximately 20 amino acids. The energy for

this transfer is supplied by a proton motive force generated by the ATP hydrolysis of Sec ATPase (Mori & Ito, 2001).

The peptides are translocated in an unfolded state since chaperones associated with the Sec system, such as SecB are unable to handle folded proteins. SecB transports the bound peptide chain to the membrane bound SecA protein (Tseng *et al.*, 2009). While it is being translocated, the signal sequence is cleaved away leaving the mature protein in the periplasm. In the periplasmic space, proteins are assembled into their final conformation utilizing the chaperones and enzymes present at the location. In the case of LT, DsbA, a disulfide isomerase, is needed for the final non-covalent assembly of the AB_5 holotoxin (Hardy *et al.*, 1988). This folding is essential for the final secretion from the secretin component, since misfolded proteins are not recognized by the T2SS machinery.

Biogenesis of the T2SS machinery starts with the formation of the outer membrane secretin. Then GspC binds secretin and firmly anchors the IMP to the outer membrane (Korotkov *et al.*, 2012). The generation of pseudopilus also occurs, but whether it precedes or follows the anchoring is not known. Folded proteins bind the periplasmic domains of GspD, GspC or to the tip of the pseudopilus (Nivaskumar & Francetic, 2014). The final extrusion of the T2SS bound protein may occur in one of two proposed ways. These two mechnisms are named, “the piston,” and the “Archimedes screw” (Campos *et al.*, 2013).

In the piston model, the assembly of the pseudopilin provides the required driving force for secretion. The folded protein is bound to the tip of the growing pseudopilus, which in turn undergoes cycles of extension and retraction powered by ATP hydrolysis.

The piston-like movement propels the exoproteins through the secretin. In this model, minor pseudopilins determine the substrate specific binding (Campos *et al.*, 2013; Nivaskumar & Francetic, 2014).

In the Archimedes model, exoproteins are bound to the base of the pseudopilin rather than the tip. Therefore, the major pseudopilin, GspG, is considered the substrate specific binding surface. The addition of new GspG subunits to the pseudopilin rotates the helical fibers similar to the rotation of the flagellar motor. The rotary movement and the addition of new GspG subunits propel the exoproteins bound to the pseudopilus through the secretin channel (Campos *et al.*, 2013). Of the two models described, experimental and biochemical data support the piston model.

T2SS in *E. coli*

Although a T2SS has been identified in *E. coli* K-12, it is not expressed under normal laboratory growth conditions (Pugsley *et al.*, 1988). Transcription of a T2SS is increased several fold in strains lacking H-NS, but still does not result in detectable levels of secreted protein (Pugsley & Francetic, 1998). STEC O157:H7 has the remnants of a T2SS. The original genes appear to be replaced by novel DNA regions. However, a T2SS is present in the large 92-kb plasmid, pO157 (Sandkvist, 2001b). This plasmid contains *gspC-gspM*, as well as the accessory gene, *gspS*. Whether STEC O157:H7 encodes a functional T2SS is unknown (Sandkvist, 2001b).

The first report of a functional T2SS in ETEC was reported by Tauschek *et al.* (2002). It was identified in the prototypic human ETEC strain H10407. This operon consists of *gspC-gspM*, and lacks *gspN*. Proximal to the transcription site of *gspC* lays

yhgG, which encodes an accessory protein for pseudopilus assembly, and *pppA*, which encodes the prepilin peptidase. These genes are absent from K-12 strains, and share little homology with the analogous components from the T2SS it possesses. However, the T2SS used by *V. cholerae* to secrete CT shares a high degree of similarity with H10407 T2SS. Deletion of T2SS components resulted in lower LT secretion by H10407 (Tauschek *et al.*, 2002).

Genomic analysis of several sequenced porcine ETEC strains has revealed the presence of a T2SS (Shepard *et al.*, 2012; Wijemanne *et al.*, 2015). This T2SS shares >90% amino acid sequence identity with the one described in H10407 by Tauschek *et al.* (2002). Furthermore, phylogenetic analysis reveals that porcine T2SS and the human T2SS tend to cluster together, revealing a common genetic origin (Wijemanne *et al.*, 2015).

Hypotheses derived from existing literature

The capacity of ETEC to secrete LT was not established until Tauschek *et al.* (2002) demonstrated that the T2SS consisting of GspC-M was required for the secretion of LT in the human prototype ETEC strain H10407. Subsequently, secretion of LT was shown to be more highly correlated with the induction of net fluid accumulation in the small intestine than total production of LT based on studies involving H10407 inoculated into rabbit ileal loops (Lasaro *et al.*, 2006). Studies in our lab demonstrating that LT production by ETEC enhances colonization (Berberov *et al.*, 2004) and the rate of weight loss (Erume *et al.*, 2008) in gnotobiotic piglets, led us to hypothesize that LT is a more

important enterotoxin than STb in the pathogenesis of enteric colibacillosis in neonatal piglets.

The study by Johnson *et al.* (2009) demonstrating that LT mediates adherence of porcine ETEC to porcine intestinal epithelial cells *in vitro*, coupled with other studies reporting that exposure of ETEC to glucose induces LT production and secretion (Gilligan *et al.*, 1979; Kunkel *et al.*, 1979), led us to hypothesize that exposure of ETEC to glucose at an optimal concentration would lead to enhanced intestinal epithelial adherence mediated by induction of LT expression. If this were true, it would establish the physiological relevance of the effects of glucose on LT production and provide a basis for how glucose intake may influence the severity of infection.

Previous studies from our lab showing that ligated jejunal loops in fasted, 6-8-week-old weaned pigs inoculated with LT^+ ETEC strains did not have detectable fluid accumulation in contrast to loops inoculated with STb^+ strains, led us to hypothesize that glucose is needed in the intestinal lumen for induction of LT expression. Similarly, we hypothesized that the significance of LT in the neonatal gnotobiotic piglet model (Berberov *et al.*, 2004; Erume *et al.*, 2008) was related to the high glucose levels in the intestines of these piglets due to the feeding of milk replacer.

The LT and STa promoters in H10407 have been shown to be regulated in a differential manner with regard to the starting concentration of glucose in the culture medium (Bodero & Munson, 2009). Based on the study by Busque *et al.* (1995) who reported that the gene for STb (*estB*) is subject to catabolite repression, we hypothesize that the *eltAB* and *estB* promoters are under a similar differential (inverse) regulatory relationship as that thought to be true for *eltAB* and *estA* in H10407.

Hence, we raised several hypotheses and tested them in a series of experiments, the results of which are included in the subsequent chapters. Three hypotheses were established for the work in Chapter 1: porcine WT ETEC strains from cases of enteric colibacillosis secrete LT; LT secretion is more highly correlated with virulence than is total LT production; and porcine WT ETEC strains contain a T2SS similar to that of human prototype ETEC strain H10407. Chapter 2 explored the hypothesis that glucose, at a concentration optimal for LT expression, enhances bacterial adherence through the promotion of LT production. Finally in Chapter 3, the hypothesis that LT and STb genes (*eltAB* and *estB*, respectively) in the same ETEC strain are simultaneously, differentially regulated by glucose concentrations was explored.

CHAPTER 2

RELATIONSHIP BETWEEN HEAT-LABILE ENTEROTOXIN SECRETION CAPACITY AND VIRULENCE IN WILD TYPE PORCINE-ORIGIN ENTEROTOXIGENIC *ESCHERICHIA COLI* STRAINS

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Abstract

Heat-labile enterotoxin (LT) is an important virulence factor secreted by some strains of enterotoxigenic *Escherichia coli* (ETEC). The prototypic human-origin strain H10407 secretes LT via a type II secretion system (T2SS). We sought to determine the relationship between the capacity to secrete LT and virulence in porcine-origin wild type (WT) ETEC strains. Sixteen WT ETEC strains isolated from cases of severe diarrheal disease were analyzed by GM1ganglioside enzyme-linked immunosorbent assay to measure LT concentrations in culture supernatants. All strains had detectable LT in supernatants by 2 h of culture and 1 strain, which was particularly virulent in gnotobiotic piglets (3030-2), had the highest LT secretion level all porcine-origin WT strains tested ($P<0.05$). The level of LT secretion (concentration in supernatants at 6-h culture) explained 92% of the variation in time-to-a-moribund-condition ($R^2 = 0.92$, $P<0.0001$) in gnotobiotic piglets inoculated with either strain 3030-2, or an ETEC strain of lesser virulence (2534-86), or a non-enterotoxigenic WT strain (G58-1). All 16 porcine ETEC strains were positive by PCR analysis for the T2SS genes, *gspD* and *gspK*, and bioinformatic analysis of 4 porcine-origin strains for which complete genomic sequences were available revealed a T2SS with a high degree of homology to that of H10407. Maximum Likelihood phylogenetic trees constructed using T2SS genes *gspC*, *gspD*, *gspE* and homologs showed that strains 2534-86 and 3030-2 clustered together in the same clade with other porcine-origin ETEC strains in the database, UMNK88 and UMN18. Protein modeling of the ATPase gene (*gspE*) further revealed a direct relationship between the predicted ATP-binding capacities and LT secretion levels as follows: H10407, -8.8 kcal/mol and 199 ng/ml; 3030-2, -8.6 kcal/mol and 133 ng/ml; and

2534-86, -8.5 kcal/mol and 80 ng/ml. This study demonstrated a direct relationship between predicted ATP-binding capacity of GspE and LT secretion, and between the latter and virulence.

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) strains are important causes of diarrhea among travelers and children <5 years old living in developing countries (Walker *et al.,* 2007), and in addition, are economically important pathogens of pigs and cattle (Nagy & Fekete, 2005; Zhang *et al.,* 2006). ETEC infections are especially severe among young swine, causing illness and deaths of nursing and post-weaned piglets (Francis, 2002). In swine, the most common and severe ETEC infections are caused by strains that express F4 (K88) fimbria (Berberov *et al.,* 2004). These strains usually produce two major enterotoxins that cause net fluid loss and diarrhea, *viz.*, heat-labile enterotoxin (LT) and heat-stable enterotoxin-b (STb) (McVeigh *et al.,* 2000; Moon *et al.,* 1971). Some strains also may produce heat stable enterotoxin-a (STa) or enteroaggregative *E. coli* heat-stable enterotoxin 1 (Berberov *et al.,* 2004)

Tauschek *et al.* (2002) discovered a type II secretion system (T2SS) in the human prototypic ETEC strain H10407 similar to the one in *Vibrio cholerae*, and demonstrated that it is functional and necessary for secretion of LT in ETEC. Both Tauschek *et al.* (2002) and Lasaro *et al.* (2006) demonstrated the presence *gspD* and *gspK* in ETEC strains from human patients and from this observation inferred that the T2SS is highly conserved among ETEC. However, to our knowledge, no porcine-origin ETEC strains have been tested for these genes and among human-origin strains only the H10407 T2SS sequence has been analyzed (Tauschek *et al.,* 2002).

Lasaro *et al.* (2006) hypothesized that strain-specific differences in production and secretion of LT by human-origin ETEC correlated with symptoms induced *in vivo*. Although these authors demonstrated that the secretion levels, in contrast to the total

amounts of LT produced were correlated with volumes of fluid accumulation in ligated rabbit ileal loops, the authors were not able to demonstrate a relationship between LT secretion and clinical symptoms in human patients. In that study, the clinical data was limited to presence or absence of diarrhea, and whether the affected children were co-infected with other pathogens was not reported.

In the present study, we sought to test the hypothesis that LT secretion is correlated with virulence using wild-type porcine-origin ETEC strains for which we had clinical and pathological records of natural cases of disease, and conclusive evidence that ETEC was the sole cause of disease. In addition, for two of these ETEC strains which differed in virulence and a non-enterotoxigenic wild-type porcine-origin control strain, we conducted experimental gnotobiotic pig inoculations and also had genomic sequence data available. Hence, using these strains, we assessed the relationship between virulence and LT secretion, and also compared the sequences of T2SS genes by bioinformatics analysis.

Materials and Methods

Strains. The strains used in this study are shown in Table S1. Included among these were 16 porcine-origin wild type (WT) LT^+ STb^+ ETEC strains from cases of severe diarrheal disease or sudden death, most with lesions of hemorrhagic enteritis (Erume *et al.,* 2010; Francis & Willgohs, 1991; Moxley *et al.,* 1998), human WT ETEC strain H10407 (O78:K80:H11, $CFA/1^+$, LT^+, STp^+, STh^+) (Evans *et al.,* 1973), and non-pathogenic LT^- STb^- *E. coli* control strains (Hanahan, 1985; Zhang *et al.,* 2006). All

strains were confirmed to have the appropriate LT (*eltAB*) and STb (*estB*) gene content by PCR using primers and methods as previously described (Berberov *et al.,* 2004).

Media and growth conditions. In order to properly assess levels of LT secretion, we needed to use optimized culture conditions, as this has been reported to have a significant effect on LT production and release in previous studies. Casamino Acids-yeast extract (CAYE) medium-Mundell (CAYE-M) (Mundell *et al.,* 1976; Ristaino *et al.,* 1983) was prepared with Bacto Casamino Acids (2.0%) and Difco yeast extract (0.6%), and glucose at a final concentration of 0.25%, and adjusted to a final pH of 8.5.

Our first experiment aimed to determine whether porcine-origin WT ETEC strains could secrete LT. This experiment utilized prototype human-origin ETEC strain H10407 as a positive control;, test strains 2534-86 and its isogenic derivatives [MUN297(LT^+), MUN299 (LT^-), MUN300 (LT^-), and MUN301 (LT^+)]; negative control K-12 strain DH5α; positive control strain MUN302 (a DH5α-based LT^+ clone); and WT porcine-origin negative controls (LT^- strains 1836-2 and G58-1). For this experiment, starter cultures were grown overnight in 15 ml of CAYE-M in a 125 ml conical flask at 37°C and 225 rpm. After overnight incubation, a 1:100 dilution of each starter culture was inoculated into fresh medium of the same kind using the same flask-to-medium ratio (8.3:1), and incubated for 18 h at 37°C and 225 rpm. Samples were then obtained for preparation of supernatants and periplasmic extracts for LT determination.

A second experiment as a test of LT secretion attempted to rule out bacterial cell lysis as a contributor to LT presence in the culture supernatant. This experiment utilized porcine-origin LT^+ WT strain 2534-86, 2 isogenic derivatives of this strain (LT^+ MUN298 and LT^- MUN299), and human-origin prototype strain H10407, with strains

grown in CAYE-M. For this experiment, starter and experimental cultures were prepared in CAYE-M as described for the first experiment, but samples were collected at 4, 8, 12 and 24 h PI. To confirm that growth rates in CAYE-M among strains did not vary significantly, results which could confound the measured LT concentrations, growth curves on all strains were conducted with samples obtained at 0, 4, 8, 12 and 24 h. On aliquots of these samples, the OD_{600}, colony-forming units (CFU)/ ml, and pH values were determined. CFU/ml were determined by serial 10-fold dilution in phosphate-buffered saline (pH 7.4, 0.1 *M*; PBS) and plating on LB agar.

A third experiment tested the capacity for 16 WT LT^+ porcine strains isolated from cases of severe disease to secrete LT (Table S1), and was conducted using the same protocol as that used in the second experiment, except that samples were collected at 2, 4, 6 and 18 h of culture.

For each of the 3 main experiments, 3 independent replicate experiments were conducted on different days using new cultures.

Supernatant and periplasmic extract preparation. To prepare cell-free supernatant, 1-ml aliquots of bacterial cultures were collected, centrifuged at 2,150 × *g* for 10 min, and passed through a 0.2 μm filter. To prepare periplasmic extracts, the corresponding cell pellets from the same aliquots were given 3 series of suspensions in 1 ml of PBS and centrifugations at 2,150 × *g* for 10 min. The final re-suspension was in 1 ml of 2 mg/ml solution of polymyxin B in PBS for 30 min in a 37°C water bath; this was centrifuged at 2,150 × *g* for 10 min, 0.2 μm-filtered and the liquid fraction was obtained as the periplasmic extract. For each sample of culture supernatant and periplasmic extract, a lack of contamination by live cells of the strain of origin was confirmed by

culturing an aliquot overnight in LB broth and on LB agar at 37°C. To test whether LT was present in culture supernatants as a result of secretion, bacterial lysis or both during culture, aliquots of culture supernatants at 4, 6, 12, 18, and 24 h and periplasmic extracts at 2, 4, and 6 h of culture were mixed with equal volumes of *p*-nitrophenyl phosphate (PNPP, Sigma), the substrate for alkaline phosphatase, which is normally present in high content in the periplasmic space in intact *E. coli* cells (Tauschek *et al.,* 2002). Mixtures of sample and PNPP in 0.2 M Tris buffer in 96-well plates were incubated at room temperature for 30 min. At that time, 25 µl of 5.0 N NaOH was added to stop the reaction, and the OD_{405} was measured.

Assays for LT production and secretion. The concentrations of LT in the periplasmic extracts and culture supernatant were determined by GM1- ganglioside enzyme-linked immunosorbent assay (GM1-ELISA) using methods previously described (Ristaino *et al.,* 1983; Tauschek *et al.,* 2002) with the following modifications. All washings were done with 1% Tween 20 in PBS. The primary antibody was a rabbit anti-CT IgG (Sigma) at a 1:2000 dilution, and the secondary antibody was alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) at a 1:16,000 dilution in the first experiment, and at a 1:8000 dilution in the second and third experiments. Incubations of 60 min at 37°C were given after the addition of each antibody. The substrate was PNPP (Sigma) in 0.2 M Tris buffer, and after its addition, plates were incubated for 30 min at room temperature and OD measured at 405 nm. Standard curves were generated in each assay plate by using 2-fold serial dilutions of purified LT (List Biological Laboratories, Product No. 165B) at a starting concentration of 400 ng/ml. Regression analysis

(R^2>0.97) was used to generate a standard curve for determination of LT concentrations in the test samples.

Culture supernatants of MUN297, MUN299, MUN300, MUN301, MUN302, H10407, 1836-2, G58-1, DH5α, 2534-86, WAM2317, and 3030-2 were tested in Y1 adrenal cell assays to confirm that LT biological activity was present or absent in samples from LT^+ and LT^- strains, respectively. Y1 adrenal cell assays were conducted by the methods of Sack and Sack (1975) with minor modifications. Y1 adrenal cells from ATCC (CCL-79) were grown in F-12K medium (ATCC) supplemented with 15% horse serum (ATCC) and 2.5 % fetal bovine serum (Sigma). For the LT assays, 100 µl of culture supernatants diluted 1:10 were added onto Y1 cell monolayers in duplicate wells in the initial rows of 96-well microtiter plates, and then were serially 2-fold diluted in the plates to 1:1,280. To the second row of inoculated wells for each sample, 100 µl of rabbit anti-CT IgG (Sigma) at a 1:100 dilution in PBS was added. At 4 and 24 h post-inoculation (PI), all wells were observed with an inverted microscope; a well was considered positive if >50% of the cells in the respective monolayer were rounded. The titer was the highest dilution at which an inoculated well was positive and the corresponding well at the same dilution containing inoculum plus anti-toxin serum was negative.

Gnotobiotic piglet experiments. Twelve F4 receptor-positive gnotobiotic piglets were inoculated at 7- 9 days of age with strains 3030-2 (n = 5), 2534-86 (n = 5), or G58-1 (n = 2) using methods previously described (Berberov *et al.,* 2004) with minor modifications to determine whether these strains differed significantly in the number of h PI for a moribund condition to occur. Experiments were approved by the University of Nebraska Institutional Animal Care and Use Committee. In these experiments, piglets

were checked at 1-4 h intervals for depression, lethargy, diarrhea, and dehydration, and were euthanized when moribund, or at 96 h PI if this condition did not occur. The h PI for a moribund condition to occur was used for linear regression analysis along with LT secretion values (ng/ml) for the same strains. Piglets were necropsied immediately after euthanasia and tissues were collected by aseptic technique and processed for culture and histopathology as previously described (Berberov *et al.,* 2004). Blood samples obtained at necropsy also were tested for endotoxin activity using the *Limulus* Amebocyte Lysate QCL-1000 assay (Lonza Walkersville, Walkersville, MD) following the manufacturer's instructions.

Analysis of porcine ETEC strains for type II secretion system. Using primers and conditions as described by Tauschek *et al.* (2002), PCR for *gspD* and *gspK* was conducted to determine the prevalence of the T2SS in the porcine ETEC strains listed in Table S1. As a further analysis of the T2SS, using Geneious 6.1.3, nucleotide and amino acid sequences of 3 porcine strains, viz., 2534-86 (Accession no. AFDS01000066), 3030-2 (Accession no. AFDT01000052), and G58-1 (AFDX01000001) were aligned with that of H10407 (Accession no. AY056599) and *Vibrio cholerae* TRH7000 (Accession no. L33796). Furthermore, they were aligned with 2 other porcine ETEC in the NCBI Database, *viz.*, UMNK88 (CP002729) and UMNF18 (AGTD00000000) (Shepard *et al.,* 2012). To construct phylogenetic trees and protein models, the following were used in addition to the ETEC strains: *Aeromonas hydrophila* AL09-71 (CP007566), *Aeromonas salmonicida* 449 (CP000644), *Aeromonas veronii* B565 (CP002607), *Burkholderia mallei* ATCC 10399 (CH899680), *Burkholderia pseudomallei* K96243 (BX571965), *Dickeya chrysanthemi* (L02214), *Dickeya dadantii* 3937 (CP002038), *Dickeya zeae*

Ech1591 (CP001655), *Erwinia pyrifoliae* Ejp617 (CP002124), *Escherichia coli* BW2952 (CP001396), *Escherichia coli* CE10 (CP001396), *Escherichia coli* EC958 (HG941718), *Escherichia coli* LF82 (CU651637), *Escherichia coli* MG1655 (U00096), *Escherichia coli* Nissle 1917 (CP007799), *Escherichia coli* NRG 857C (CP001855), *Escherichia coli* W3110 (AP009048), *Klebsiella oxytoca* HKOLP1 (CP004887), *Klebsiella pneumoniae* ATCC BAA-2146 (CP006659), *Legionella longbeachae* D-4968 (ACZG01000001), *Pectobacterium carotovorum* (X70049) *Pseudomonas aeruginosa* PA1 (CP004054), *Pseudomonas putida* H8234 (CP005976), *Shewanella amazonensis* SB2B (CP000507), *Shewanella loihica* PV-4 (CP000606), *Shewanella putrefaciens* 200 (CP002457), and *Vibrio vulnificus* (CP002469). Protein models for all the strains were created using the SWISS-MODEL server platform (swissmodel.expasy.org) using *V. cholerae, V. vulnificus* and *E. coli* T2SS protein crystal structures in the Protein Data Bank. PyRx Python Prescription 0.8 was used to analyze the binding capacities of all the ATPases using models generated by the SWISS-MODEL server as the substrate and ATP as the ligand. Phylogenetic analyses of *gspC*, *gspD*, *gspE* and homologs were conducted using MEGA6 (Tamura *et al.,* 2001). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura & Nei, 1993). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. Trees were drawn to scale, with branch lengths measured in the number of substitutions per site. Codon positions included were 1st+2nd+3rd+noncoding, with all positions containing gaps and missing data eliminated.

E. coli ATCC 25922 16S rRNA (DQ360844.1:86278349) was used as an out group. For protein alignment studies, PyMOL Molecular Graphics System version 1.3 was used.

Statistical analyses. Statistical Analysis System (SAS, Version 9.4, Cary, NC) software was used to analyze the data for effect of strain on secretion, and strain on virulence. A test for a linear association between LT secretion and h-to-a-moribund-condition was run for each of the 2 times at which culture supernatant LT concentrations (ng/ml) were measured (6 and 18 h), and tested for lack of fit. The coefficient of determination (R^2) was calculated for each regression analysis. In addition, the linear regressions for each time were compared to see if the slopes were different. Analysis of variance for each time was conducted for comparisons between human and porcine strains as well as among the WT porcine strains and separated using a protected least significant difference test. Calculated *P* values of < 0.05 were considered significant.

Results

LT secretion by porcine ETEC strains. As an initial test of the capacity for porcine-origin WT strains to secrete LT, supernatants from 18-h cultures of 2534-86, 3030-2, and derivatives of 2534-86 grown in CAYE-M medium were analyzed by GM1-ELISA and the Y-1 adrenal cell assay. Culture supernatants of all ETEC strains with detectable GM1-ELISA activity also had activity in the Y1 assay after 24 h PI, whereas non-enterotoxigenic *E. coli* strains lacked activity in both assays (Table S2). Y1 activity was not present at 4 h PI with any of the strains, suggesting that activity seen at 24 h PI was not due to other toxic effects. Cell rounding was inhibited by incubation of culture supernatants with anti-CT antiserum prior to inoculation. A lack of contamination of each

of the culture supernatants with the respective strains under growth also was confirmed by LB broth and agar cultures. Although these results confirmed secretion of biologically active LT, the results were largely qualitative, and involved the testing of only 2 porcine-origin WT ETEC strains. The secondary antibodies used in the GM1-ELISA had not been optimized at the time this experiment was done, so results were interpreted as positive or negative as reported in Table S2, and results were only semi-quantitative in the case of the Y1 assay. In addition, use of 18-h cultures raised the question that some of the LT activity in the culture supernatant could have resulted from bacterial lysis. To confirm that strain 2534-86 secreted LT and to compare secreted levels with that of H10407, 2534-86, isogenic derivatives MUN298 (LT^+) and MUN299 (LT^-), and H10407 grown in CAYE-M were sampled at 4, 8, 12, and 24 h, and LT concentrations in culture supernatants and periplasmic extracts determined with an optimized GM1-ELISA. LT concentrations in the culture supernatant were significantly higher for H10407 ($P<0.05$), whereas no significant difference was detected between 2534-86 and MUN298, and MUN299 was confirmed to have no detectable LT activity (Fig. S1). Periplasmic LT concentrations were also highest for H10407 ($P<0.05$), and peaked at 4 h of culture for this and the other 2 LT^+ strains (Fig. S2). Growth curves (Fig. S3) demonstrated that the CFU/ml did not significantly differ among strains, and all exhibited the same drop in pH at 4 h and increase in pH thereafter, which reflected utilization of glucose through aerobic fermentation, the production and accumulation of organic acids, and afterward their utilization as energy sources.

To confirm that other porcine-origin ETEC strains could secrete LT and determine whether LT secretion levels were detectably related to virulence, 16 ETEC

strains isolated from pigs with severe disease (Table S1) were compared with that of prototypic human-origin strain H10407. All strains were cultured in CAYE-M and sampled at 2, 4, and 6 h of culture to avoid significant contribution of LT to the supernatant by bacterial lysis. In addition, the supernatants at 18 h of culture were tested to determine whether any differences among strains remained so throughout the growth curve. LT was detected in the culture supernatants of all porcine WT strains at 2, 4 and 6 h, confirming the capacity to secrete the toxin (Fig. 1). At 2 h, the concentration of LT in culture supernatant for each of the strains did not differ significantly from that of H10407; however, at 4 and 6 h the concentrations were significantly lower than that of H10407 ($P<0.05$; Fig. 1). A notable finding was that by 6 h, strain 3030-2 had secreted a significantly higher concentration of LT into the supernatant compared to that of all other porcine WT strains tested ($P<0.05$) and this difference remained so through 18 h of culture (Fig. 1). Over all time points, all strains tested had a higher concentration of LT in the supernatant than in the periplasm (Fig. 2). As in the previous experiment, the highest concentration of LT in the periplasm was at 4 h (Fig. 2), and at this time the 3030-2 periplasmic LT concentration was significantly higher than that of the other WT porcine strains ($P<0.05$). To confirm that bacterial lysis was not a significant contributor to LT in the culture supernatant nor resulted in loss of LT from the periplasm during the exponential growth phase, each fraction was tested for alkaline phosphatase activity in the samples at 4 and 6 h of culture. No alkaline phosphatase activity was detected in the culture supernatants of any of the 16 WT porcine strains at either time interval, and all periplasmic extracts contained alkaline phosphatase activity, as expected. In contrast, at 12, 18 and 24 h of culture, alkaline phosphatase activity was present in both the culture

supernatant and periplasmic fractions. These results confirmed that LT was present in the supernatants during the exponential phase by secretion and not by bacterial lysis, but during the stationary and death phases, a portion of the LT activity was present in the supernatants as a result of cell lysis.

Virulence of porcine-origin ETEC strains in gnotobiotic piglets. Gnotobiotic piglets inoculated with strains 3030-2 or 2534-86 had an onset of diarrhea at 6 or 12 h PI, respectively, with subsequent passage of watery, clear-yellow fecal material. All piglets inoculated with strain 3030-2 or 2534-86 rapidly developed severe weight loss and dehydration, and became moribund; however, the clinical course with 3030-2 was more rapid. The mean time-to-a-moribund-condition with 3030-2 was 14.4 h PI, in contrast to 71.2 h PI for 2534-86 (P<0.001). Hence, among these 2 strains, 3030-2 was significantly more virulent. Non-enterotoxigenic strain G58-1 did not induce clinical illness, and piglets inoculated with this strain were euthanized at 96 h PI since their survival time would have been indefinite. A linear regression between LT secretion at 6 h of culture for these 3 strains and h-to-a-moribund-condition in inoculated gnotobiotic piglets was conducted. The model-adjusted coefficient of determination (R^2) was 0.92 ($P < 0.0001$), indicating that approximately 92% of the variation in the time-to-a-moribund condition could be explained by the LT secretion level of the inoculum strain (Fig. 3). A second linear regression based on LT concentrations at 18 h of culture yielded a model-adjusted R^2 of 0.8842 ($P < 0.0001$). This lower R^2 value supported the hypothesis by Lasaro et al. (2006) that virulence is more highly correlated with secretion than total production of LT, since a portion of the LT activity in the supernatants from 18 h cultures originated from lysed cells in the stationary-to-death phases.

All piglets inoculated with 3030-2 or 2534-86 developed gross and microscopic lesions in the intestines compatible with shock (Figs. 4-8). Both small and large intestines were affected with hyperemia, hemorrhage and necrosis; these lesions were most severe in the mucosa, and were compatible with what we have previously described as hemorrhagic enteritis (Berberov *et al.,* 2004). Histologically, necrotic epithelial cells in affected intestines had either sloughed or were in the process of it with formation of subepithelial clefts and exposure of the basal lamina. Intact small intestinal epithelium in all pigs inoculated with 2534-86 and 3030-2, but none inoculated with G58-1, had *E. coli* cells adherent to their apical surfaces, and in the case of pigs with lesions of shock had bacteria adherent to the exposed basal lamina. Platelet-fibrin thrombi and intravascular bacteria were seen in mucosal capillaries and venules of all piglets inoculated with 2534-86 and 3030-2, but in none of the piglets inoculated with G58-1. In all piglets inoculated with 2534-86 or 3030-2 but none inoculated with G58-1, either the respective inoculum strain, endotoxin activity or both were detected in blood samples obtained at necropsy. Mean endotoxin activity measured 0.49 ± 0.23 endotoxin units per ml in blood samples testing positive.

Presence of type II secretion system in porcine ETEC strains. PCR analysis revealed the presence of both *gspD* and *gspK* genes in all porcine-origin WT strains shown in Table S1 with H10407 as a positive control (Fig. S4). Bioinformatic analyses of the genomic sequences of porcine LT^+ WT strains 2534-86 and 3030-2, as well as that of porcine LT^- WT strain G58-1, further revealed that all 3 strains have a complete *gspC-M* operon with a high degree of similarity to that of H10407. A comparison of the *gspC-M* sequences revealed that 2534-86 shared 38.6-45.0% nucleotide and 94.3-99.8% amino

acid identity with H10407, and 38.5-45.2 % nucleotide and 27.3-76.2% amino acid identity with *V. cholerae* T2SS (Table 1). Similarly, 3030-2 shared 37.9-42.6% nucleotide and 20.0-76.2% amino acid identity with *V. cholerae* T2SS while having 37.8-44.7% nucleotide and 95.5-99.7% amino acid identity with the human ETEC strain H10407 (Table 1). Strain G58-1 shared 39.1-43.5 % nucleotide and 15.7-61.2% amino acid identity with 2534-86, 37.3-65.1% nucleotide and 16.1-61.9% amino acid identity with H10407, 37.0-43.3% nucleotide and 16.7-64.6% amino acid identity with 3030-2, and 37.6-61.4% nucleotide and 16.3-62.6 % amino acid identity with the *V. cholerae* T2SS (Table 2). Two other porcine ETEC strains in the NCBI database, UMNK88 and UMNF18, shared ≥95.7% nucleotide and ≥93.8% amino acid identity with 2534-86, whereas they collectively shared only 37.0-43.9 % and 16.1-61.9 % nucleotide and amino acid identity, respectively, with G58-1 (Table 3).

Maximum Likelihood phylogenetic trees were constructed using genetic sequences of *gspC* (Table S3; Fig. S5), *gspD* (Table S4; Fig. S6), *gspE* (Table S5; Fig. 9) and corresponding homologs. In addition to *V. cholerae* TRH7000, porcine- and human-origin ETEC, and a subset of other bacterial strains that have been described in the literature to possess *gspC-M* homologs were used. All three trees generated showed that all four porcine ETEC strains (2534-86, 3030-2, UMNK88 and UMN18) clustered together in the same clade with high bootstrap values (≥ 60%). G58-1, however, clustered away from these ETEC strains. In trees generated by homologs of *gspD* and *gspE*, G58-1 clustered closer to H10407 and *V. cholerae* TRH7000.

Protein models were constructed for the 2534-86, 3030-2 and G58-1 predicted Gsp proteins based on structures of homologs in the Protein Data Bank (Table 4). The

Qualitative Model Energy ANalysis (QMEAN)4 scores, which signify the quality of the model and its relatedness to the template used, showed that GspC, D, E, F, G, K and M have higher scores (i.e. closer to 1) while only GspL has a low QMEAN4 score and a very low negative Z-score value (Benkert *et al.,* 2009a; Benkert *et al.*, 2009b; Benkert *et al.,* 2008). Similar results were obtained for protein models constructed with 3030-2 (Table 4). Both 2534-86 and 3030-2 had consistently higher QMEAN scores compared to those of G58-1. Furthermore, G58-1 protein modeling yielded structures with negative Z-scores in all instances, while both 2534-86 and 3030-2 had proteins that yielded positive or very low negative Z-scores which signified that the model elucidated is statistically better than the average models for those proteins.

Proteins in T2SS that function as ATPases, in addition to the proton motive force, have been identified as the source of energy required for secretion (Cambert & Sandkvist, 2005; Nivaskumar & Francetic, 2014). Hence, we hypothesized that the ATP binding capacity of GspE for each of the strains tested would be related to LT secretion levels and virulence. To address this hypothesis, the protein model of 2534-86 GspE was aligned with that of H10407 GspE and *V. cholerae* TRH7000 EpsE using PyMol. While H10407 GspE and 2534-86 GspE both aligned with *V. cholerae* TRH7000 EpsE with a relative mean square (RMS) value of 0.083, H10407 and 2534-86 ATPases aligned with each other with a RMS of 0.001. PyRx was used to analyze the predicted ATP-binding capacities of the T2SS ATPases of each of the strains listed in the phylogenetic trees (Table 5). Of those strains which we had corresponding quantitative data for LT secretion, the predicted binding capacities, from highest to lowest, were H10407 (-8.8 kcal/mol), 3030-2 (-8.6 kcal/mol), 2534-86 (-8.5 kcal/mol), and G58-1 (-8.0 kcal/mol).

MG1655, a K-12 *E. coli* strain, which was the origin of MUN302 used in this study, had a predicted binding capacity of -7.3 kcal/mol. For those strains for which we had virulence data (3030-2, 2534-86, and G58-1), a direct relationship was seen between virulence and the predicted ATP-binding capacity of the GspE.

Discussion

In the present study, we found that WT ETEC strains varied in LT secretion capacity, and this played a major role in determining virulence. One strain (3030-2) secreted significantly more LT than any other WT porcine strain tested, and also was significantly more virulent. When combining data for 3 strains that varied in virulence, we found that LT secretion, based on concentrations in supernatants from 6-h cultures explained 92% of the variation in time-to-a-moribund-condition. If the regression was run using LT concentrations in supernatants from 18-h cultures, the coefficient of determination was 89%. This lower R^2 value supports the hypothesis by Lasaro *et al.* (2006) that virulence is more highly correlated with secretion than total production of LT, since a portion of the LT activity in the supernatants from 18-h cultures originated from lysed cells. In a previous study, we found that approximately 58% of the variation in the rate of weight loss was explained by the LT production levels of the respective inoculum strain (Erume *et al.,* 2008). However, in that study, LT secretion per se was not measured, and the strains only included isogenic derivatives of 2534-86. The decision to euthanize gnotobiotic piglet experiments at 96 h PI if a moribund condition did not occur was somewhat arbitrary, but mainly based on animal welfare.

In previous studies, we determined that LT contributed more than STb to the severity of disease in 9-day-old gnotobiotic piglets inoculated with isogenic derivatives of strain 2534-86 (Erume *et al.,* 2008). In those studies, death phase (48- or 72-h) cultures treated with polymyxin B which caused further cell lysis were used to test for levels of LT production; hence, the capacity for the strain to secrete LT was not measured. In a more recent study, we found that these same strains secreted LT in 18-h cultures, but these studies did not test for attribution of LT secretion to virulence (Erume *et al.,* 2013). To our knowledge, as reported in the published literature, only 4 other porcine-origin strains had been tested for LT secretion, and the authors of these studies postulated that "the intact holotoxin transverses the outer membrane with newly synthesized LPS and becomes a component of the outer surface of *E. coli*" (Kunkel & Robertson, 1979). Besides the conclusion that LT is not fully secreted, this latter study provided no information about the virulence of the strains being tested, or the relationship, if any, between LT secretion and virulence.

In the present study, we detected LT in the supernatant by an optimized GM1-ELISA as early as 2 h of culture, and detected LT secretion by all 16 porcine WT ETEC strains tested, each isolated from cases of severe disease. Although 1 porcine strain was found to secrete relatively high levels of LT, we, similar to Gilligan and Robertson (1979) also found that the levels of LT produced by porcine ETEC strains were lower than that of the prototypic human strain, H10407. Lasaro *et al.* (2006), who analyzed 26 human ETEC isolates, reported that the levels of LT secreted by human strains can vary by as much as 50-fold. Although the culture media, conditions, and analytical methods used in our study differed in several respects, the levels of LT secreted by porcine ETEC

strains in our study were within the general range of that of the human strains in the Lasaro et al. (2006) study, with the exception of H10407. H10407 was originally isolated from a patient with severe, cholera-like diarrhea (Evans *et al.,* 1973), and was found to secrete more LT than any of the 26 human test strains in the Lasaro *et al.* (2006) study. In contrast to the conclusion by Gilligan and Robertson (1979) that porcine ETEC strains produce less LT in complex medium than human strains, we conclude that strains from pigs and humans produce and secrete LT levels that are in general comparable to one another, whereas H10407 is more of an outlier.

We used a culture medium that had previously been shown to be optimal for LT secretion by H10407 and other human-origin strains (Hegde *et al.*, 2009; Mundell *et al.*, 1976) for detection of LT secretion by porcine ETEC strains. CAYE-M medium containing 0.25% glucose and adjusted to pH 8.5 has yielded the highest LT concentrations in culture supernatants in studies involving human-origin strains (Hegde *et al.,* 2009; Mundell *et al.,* 1976). The molecular basis to explain the optimal nature of this medium for LT production and secretion has been apparent in recent studies. The cAMP repressor protein (CRP) is a repressor of *eltAB* transcription; glucose causes derepression of the *eltAB* promoter in H10407 by suppressing synthesis of cAMP, thereby decreasing cAMP availability to bind to the CRP and increasing transcription (Bodero & Munson, 2009). In contrast, CRP is a positive regulator of LT secretion and alkaline pH is a signal optimal for production and secretion of LT (Gonzales *et al.,* 2013). Based on the results reported herein, porcine ETEC strains would be expected to be affected by glucose and alkaline pH in the same manner as H10407; however, experiments specifically testing these hypotheses were not conducted.

Similar to the study of Tauschek *et al.* (2002) with the prototypic human-origin strain H10407, we found that in porcine ETEC strains most of the LT secreted into the supernatant is not retained in the periplasm. The time at which the highest concentration of LT was detected in the periplasm by any strain tested was at 4 h PI. Furthermore, the LT levels in the supernatant did not increase significantly between 4 and 6 h, with some WT porcine strains showing similar or lower levels compared to the 4-h LT levels.

We found that all porcine-origin ETEC strains that had been genomically sequenced contained a T2SS with a high degree of amino acid identity to that of H10407, supporting the inference by Tauschek *et al.* (2002) that the T2SS is highly conserved in ETEC. Interestingly, porcine-origin non-enterotoxigenic strain G58-1 was also found to contain the T2SS. This strain was originally isolated from a piglet with diarrhea, and is of a serotype (O101:K28) that is commonly enterotoxigenic, usually expressing K99 (F5) and/or 987P (F6) fimbria and STa (Chen *et al.,* 1985; Gunee *et al.,* 1977; Moon *et al.,* 1980). Hence, this strain may have been an ETEC that lost one or more plasmids containing enterotoxin and fimbrial genes. The T2SS in G58-1 shares a similar level of nucleotide identity with both H10407 and 2534-86 but a much lower level of amino acid identity with the respective components of the secretion system. In addition, 3030-2, as well as the other 2 porcine ETEC strains in the NCBI database, all shared a very high degree of amino acid and nucleotide identity with each other, typically >93%, but a low level of nucleotide identity with the human ETEC strain H10407. This fact is emphasized by the phylogenetic trees generated using *gspC*, *gspD* and *gspE* , which showed that porcine ETEC have a similar clonal origin to each other while G58-1 clustered closer to H10407 and *V. cholerae* TRH7000. Since *gspC* and *gspD* encode substrate-specific

components of the secretion pathway, this suggests a parallel evolution of the same secretion system, which is not surprising since all these organisms are subjected to the same evolutionary pressures that force them to evolve a functioning secretion system to take advantage of their enterotoxin (Bouley *et al.,* 2001). It could be argued that both 2534-86 and 3030-2 developed a T2SS more efficient for recognizing the leader sequences of the enterotoxin subunits and their subsequent processing in the periplasm. This may have happened after the acquisition of the LT containing plasmid resulting in a secretion system that shares a high protein identity with H10407, while G58-1 which shares a closer clonal match to H10407, has a low protein identity with the H10407 T2SS.

GspE (EpsE) binds and hydrolyzes ATP, thereby providing energy for pseudopilus assembly and protein secretion (Camberg & Sandkvist, 2005; Nivaskumar & Francetic, 2014). The results of protein modeling of these ATPases showed that different T2SS have different predicted binding capacities, which might in part explain the different secretion capabilities of different strains of both animal and human ETEC. However, further experiments are needed to test this hypothesis. The predicted binding affinities were generated by using the respective GspE monomer of the T2SS. However, it has been shown that the hexameric form of GspE has a much higher ATPase activity than the monomeric one, and is thought to be the functional form in nature (Camberg & Sandkvist, 2005; Nivaskumar & Francetic, 2014). Therefore, it can be assumed that the ATPase activities of GspE in the strains tested herein might be different in nature from their predicted values based on protein models.

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Table 1. Comparison of type II secretion system nucleotide and amino acid sequences of *Escherichia coli* strains 2534-86 and 3030-2 with that of *E. coli* strain H10407 and *Vibrio cholerae* strain TRH7000[a].

ETEC[b] / *Vibrio cholerae* gene	***Vibrio cholerae* (TRH7000)**				***Escherichia coli* H10407**			
	% Nucleotide Identity		**% Amino acid Identity**		**% Nucleotide Identity**		**% Amino Acid Identity**	
	2534-86	**3030-2**	**2534-86**	**3030-2**	**2534-86**	**3030-2**	**2534-86**	**3030-2**
gspC/espC	42.4	39.3	31.7	29.5	45.0	44.7	99.3	98.2
gspD/espD	40.9	38.9	51.6	42.8	42.9	42.8	99.8	99.1
gspE/espE	44.2	37.9	66.4	69.3	44.6	37.8	99.4	99.7
gspF/espF	43.6	42.6	56.2	56.2	43.0	42.6	98.5	98.8
gspG/espG	43.0	41.6	76.2	76.2	40.4	40.6	98.0	98.7
gspH/espH	40.8	39.8	32.0	26.2	41.8	42.1	96.6	98.9
gspI/espI	40.7	40.3	42.4	39.0	43.2	42.2	94.3	96.7
gspJ/espJ	40.7	43.5	44.3	36.0	38.6	40.3	98.4	98.9
gspK/espK	45.2	42.5	39.6	39.6	43.2	42.7	98.5	99.7
gspL/espL	38.5	38.0	27.3	20.0	39.7	39.4	97.9	99.7
gspM/espM	43.9	42.4	29.0	28.3	42.0	41.4	94.9	95.5

[a]Accession numbers: 2534-86, AFDS01000066; 3030-2, AFDT01000052; H10407, AY056599; *Vibrio cholerae* TRH7000, L33796.

[b]ETEC: enterotoxigenic *Escherichia coli*.

Table 2. Comparison of type II secretion system nucleotide and amino acid sequences of *Escherichia coli* strain G58-1 with that of *E. coli* strains 2534-86, 3030-2, H10407, and *Vibrio cholerae* strain TRH7000.[a]

ETEC[b] / *Vibrio cholerae* gene	***V. cholerae* TRH7000**		***E. coli* H10407**		***E. coli* 2534-86**		***E. coli* 3030-2**	
	% NA ID[c]	**% AA ID[c]**	**% NA ID**	**% AA ID**	**% NA ID**	**% AA ID**	**% NA ID**	**% AA ID**
gspC/espC	37.6	37.6	37.3	19.4	39.1	22.7	39.4	22.7
gspD/espD	53.9	42.8	50.2	44.5	42.5	43.9	41.4	42.8
gspE/espE	58.2	57.5	59.5	57.0	43.3	57.4	37.0	64.6
gspF/espF	41.4	43.6	42.4	45.1	42.9	44.7	42.7	44.8
gspG/espG	61.4	62.6	65.1	61.9	39.8	61.2	39.4	61.2
gspH/espH	43.4	26.1	40.7	19.8	43.5	20.7	42.3	20.3
gspI/espI	43.1	25.2	45.8	26.1	42.4	23.5	42.0	25.2
gspJ/espJ	44.2	22.7	43.2	21.8	42.8	21.3	43.3	21.8
gspK/espK	49.6	32.8	47.4	32.6	42.6	32.6	43.2	32.6
gspL/espL	43.4	18.3	41.1	19.9	40.6	15.7	39.9	18.4
gspM/espM	44.0	16.3	40.5	16.1	41.7	17.2	41.9	16.7

[a]Accession numbers: G58-1, AFDX01000001; 2534-86, AFDS01000066; 3030-2, AFDT01000052; H10407, AY056599; *Vibrio cholerae* TRH7000, L33796.

[b]ETEC: enterotoxigenic *Escherichia coli*.

[c]% NA ID = percent nucleotide identity; % AA ID = percent amino acid identity.

Table 3. Comparison of type II secretion system nucleotide and amino acid sequences of *Escherichia coli* strains G58-1, 2534-86, H10407, UMNK88, and UMNF18.[a]

Gene	G58-1				H10407				2534-86			
	% Nucleotide Identity		% Amino Acid Identity		% Nucleotide Identity		% Amino Acid Identity		% Nucleotide Identity		% Amino Acid Identity	
	UMNK88	UMNF18	UMNK88	UMNF18	UMNK88	UMNF18	UMNK88	UMNF18	UMNK88	UMNF18	UMNK88	UMNF18
gspC	38.7	39.9	22.1	22.2	44.7	44.0	97.1	95.3	97.5	96.1	97.8	95.3
gspD	43.0	43.9	43.9	43.9	43.3	42.2	99.7	99.7	98.6	97.2	99.9	99.9
gspE	43.6	43.7	57.2	57.2	43.9	44.7	99.2	99.2	98.6	98.2	99.4	99.4
gspF	41.9	42.6	44.8	45.1	42.1	41.5	98.8	99.5	97.6	97.3	99.3	98.5
gspG	39.9	39.0	61.9	61.9	40.5	40.5	98.7	99.3	97.6	98.2	98.0	98.7
gspH	43.3	43.7	21.2	20.7	40.7	40.4	95.5	94.9	98.7	98.9	98.9	98.3
gspI	37.0	41.2	25.2	26.1	42.2	43.9	95.1	99.2	97.3	96.0	95.9	95.1
gspJ	42.9	43.4	21.3	22.0	39.1	40.3	98.9	99.5	95.7	95.7	98.4	97.8
gspK	42.9	42.7	32.3	32.6	43.0	42.8	98.5	99.1	97.3	97.8	98.8	99.4
gspL	42.5	41.8	19.9	20.4	43.1	43.0	100.0	98.5	98.5	98.5	97.9	97.9
gspM	41.1	39.9	16.1	16.1	40.1	40.2	100.0	98.9	96.1	95.7	94.9	93.8

[a]Accession numbers: G58-1, AFDX01000001; 2534-86, AFDS01000066; 3030-2, AFDT01000052; H10407, AY056599; *Vibrio cholerae* TRH7000, L33796; UMNK88, CP002729; UMNF18, AGTD00000000.

Table 4. Protein modeling data for 2534-86, 3030-2 and G58-1 type II secretion system proteins GspC-M.

Protein	**Model Template**[a]	**2534-86**		**3030-2**		**G58-1**	
		QMEAN4 Score[b]	**Z-Score**[c]	**QMEAN4 Score**[b]	**Z-Score**[c]	**QMEAN4 Score**[b]	**Z-Score**[c]
GspC	EpsC (2I4S)	0.971	1.37	0.971	1.37	0.657	-1.30
GspD	GspD (3EZJ)	0.895	0.85	0.801	0.12	0.729	-0.85
GspE	EpsE (1P9R)	0.754	-0.52	0.741	-0.63	0.685	-1.34
GspF	EpsF (2VMB)	0.662	-1.38	0.662	-1.38	0.647	-1.55
GspG	EpsG (3GN9)	0.894	0.85	0.899	0.90	0.671	-1.28
GspH	EpsH (2QV8)	0.579	-2.37	0.596	-2.18	0.688	-1.19
GspI	EpsI (2RET)	0.475	-2.34	0.491	-2.23	0.522	-1.92
GspJ	EpsJ (2RETE)	0.479	-3.61	0.502	-3.33	0.495	-3.28
GspK	EpsK (3CIO)	0.695	-1.24	0.699	-1.19	0.556	-3.31
GspL	EpsL (1W97)	0.214	-6.08	0.211	-6.11	0.429	-4.29
GspM	EpsM (1UV7)	0.750	-0.33	0.740	-0.40	0.608	-1.21

[a]Source of model template: EpsC, EpsE, EpsF, EpsH, EpsJ-M, *Vibrio cholerae* (strain not stated in literature); GspD, *E. coli* strain H10407; EpsG, EpsI, *V. vulnificus* (strain not stated in literature). Letters in parentheses are the Protein Data Bank (PDB) identification for the respective protein.

[b]QMEAN4 score (Range 0 -1) is a composite score consisting of a linear combination of 4 statistical potential terms: (1) C-beta interaction energy, (2) all-atom pairwise energy, (3) solvation energy, and (4) torsion angle energy.

[c]Z-score: an estimate of the "degree of nativeness" of the structural features observed in a model by describing the likelihood that a model is of comparable quality to high-resolution experimental structures; it provides an estimate of the absolute quality of a model by relating it to reference structures solved by X-ray crystallography.

Table 5. Predicted ATP-binding affinities of GspE homologs in different bacteria.

Strain	**Predicted Binding Affinity (kcal/mol)**
Burkholderia mallei ATCC 10399	-9.2
Dickeya dadantii 3937	-9.2
Dickeya zeae Ech1591	-9.2
Pectobacterium carotovorum	-9.2
Shewanella putrefaciens 200	-9.1
Pseudomonas putida H8234	-9.0
Klebsiella pneumoniae ATCC BAA-2146	-8.9
Shewanella amazonensis SB2B	-8.9
Shewanella loihica PV-4	-8.9
Burkholderia pseudomallei K96243	-8.8
Dickeya chrysanthemi	-8.8
Escherichia coli BW2952 (K-12)	-8.8
Escherichia coli H10407 (hETEC)	-8.8
Escherichia coli CE10 (NMEC)	-8.7
Escherichia coli NRG 857C (AIEC)	-8.7
Escherichia coli UMNF18 (pETEC)	-8.7
Escherichia coli W3110 (K-12)	-8.7
Escherichia coli 3030-2 (pETEC)	-8.6
Pseudomonas aeruginosa PA1	-8.6
Escherichia coli 2534-86 (pETEC)	-8.5

Erwinia pyrifoliae Ejp617	-8.2
Escherichia coli LF82 (AIEC)	-8.2
Legionella longbeachae D-4968	-8.1
Vibrio vulnificus	-8.1
Aeromonas salmonicida 449	-8.0
Escherichia coli G58-1	-8.0
Escherichia coli EC958 (UPEC)	-7.9
Aeromonas hydrophila AL09-71	-7.7
Aeromonas veronii B565	-7.6
Escherichia coli Nissle 1917	-7.4
Escherichia coli MG1655 (K-12)	-7.3
Vibrio cholerae TRH7000	-7.3
Klebsiella oxytoca HKOLP1	-6.7
Escherichia coli UMNK88 (pETEC)	-6.5

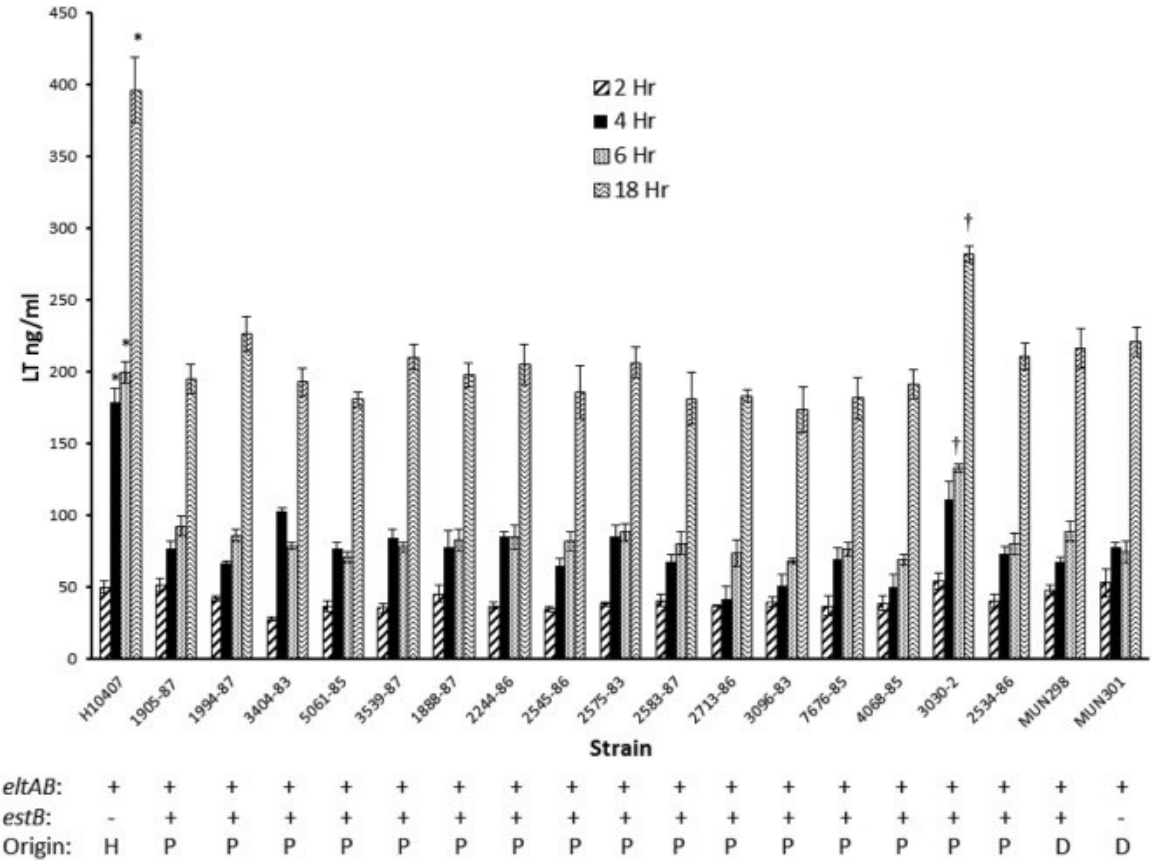

Figure 1. Heat-labile enterotoxin (LT) secretion into culture supernatant over time by human- and porcine-origin enterotoxigenic *E. coli* strains grown in CAYE-M medium**.** Strains were cultured at 37°C and 225 rpm in CAYE-M using a flask-to-medium ratio of 8.3:1. Samples of culture supernatants were obtained at 2, 4, 6 and 18 h of incubation, and LT concentrations in supernatant samples at each time interval were determined by GM1-ELISA. *LT concentrations in H10407 culture supernatant are significantly different (P<0.05) from that of all other strains at the corresponding time interval. †LT concentrations in 3030-2 culture supernatant are significantly different (P<0.05) from that of all other porcine or 2534-86 derivative strains at the corresponding time interval.

eltAB: strain is positive for LT genes by PCR. *estB*: strain is positive for STb gene by PCR. H = human-origin strain, P = porcine-origin strain, D = 2534-86 derivative strain.

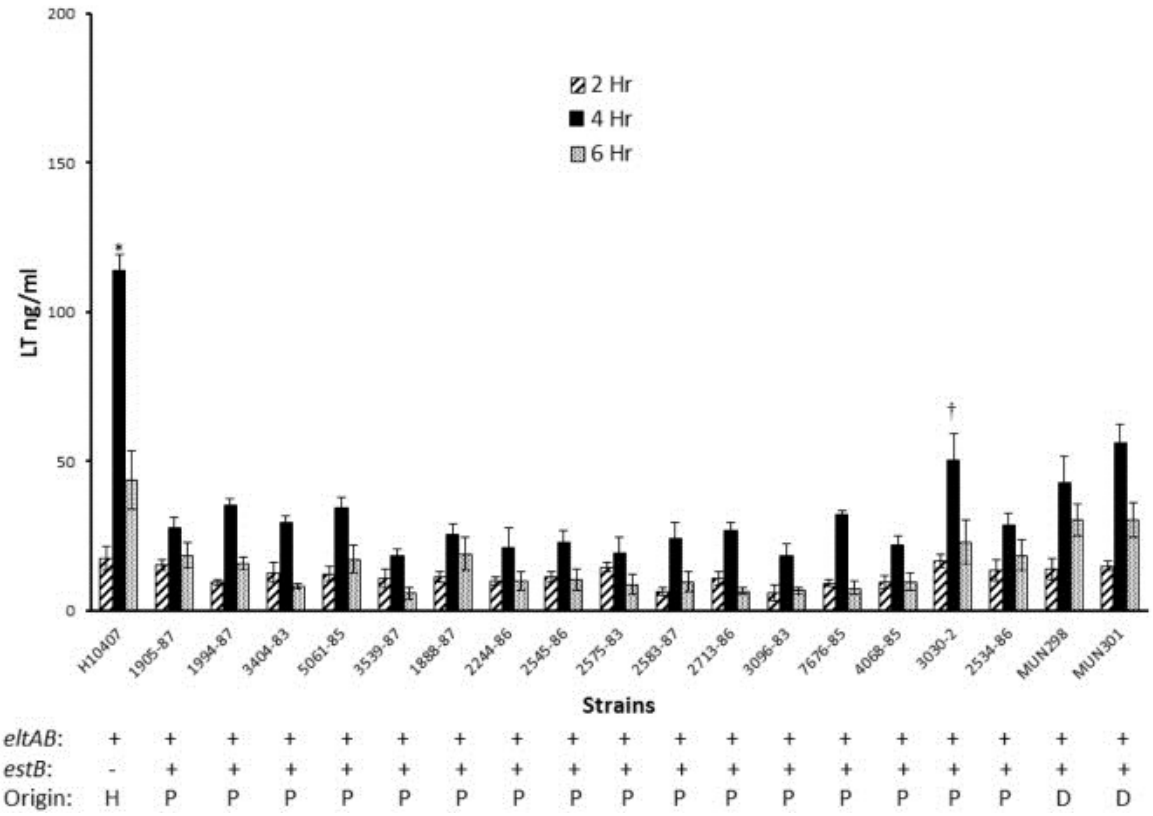

Figure 2. Heat-labile enterotoxin (LT) secretion into the periplasm over time by human- and porcine-origin enterotoxigenic *E. coli* strains grown in CAYE-M medium. Strains were cultured at 37°C and 225 rpm in CAYE-M using a flask-to-medium ratio of 8.3:1. Periplasmic extracts were prepared from samples of cell pellets obtained at 2, 4 and 6 h of culture, and LT concentrations in these extracts at each time interval were determined by GM1-ELISA. *LT concentrations in H10407 periplasmic extracts are significantly different ($P<0.05$) from that of all other strains at the corresponding time interval. †LT concentrations in 3030-2 periplasmic extracts are significantly different ($P<0.05$) from that of all other porcine or 2534-86 derivative strains at the corresponding time interval. *eltAB*: strain is positive for the LT genes by PCR. *estB*: strain is positive for STb gene by PCR. H = human-origin strain, P = porcine-origin strain, D = 2534-86 derivative strain.

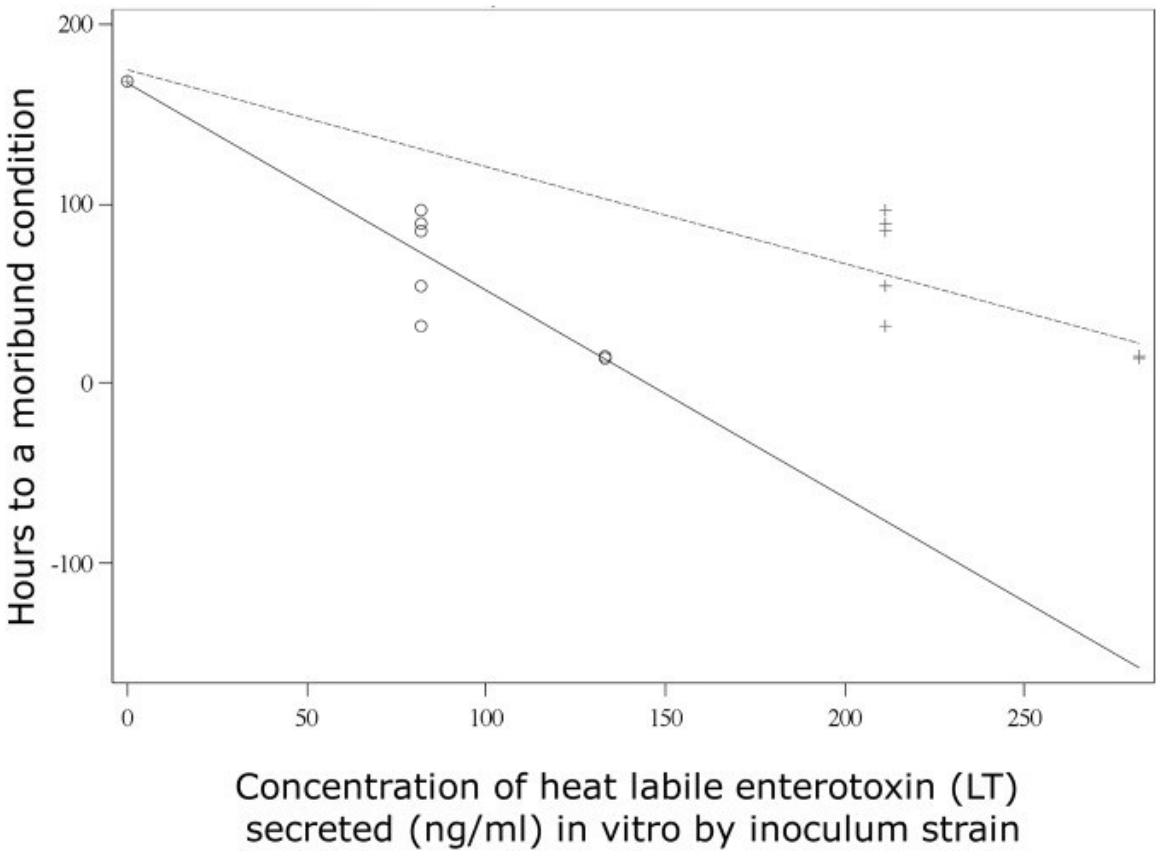

Figure 3. Linear regression between heat-labile enterotoxin (LT) secretion in culture and h to a moribund condition in gnotobiotic piglets inoculated with the corresponding *E. coli* strain. Concentration of LT in supernatants of 6-h cultures of strains G58-1 (LT^-), 2534-86 (LT^+), and 3030-2 (LT^+; data shown in Fig. 1) and h-to-a-moribund-condition in piglets after inoculation with the same strains was used in the regression analysis.

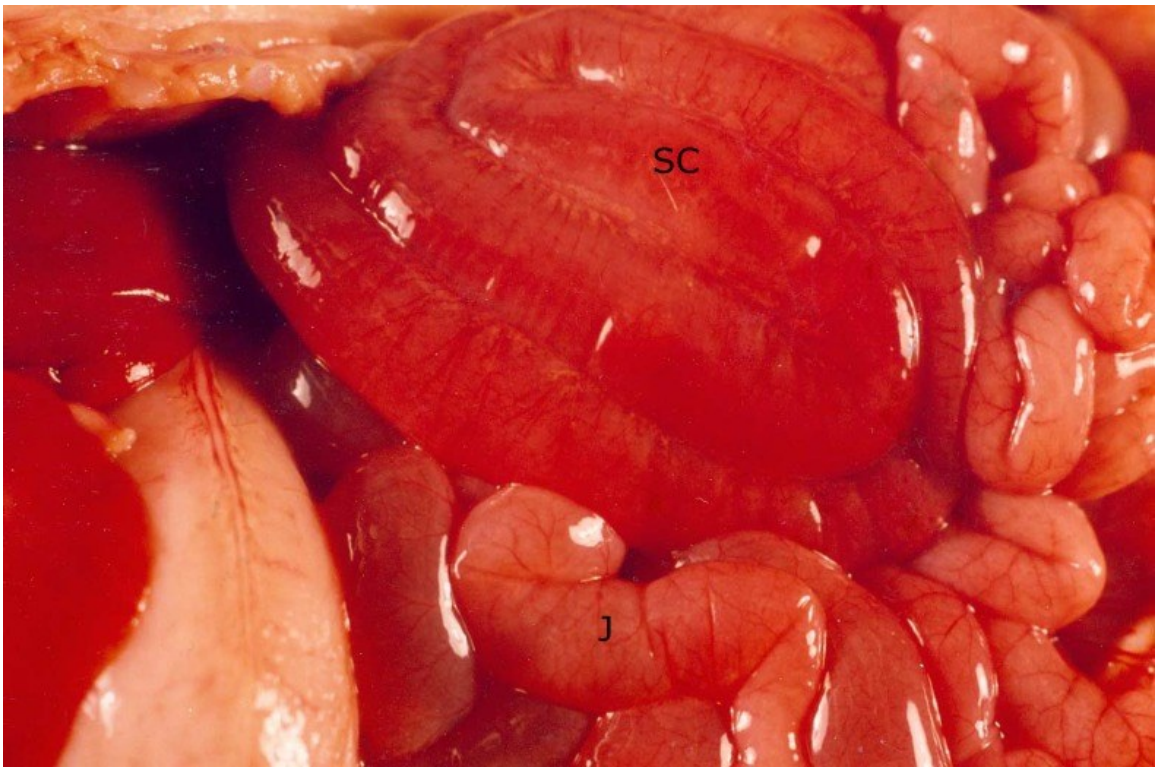

Figure 4. Photograph at necropsy of piglet 15 h after inoculation with enterotoxigenic *E. coli* strain 3030-2. Spiral colon (SC) is diffusely hemorrhagic, and jejunum (J) is hyperemic; both spiral colon and jejunum are distended with watery ingesta.

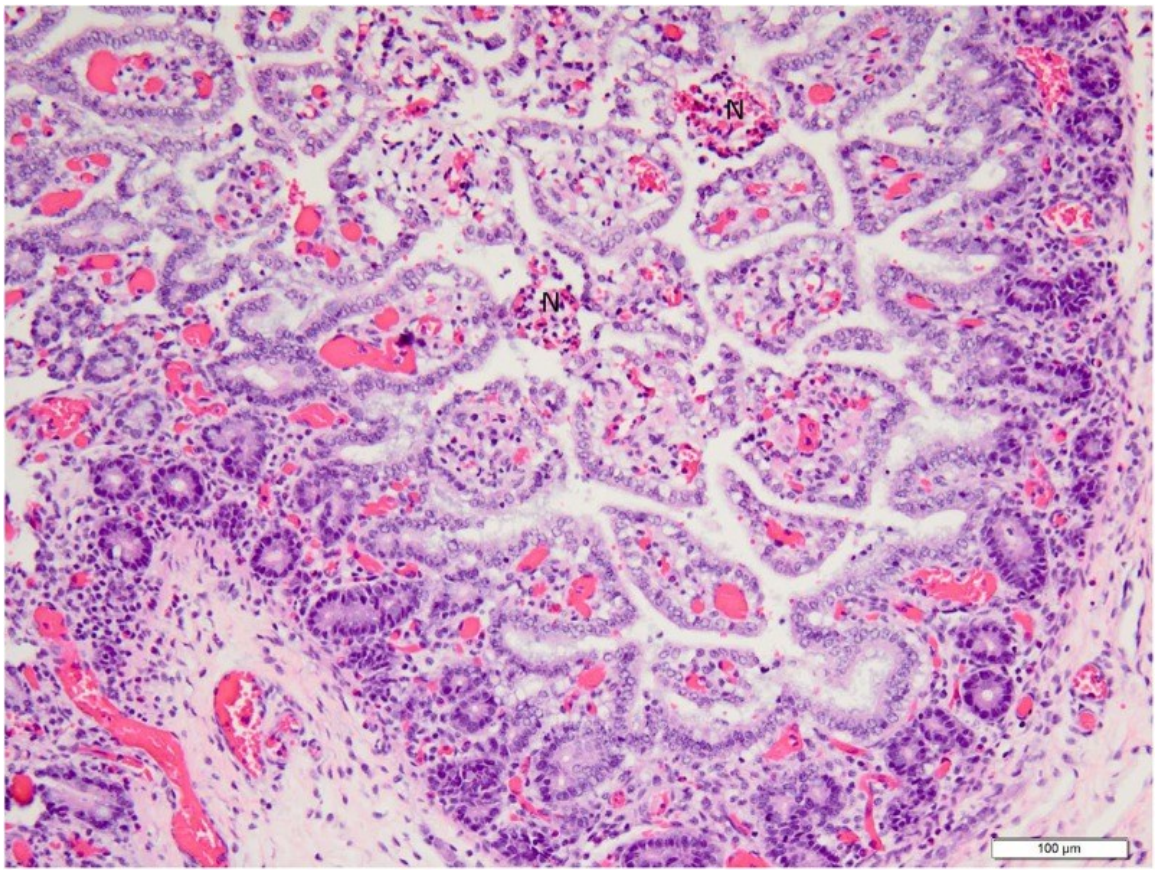

Figure 5. Low (20X objective) magnification photomicrograph of jejunum of piglet shown in Figure 4. Mucosa and submucosa are diffusely hyperemic, and villi multifocally are necrotic. Necrotic villi (N) have hemorrhage in the lamina propria and loss of absorptive epithelium. Photomicrograph was taken of 4 µm-thick section of 10% neutral-buffered formalin-fixed, paraffin-embedded jejunal tissue stained with hematoxylin and eosin. Bar = 100 µm.

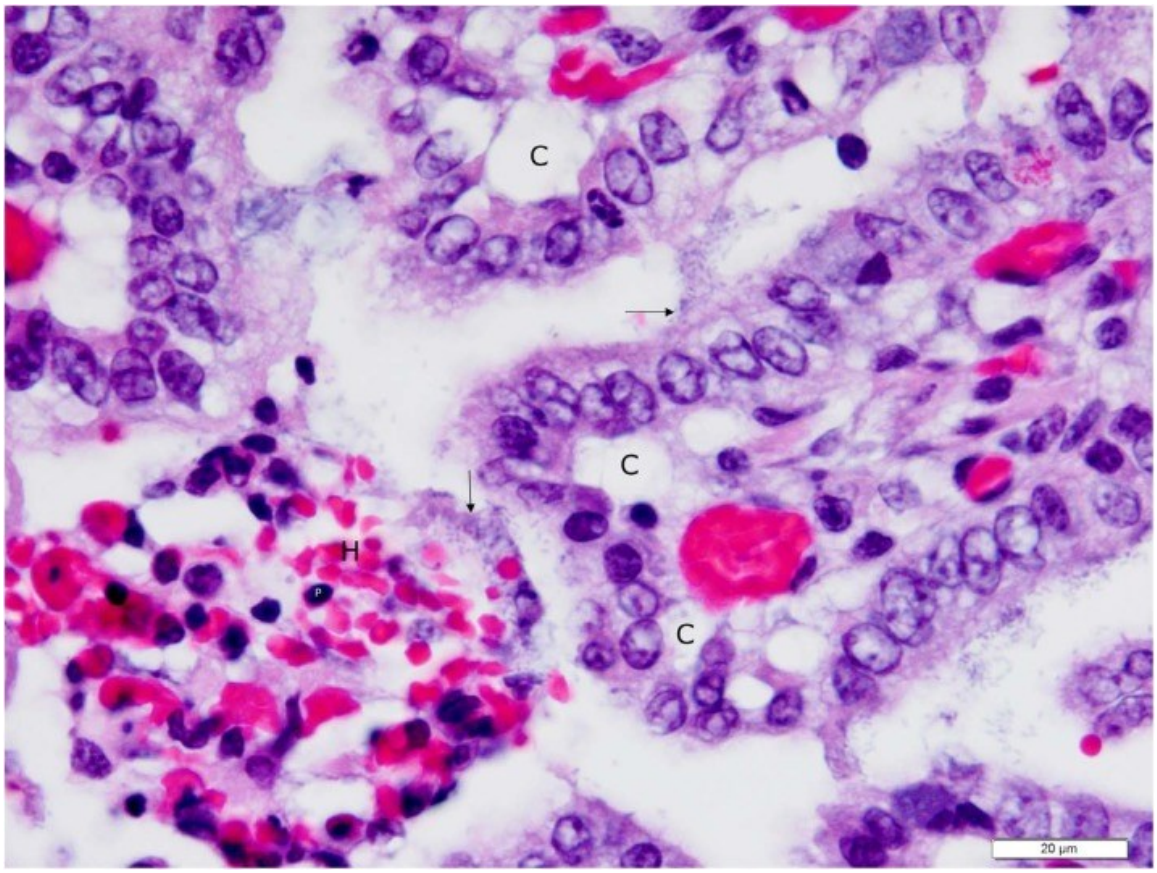

Figure 6. Higher (100X objective) magnification photomicrograph of jejunum shown in Fig. 5 with detail of necrotic and intact villi. The lamina propria of a necrotic villus is hemorrhagic (H), and contains numerous cells with pyknotic nuclei (P), indicating coagulation necrosis. The epithelium overlying this villus is absent, due to necrosis and sloughing of epithelial cells into the intestinal lumen. The exposed basal lamina of the necrotic villus is colonized with enterotoxigenic *E. coli* (vertical arrow), with bacteria having penetrated into the lamina propria with access to the microcirculation. The villi above and to the right of the necrotic villus have ETEC bacteria (horizontal arrow) colonizing the apical surfaces of absorptive epithelial cells. Many of these epithelial cells are in the process of sloughing as evidenced by the presence of clefts (C) between their

basolateral surfaces and the underlying basal lamina. Photomicrograph was taken of 4 μm-thick section of 10% neutral-buffered formalin-fixed, paraffin-embedded jejunal tissue stained with hematoxylin and eosin. Bar = 20 μm.

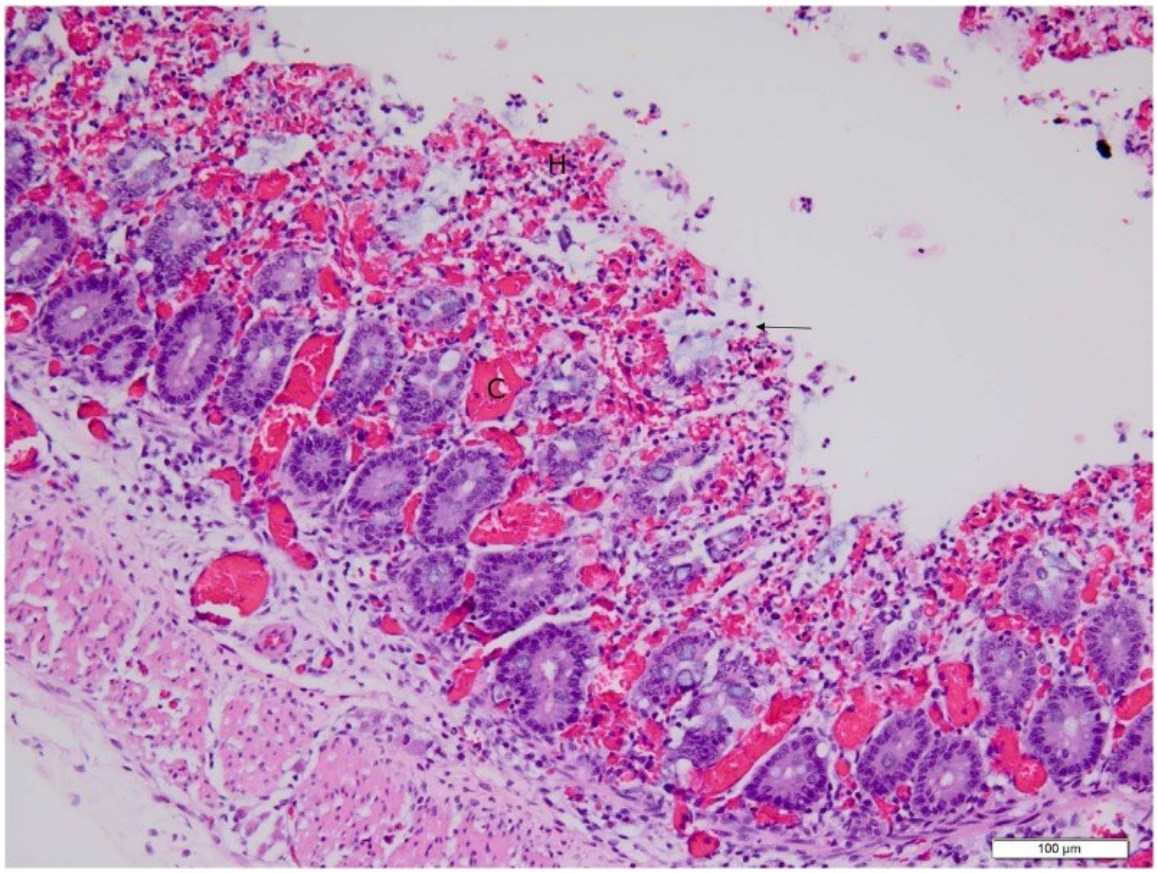

Figure 7. Low (20X objective) magnification photomicrograph of spiral colon of piglet shown in Figure 4. Mucosa and submucosa are diffusely hyperemic and hemorrhagic; a markedly hyperemic venule (C) in the center of the field is seen. The epithelium on the mucosal surface is almost completely absent with the exception of a few cells (arrow) that are in the process of sloughing; architectural detail in the lamina propria and deep crypt epithelium are still intact at this point. Photomicrograph was taken of 4 µm-thick section of 10% neutral-buffered formalin-fixed, paraffin-embedded spiral colonic tissue stained with hematoxylin and eosin. Bar = 100 µm.

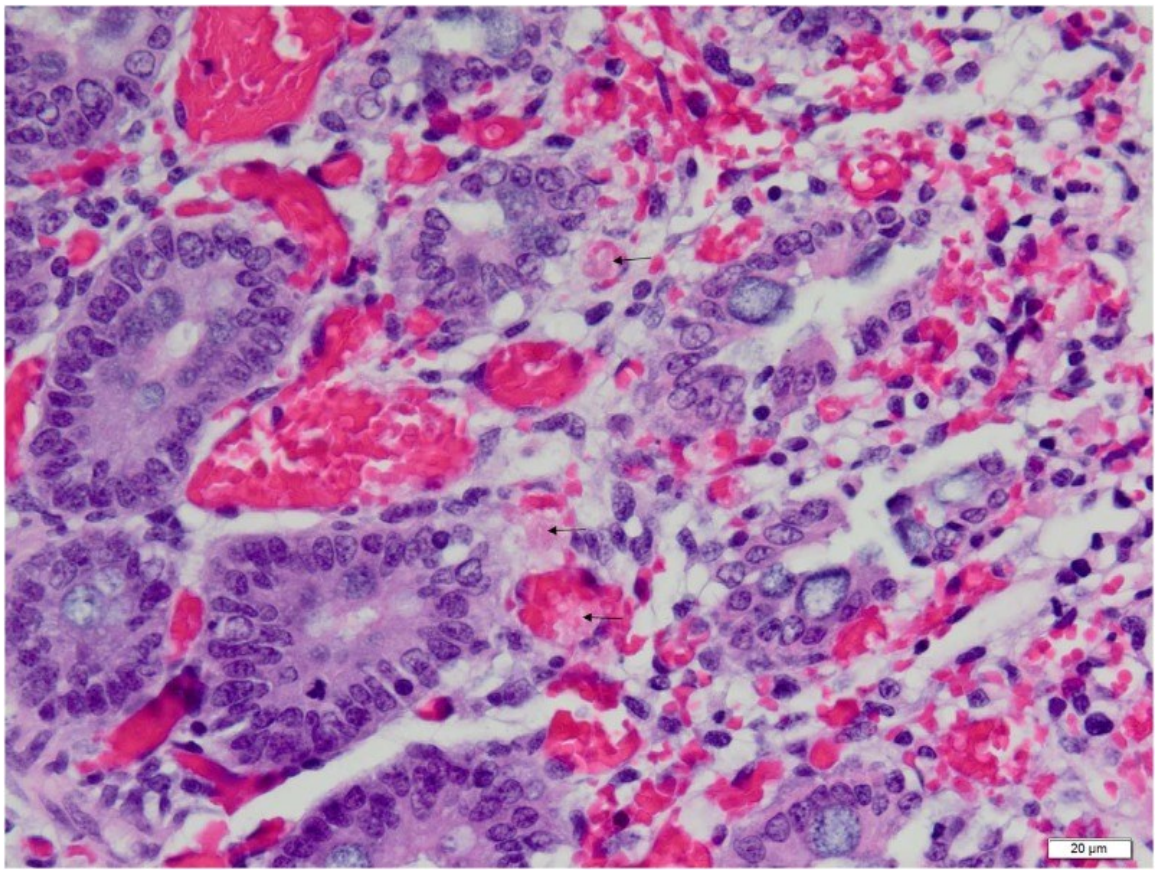

Figure 8. Higher (60X objective) magnification photomicrograph of the same field as that shown in Figure 7. Eosinophilic platelet-fibrin thrombi (arrows) are seen in hyperemic capillaries and venules. Photomicrograph was taken of 4 µm-thick section of 10% neutral-buffered formalin-fixed, paraffin-embedded spiral colonic tissue stained with hematoxylin and eosin. Bar = 20 µm.

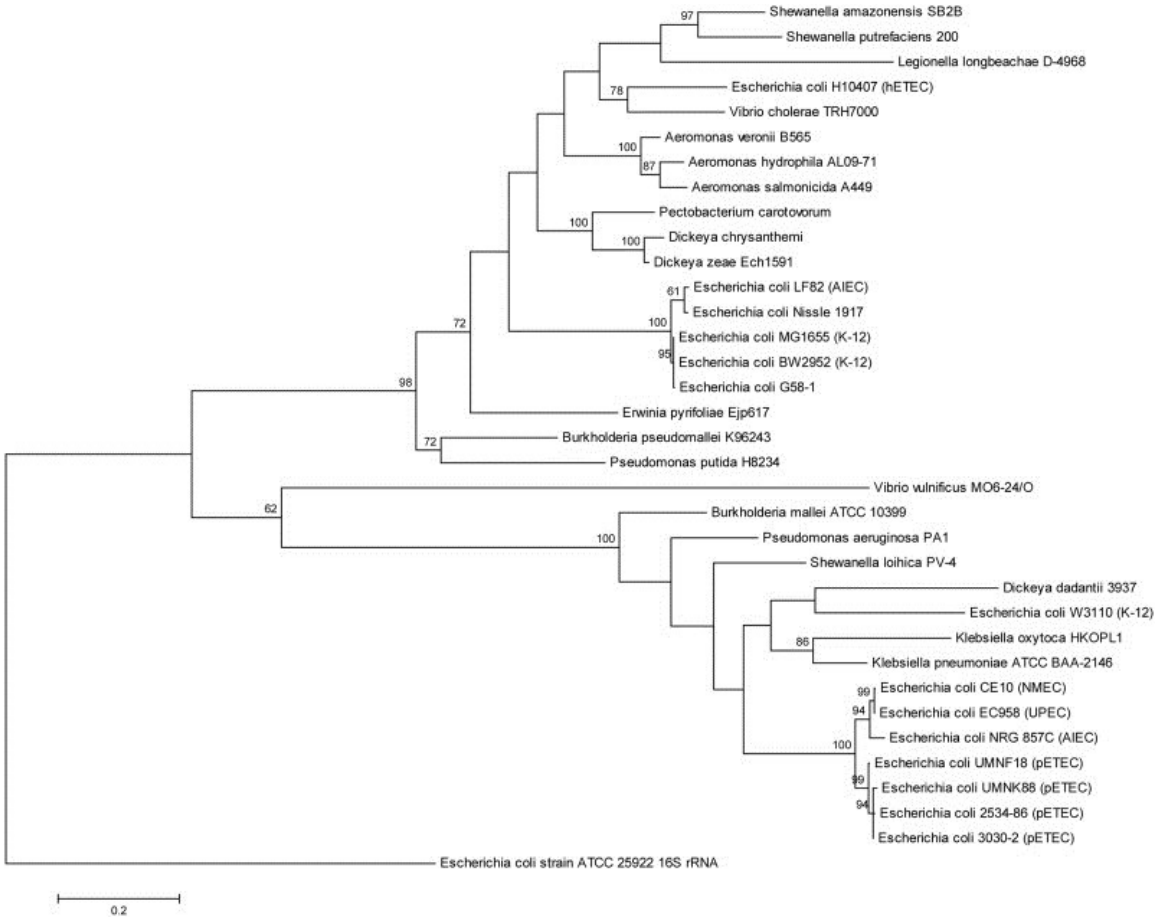

Figure 9. Maximum Likelihood phylogenetic tree generated by analyses of *gspE* and homolog sequences listed in Table S5 using MEGA6.

Table S1. *Escherichia coli* strains used in this study.

Strain	Description[a]	Reference
H10407	O78:K80:H11, CFA/1$^+$, LT$^+$, STp$^+$, STh$^+$, HWT (adult, cholera-like D)	Evans *et al.*, 1973.
2534-86	O8:K87:H$^-$, F4ac$^+$, LT$^+$, STb$^+$, PWT (2 wks, D, HE)	Moxley *et al.,* 1998.
WAM2317	O8:K87:H$^-$, F4ac$^+$, LT$^+$, STb$^+$, spontaneous Nal^r mutant of 2534-86	Moxley *et al.,* 1998.
MUN297	O8:K87:H$^-$, F4ac$^+$, LT$^+$, STb$^-$, Nal^r, Km^r, Δ*estB*::Km^r derivative of WAM2317	Erume *et al.*, 2008
MUN298	O8:K87:H$^-$, F4ac$^+$, LT$^+$, STb$^+$, Nal^r, Km^r, Amp^r, MUN297/pBR322::*estB*	Erume *et al.*, 2008
MUN299	O8:K87:H$^-$, F4ac$^+$, LT$^-$, STb$^+$, Nal^r, Km^r, Δ*eltAB*::Km^r derivative of WAM2317	Erume *et al.*, 2008
MUN300	O8:K87:H$^-$, F4ac$^+$, LT$^-$, STb$^-$, Nal^r, Km^r, Cm^r, Δ*estB*::Cm^r derivative of MUN299	Erume *et al.*, 2008
MUN301	O8:K87:H$^-$, F4ac$^+$, LT$^+$, STb$^-$, Nal^r, Km^r, Cm^r, Amp^r, MUN300/pBR322::*eltAB*)	Erume *et al.*, 2008
MUN302	LT$^+$, STb$^-$, DH5α/pBR322::*eltAB*	Erume *et al.*, 2008
MUN303	LT$^-$, STb$^-$, DH5α/pBR322	Erume *et al.*, 2008
DH5α	K-12 laboratory strain, F$^-$ Φ80*lac*ZΔM15 Δ(*lacZYA-argF*) U169 *recA1 endA1 hsdR17* (rK$^-$, mK$^+$) *phoA supE44* λ$^-$ *thi*-1 *gyrA96 relA1*	Hanahan, 1985
1836-2	O8:H4:F4ac, LT$^-$, STb$^-$, PWT (piglet of unknown age, ND)	Zhang *et al.,* 2006
G58-1	O101:K28:H$^-$:F$^-$, LT$^-$, STb$^-$, PWT (piglet of unknown age, D)	Chen *et al.*, 1985
1905-87	O8:H19:F4, LT$^+$, STb$^+$, PWT (3 wks, D)	Erume *et al.*, 2008
1994-87	O8:H19:F4, LT$^+$, STb$^+$, PWT (4 wks, D, SD, HE)	Erume *et al.*, 2008
3404-83	O149:H19:F4, LT$^+$, STb$^+$ PWT (8 wks, D, HE)	Erume *et al.*, 2008
5061-85	O149:H$^-$:F4, LT$^+$, STb$^+$, PWT (3-5 d, D, HE)	Erume *et al.*, 2008
3539-87	O157:H$^-$:F4, LT$^+$, STb$^+$, PWT (1-3 d, D)	Erume *et al.*, 2008
1888-87	O149:H19:F4, LT$^+$, STb$^+$, PWT (8 wks, D, HE)	Erume *et al.*, 2008
2244-86	O149:H19:F4, LT$^+$, STb$^+$, PWT (4 wks, SD, HE)	Erume *et al.*, 2008

2545-86	O149:H19:F4, LT^+, STb^+, PWT (5-6 wks, D, HE)	Erume *et al/*, 2008
2575-83	O149:H^-:F4, LT^+, STb^+, PWT (2-3 wks, D, SD, HE)	Erume *et al.*, 2008
2583-87	O157:H^-:F4, LT^+, STb^+, PWT (5 wks, D, SD, HE)	Erume *et al.*, 2008
2713-86	O149:H19:F4, LT^+, STb^+, PWT (2 d, D, HE)	Erume *et al.*, 2008
3096-83	O149:H19:F4, LT^+, STb^+, PWT (3 wks, SD, HE)	Erume *et al.*, 2008
7676-85	O149:H^-:F4, LT^+, STb^+, PWT (4 wks, SD, HE)	Erume *et al.*, 2008
4068-85	O149:H^-:F4, LT^+, STb^+, PWT (4 wks, SD, HE)	Erume *et al.*, 2008
3030-2	O157:H^-:F4ac, LT^+, STb^+, PWT (weanling, D, HE)	Francis & Willgohs, 1991

[a]O antigen: K antigen (if known): H antigen, F (fimbrial) antigen (subtype included if known); CFA, colonization factor antigen; LT, heat-labile enterotoxin; STp, heat-stable enterotoxin-a, porcine; STh, heat-stable enterotoxin-a, human; STb, heat-stable enterotoxin-b; HWT, human-derived wild type; PWT, porcine-derived wild type; wks or d, age of pig in weeks or days from which strain was isolated; D, diarrhea; ND, no diarrhea; SD, sudden death; HE, hemorrhagic enteritis was detected grossly and/or histologically in the piglet from which the strain was originally isolated or experimentally in gnotobiotic piglets subsequently inoculated with the strain.; Nal^r, nalidixic acid-resistant; Km^r, kanamycin-resistant; Cm^r, chloramphenicol-resistant; Amp^r, ampicillin-resistant; *eltAB*, genes encoding LT; *estB*, gene encoding STb.

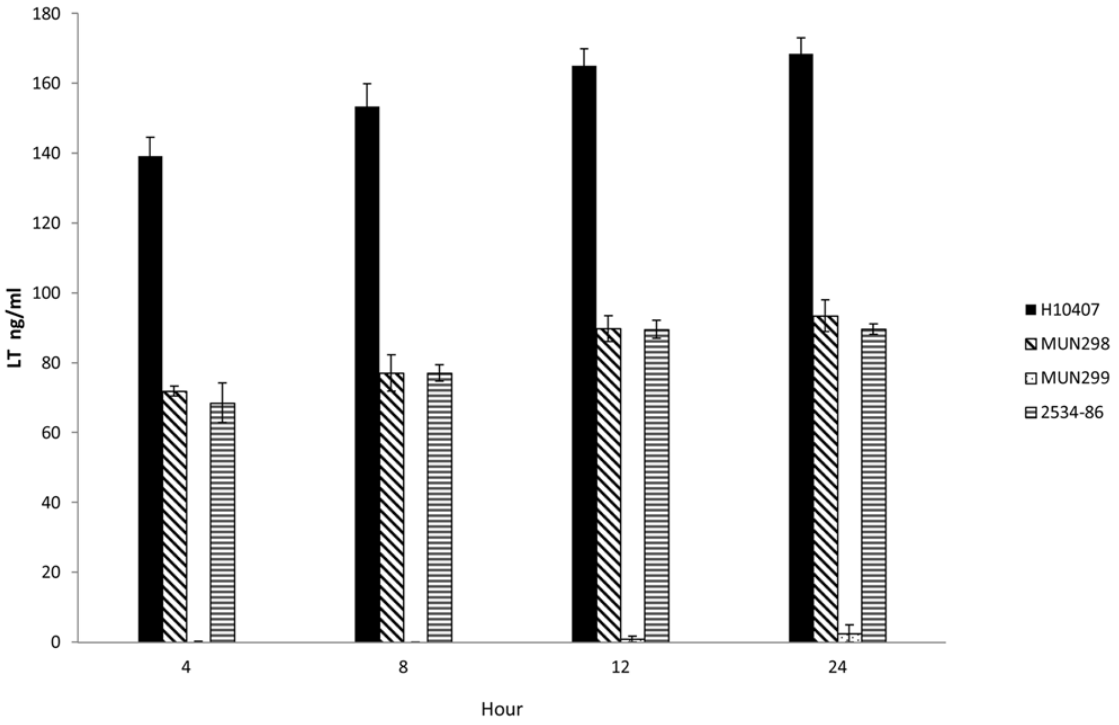

Figure S1. Heat-labile enterotoxin (LT) concentrations in supernatants of cultures of human- and porcine-origin enterotoxigenic *E. coli*. Strains were cultured at 37°C and 225 rpm in Casamino Acids yeast extract medium-Mundell (CAYE-M) medium containing 0.25% glucose, pH 8.5 using a flask-to-medium ratio of 8.3:1. Samples of culture supernatant were obtained at 4, 8, 12, and 24 h of incubation, and LT concentrations in these samples were measured by GM1-ELISA. A human-origin strain is represented by H10407, whereas porcine-origin strains are represented by wild type 2534-86 and derivative strains, MUN298 (LT^+, Δ*estB*, pBR322::*estB*) and MUN299 (LT^- Δ*eltAB*).

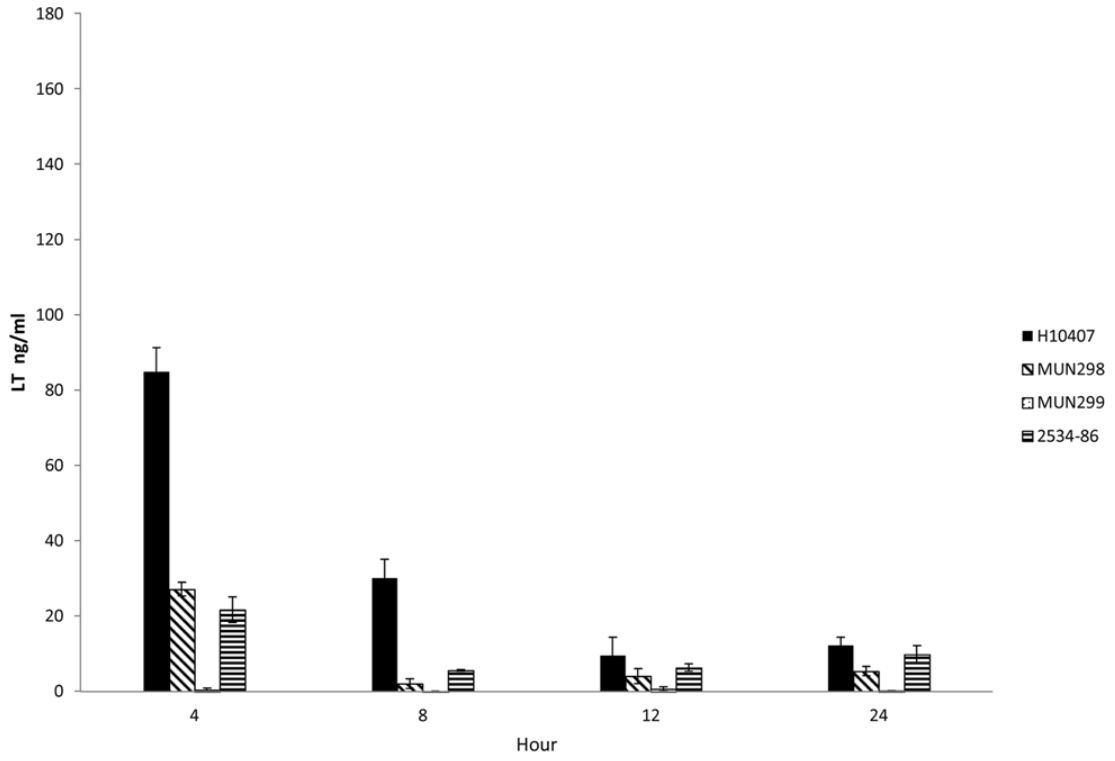

Figure S2. Heat-labile enterotoxin (LT) concentrations in periplasmic extracts of human- and porcine-origin enterotoxigenic *E. coli* strains. Strains were cultured at 37°C and 225 rpm in Casamino Acids yeast extract medium-Mundell (CAYE-M) medium containing 0.25% glucose, pH 8.5 using a flask-to-medium ratio of 8.3:1. Periplasmic extracts were prepared from cell pellets of samples obtained at 4, 8, 12, and 24 h of culture, and LT concentrations were measured by GM1-ELISA. A human-origin strain is represented by H10407, whereas porcine-origin strains are represented by wild type 2534-86 and derivative strains, MUN298 (LT^+, Δ*estB*, pBR322::*estB*) and MUN299 (LT^- Δ*eltAB*).

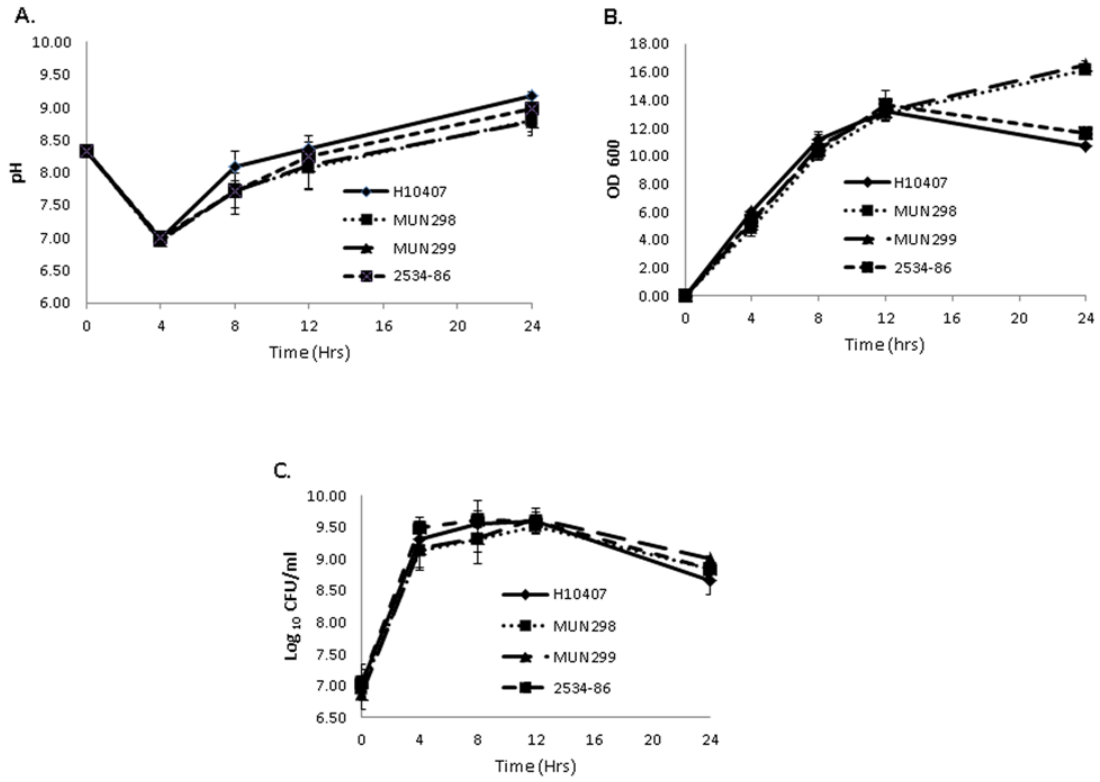

Figure S3. Growth curves of human- and porcine-origin enterotoxigenic *E. coli* strains. Strains were cultured at 37°C and 225 rpm in Casamino Acids yeast extract medium-Mundell (CAYE-M) medium containing 0.25% glucose, pH 8.5 using a flask-to-medium ratio of 8.3:1. Samples were obtained at 0, 4, 8, 12 and 24 h of culture and from these samples the OD_{600}, colony-forming units (CFU)/ml and pH values were determined. The CFU/ml were determined by serial 10-fold dilution and plating on LB agar. A human-origin strain is represented by H10407, whereas porcine-origin strains are represented by wild type 2534-86 and derivative strains, MUN298 (LT^+, Δ*estB*, pBR322::*estB*) and MUN299 (LT^- Δ*eltAB*).

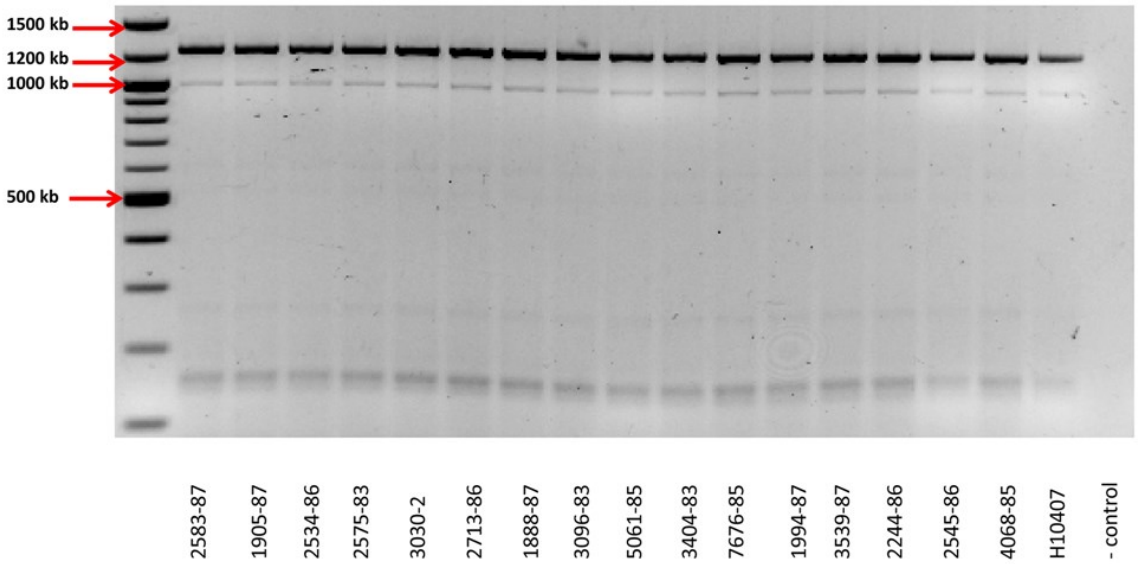

Figure S4. Detection of *gspD* and *gspK* in wild type porcine-origin enterotoxigenic *E. coli* strains (ETEC) by polymerase chain reaction (PCR) assay. PCR assays to determine the existence of the T2SS in porcine ETEC stains were conducted using primers gspDF (5-TTCGGAAATCGCCCGCGTGC) and gspDR (5-TCCACCTTCGAGACTTCC) to generate a 1.0-kb fragment of *gspD*, and primers gspKF (5-GCAGCAGGTGACTAACGGC) and gspKR (5-CAGGGCTTAACCACGGGTC) to generate a 1.2-kb fragment of *gspK* [34]. PCR reactions were conducted using a 95°C initial denaturation for 1 min, followed by 30 cycles of 95°C (30 sec), 60°C (30 sec), and 68°C (90 sec), and a final extension at 72°C for 10 min. Electrophoresis was performed using a 2% agarose- tris acetate ethanol (TAE) gel, supplemented with 0.5 µg/ ml of ethidium bromide. Human-origin strain H10407 was used as a positive control for the presence of *gspD* and *gspK*, and a lane lacking DNA was used as negative control. Amplicons of the appropriate sizes for *gspD* and *gspK* were seen in the lanes containing DNA from H10407 and all porcine-origin ETEC strains (arrows), but not in the negative control lane.

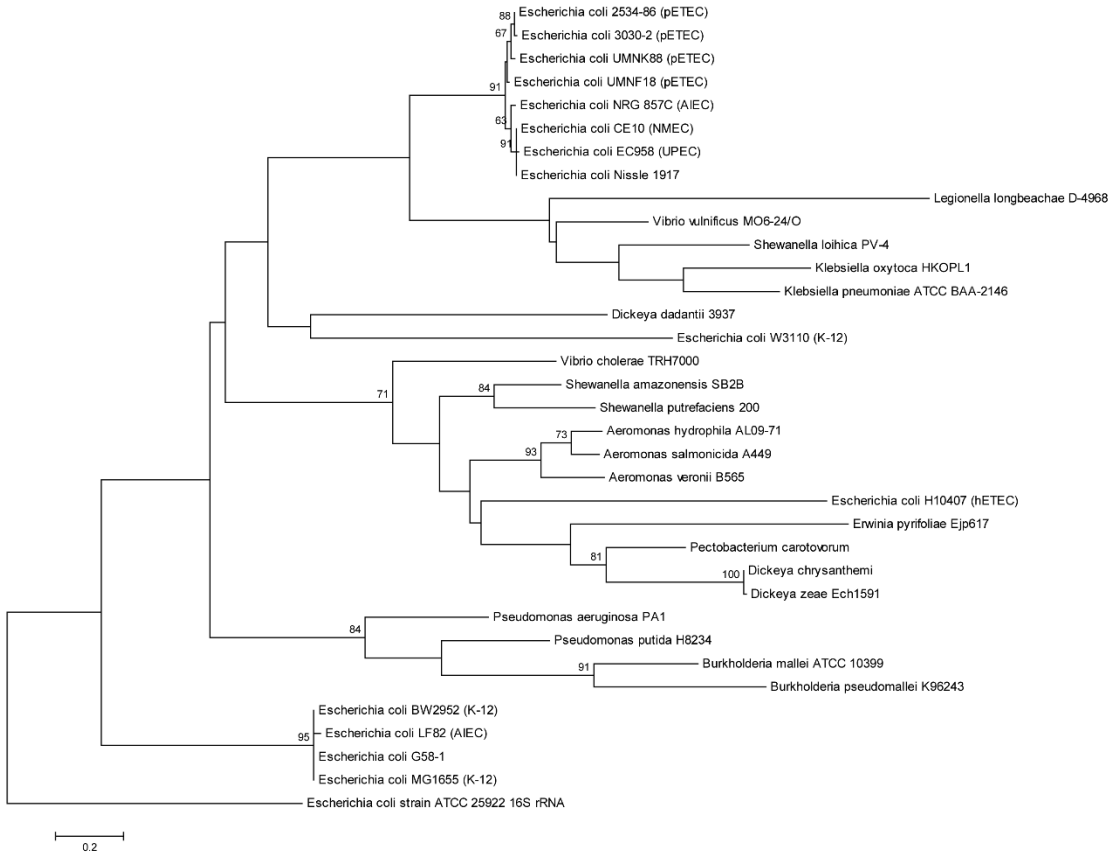

Figure S5. Maximum Likelihood phylogenetic tree generated by analyses of *gspC* and homolog sequences listed in Table S3 using MEGA6.

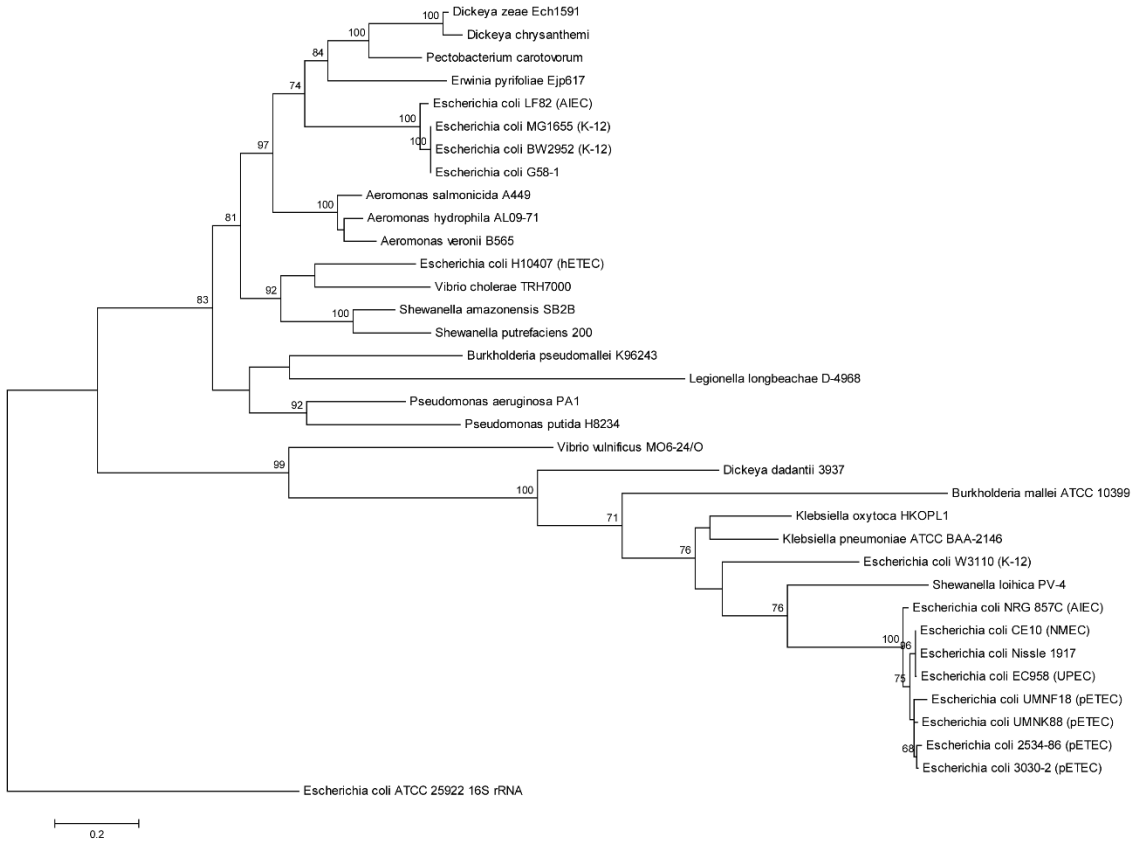

Figure S6. Maximum Likelihood phylogenetic tree generated by analyses of *gspD* and homolog sequences listed in Table S4 using MEGA6.

Table S2. Y1 adrenal cell assay results.

Strain	**GM1 ELISA activity**	**Titer in Y1 cells***	
		After 4 hours	**After 24 hours**
MUN297	+	-	1:40
MUN299	-	-	
MUN300	-	-	
MUN301	+	-	1:20
MUN302	+	-	1:20
H10407	+	-	1:40
1836-2	-	-	
G58-1	-	-	
DH5α	-	-	
2534-86	+	-	1:20
WAM2317	+	-	1:20
3030-2	+	-	1:20

*Titer reflects the highest dilution at which cell rounding involving >50% of the cells was detected.

Table S3. Genetic sequences of *gspC* homologs used for generating the Maximum Likelihood phylogenetic tree.[a]

Strain	**GenBank Accession No.**
Aeromonas hydrophila AL09-71	CP007566.1|:638829-639674
Aeromonas salmonicida 449	CP000644.1|:4067315-4068187
Aeromonas veronii B565	CP002607.1|:3257300-3258139
Burkholderia mallei ATCC 10399	CH899680.1|:193836-194246
Burkholderia pseudomallei K96243	BX571965.1|: 13452-13862
Dickeya chrysanthemi	L02214.1|ERWOUTCM:730-1548
Dickeya dadantii 3937	CP002038.1|:2888814-2889302
Dickeya zeae Ech1591	CP001655.1|:1473340-1474158
Erwinia pyrifoliae Ejp617	CP002124.1|:358437-358898
Escherichia coli 2534-86 (pETEC)	AFDS01000066.1|:14963-15793
Escherichia coli 3030-2 (pETEC)	AFDT01000052.1|:16960-17790
Escherichia coli BW2952 (K-12)	CP001396.1|:3340757-3341572
Escherichia coli CE10 (NMEC)	CP001396.1|:3340757-3341572
Escherichia coli EC958 (UPEC)	HG941718.1|:3389867-3390697
Escherichia coli G58-1	AFDX01000036.1|:16094-16807
Escherichia coli H10407 (hETEC)	AY056599.1|:2234-3064
Escherichia coli LF82 (AIEC)	CU651637.1|:3498687-3499502
Escherichia coli MG1655 (K-12)	U00096.3|:3455578-3456393
Escherichia coli Nissle 1917	CP007799.1|:3440567-3441526

Escherichia coli NRG 857C (AIEC)	CP001855.1|:3112931-3113890
Escherichia coli UMNF18 (pETEC)	AGTD01000001.1|:3643451-3644281
Escherichia coli UMNK88 (pETEC)	CP002729.1|:3592465-3593295
Escherichia coli W3110 (K-12)	AP009048.1|:4184023-4184838
Klebsiella oxytoca HKOLP1	CP004887.1|:5024742-5025233
Klebsiella pneumoniae ATCC BAA-2146	CP006659.1|:957129-957971
Legionella longbeachae D-4968	ACZG01000001.1|:936613-937122
Pectobacterium carotovorum	X70049.1|:74-931
Pseudomonas aeruginosa PA1	CP004054.1|:1075982-1076638
Pseudomonas putida H8234	CP005976.1|:1106032-1106457
Shewanella amazonensis SB2B	CP000507.1|:197297-198220
Shewanella loihica PV-4	CP000606.1|:4317974-4318891
Shewanella putrefaciens 200	CP002457.1|:425346-426272
Vibrio cholerae TRH7000	L33796.1|VIBEPSCN:213-1130
Vibrio vulnificus	CP002469.1|:3073737-3074666
Escherichia coli strain ATCC 25922 16S rRNA	DQ360844.1:86278349

[a]pETEC: porcine-derived enterotoxigenic *Escherichia coli*; NMEC: neonatal meningitis *E. coli*; UPEC: uropathogenic *E. coli*; hETEC: human-derived enterotoxigenic *E. coli*; AIEC: adherent-invasive *E. coli*.

Table S4. Genetic sequences of *gspD* homologs used for generating the Maximum Likelihood phylogenetic tree.[a]

Strain	**GenBank Accession No.**
Aeromonas hydrophila AL09-71	CP007566.1|:639696-641732
Aeromonas salmonicida 449	CP000644.1|:4068209-4070245
Aeromonas veronii B565	CP002607.1|:3258161-3260200
Burkholderia mallei ATCC 10399	CH899680.1|:197030-199282
Burkholderia pseudomallei K96243	BX571965.1|:8395-10668
Dickeya chrysanthemi	L02214.1|ERWOUTCM:1550-3688
Dickeya dadantii 3937	CP002038.1|:2886708-2888798
Dickeya zeae Ech1591	CP001655.1|:1474160-1476310
Erwinia pyrifoliae Ejp617	CP002124.1|:358927-360864
Escherichia coli 2534-86 (pETEC)	AFDS01000066.1|:12873-14933
Escherichia coli 3030-2 (pETEC)	AFDT01000052.1|:15359-16930
Escherichia coli BW2952 (K-12)	CP001396.1|:3341556-3343508
Escherichia coli CE10 (NMEC)	CP001396.1|:3558481-3560541
Escherichia coli EC958 (UPEC)	HG941718.1|:3387777-3389837
Escherichia coli G58-1	AFDX01000036.1|:16791-18743
Escherichia coli H10407 (hETEC)	AY056599.1|:3304-5154
Escherichia coli LF82 (AIEC)	CU651637.1|:3499486-3501438
Escherichia coli MG1655 (K-12)	U00096.3|:3456377-3458329
Escherichia coli Nissle 1917	CP007799.1|:3438477-3440537

Escherichia coli NRG 857C (AIEC)	CP001855.1|:3110841-3112901
Escherichia coli UMNF18 (pETEC)	AGTD01000001.1|:3641361-3643421
Escherichia coli UMNKK88 (pETEC)	CP002729.1|:3590375-3592435
Escherichia coli W3110 (K-12)	AP009048.1|:4182087-4184039
Klebsiella oxytoca HKOLP1	CP004887.1|:5022762-5024741
Klebsiella pneumoniae ATCC BAA-2146	CP006659.1|:955146-957119
Legionella longbeachae D-4968	ACZG01000001.1|:527152-529512
Pectobacterium carotovorum	X70049.1|:1020-2969
Pseudomonas aeruginosa PA1	CP004054.1|:1076643-1078616
Pseudomonas putida H8234	CP005976.1|:1106479-1108227
Shewanella amazonensis SB2B	CP000507.1|:198247-200358
Shewanella loihica PV-4	CP000606.1|:4315828-4317951
Shewanella putrefaciens 200	CP002457.1|:426297-428414
Vibrio cholerae TRH7000	L33796.1|VIBEPSCN: 1176-3200
Vibrio vulnificus	CP002469.1|:3070180-3071679
Escherichia coli strain ATCC 25922 16S rRNA	DQ360844.1:86278349

[a]pETEC: porcine-derived enterotoxigenic *Escherichia coli*; NMEC: neonatal meningitis *E. coli*; UPEC: uropathogenic *E. coli*; hETEC: human-derived enterotoxigenic *E. coli*; AIEC: adherent-invasive *E. coli*.

Table S5. Genetic sequences of *gspE* homologs used for generating the Maximum Likelihood phylogenetic tree.[a]

Strain	**GenBank Accession No.**
Aeromonas hydrophila AL09-71	CP007566.1|:641732-643237
Aeromonas salmonicida 449	CP000644.1|:4070245-4071750
Aeromonas veronii B565	CP002607.1|:3260200-3261705
Burkholderia mallei ATCC 10399	CH899680.1|:195540-197033
Burkholderia pseudomallei K96243	BX571965.1|:10665-12158
Dickeya chrysanthemi	L02214.1|ERWOUTCM:3685-5181
Dickeya dadantii 3937	CP002038.1|:2885230-2886708
Dickeya zeae Ech1591	CP001655.1|:1476307-1477803
Erwinia pyrifoliae Ejp617	CP002124.1|:360864-362330
Escherichia coli 2534-86 (pETEC)	AFDS01000066.1|: 11380-12873
Escherichia coli 3030-2 (pETEC)	AFDT01000006.1|:81778-83163
Escherichia coli BW2952 (K-12)	CP001396.1|:3343518-3344999
Escherichia coli CE10 (NMEC)	CP001396.1|:3556988-3558481
Escherichia coli EC958 (UPEC)	HG941718.1|:3386284-3387777
Escherichia coli G58-1	AFDX01000036.1|:18753-20234
Escherichia coli H10407 (hETEC)	AY056599.1|:5154-6647
Escherichia coli LF82 (AIEC)	CU651637.1|:3501448-3502929
Escherichia coli MG1655 (K-12)	U00096.3|:3458339-3459820
Escherichia coli Nissle 1917	CP007799.1|:3819104-3820585

Escherichia coli NRG 857C (AIEC)	CP001855.1|:3109348-3110841
Escherichia coli UMNF18 (pETEC)	AGTD01000001.1|:3639868-3641361
Escherichia coli UMNKK88 (pETEC)	CP002729.1|:3588882-3590375
Escherichia coli W3110 (K-12)	AP009048.1|:4180596-4182077
Klebsiella oxytoca HKOLP1	CP004887.1|:5021257-5022762
Klebsiella pneumoniae ATCC BAA-2146	CP006659.1|:953656-955149
Legionella longbeachae D-4968	ACZG01000001.1|:529512-530999
Pectobacterium carotovorum	X70049.1|:2966-4462
Pseudomonas aeruginosa PA1	CP004054.1|:1074203-1075711
Pseudomonas putida H8234	CP005976.1|:1108227-1109675
Shewanella amazonensis SB2B	CP000507.1|:200369-201931
Shewanella loihica PV-4	CP000606.1|:4314264-4315835
Shewanella putrefaciens 200	CP002457.1|:428407-429972
Vibrio cholerae TRH7000	L33796.1|VIBEPSCN:3197-4708
Vibrio vulnificus	CP002469.1|:3068963-3070180
Escherichia coli strain ATCC 25922 16S rRNA	DQ360844.1:86278349

[a]pETEC: porcine-derived enterotoxigenic *Escherichia coli*; NMEC: neonatal meningitis *E. coli*; UPEC: uropathogenic *E. coli*; hETEC: human-derived enterotoxigenic *E. coli*; AIEC: adherent-invasive *E. coli*.

CHAPTER 3

GLUCOSE SIGNIFICANTLY ENHANCES ENTEROTOXIGENIC *ESCHERICHIA COLI* ADHERENCE TO INTESTINAL EPITHELIAL CELLS THROUGH ITS EFFECTS ON HEAT-LABILE ENTEROTOXIN PRODUCTION

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Abstract

The present study tested whether exposure of enterotoxigenic *Escherichia coli* (ETEC) to glucose at different concentrations in the media results in increased bacterial adherence to host cells through increased heat-labile enterotoxin (LT) production, thereby suggesting the effects are physiological. Porcine-origin ETEC strains grown in Casamino acid yeast extract medium containing different concentrations of glucose were washed and inoculated onto IPEC-J2 porcine intestinal epithelial cells to test for effects on adherence and host cell cAMP concentrations. Consistent with previous studies, all LT^+ strains had higher ETEC adherence to IPEC-J2 cells than did LT^- strains. Adherence of the LT^- but not the LT^+ strains was increased by pre-incubating the IPEC-J2 cells with LT and decreased by co-incubation with GM1 ganglioside in a dose-dependent manner ($P<0.05$). To determine whether the glucose concentration of the cell culture media has an effect on adherence, IPEC-J2 cells were inoculated with LT^+ or LT^- strains in cell culture media containing a final glucose concentration of 0, 0.25, 0.5, 1.0 or 2.0%, and incubated for 4 h. Only media containing 0.25% glucose resulted in increased adherence and cAMP levels, and this was limited to IPEC-J2 cells inoculated with LT^+ strains. This study supports the hypothesis that glucose, at a concentration optimal for LT expression, enhances bacterial adherence through the promotion of LT production. Hence, these results establish the physiological relevance of the effects of glucose on LT production, and provide a basis for how glucose intake may influence the severity of ETEC infection.

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is the most common bacterial cause of diarrhea in humans in the world (Walker *et al.,*2007) and an important cause of diarrhea and death in livestock, especially cattle and swine (Nagy & Fekete, 2005; Zhang *et al.,*2007). ETEC cause diarrhea primarily through the effects of enterotoxins (Sears & Kaper, 1996). Many highly virulent ETEC isolates from swine produce F4ac (K88ac) fimbria and both heat-labile enterotoxin-I (LT) and heat-stable enterotoxin-b (STb) (Berberov *et al.,* 2004), with the genes for the two toxins linked on a large, transmissible plasmid (Picken *et al.,*1983).

Environmental conditions are known to affect the production of LT, heat stable enterotoxin-a (STa) and STb (Mundell *et al.,*1976; Gilligan & Robertson, 1979; Kunkel & Robertson, 1979; Busque *et al.,*1995; Hedge *et al.,*2009). Glucose increases LT production through de-repression of the *eltAB* (LT) promoter (Bodero & Munson, 2009). Glucose suppresses synthesis of cyclic AMP (cAMP), which prevents cAMP receptor protein (CRP)-cAMP complex formation and binding to sites upstream and within the *eltA* promoter (Bodero & Munson, 2009). Interestingly, glucose also prevents transcription of *estB* (STb) and *estA* (STa) through catabolite repression (Busque *et al.,*1995; Bodero & Munson, 2009).

In addition to causing severe diarrhea and weight loss, LT produced by ETEC enhances the organism's capacity to colonize the small intestines (Berberov *et al.,* 2004; Allen *et al.,* 2006), although this effect has not always been reproducible with isogenic LT^- mutant ETEC strains (Erume *et al.*, 2008). LT promotes adherence of ETEC to intestinal epithelial cells (Johnson *et al.,* 2009), and this effect is mediated both by

enzymatic and non-enzymatic properties of the toxin (Fekete *et al.,* 2013). Conversely, possession of *estB* has not resulted in increased colonization in inoculated 9-day-old gnotobiotic piglets (Erume *et al.,*2008), but instead has been associated with reduced ETEC adherence in ligated jejunal loops in fasted 5- to 8-week-old weaned pigs in association with increased fluid accumulation (Erume *et al.,* 2013).

Mudrak and Kuehn (Mudrak & Kuehn, 2010) raised the hypothesis that exposure of ETEC to glucose in the proximal intestine may induce the production of LT to aid adherence and colonization of the ileum, where most ETEC cells are found in a mouse model of colonization (Allen *et al.,*2006). However, to our knowledge, no studies have tested whether exposure of LT^+ ETEC to glucose leads to increased colonization or epithelial adherence. The present study addressed the latter question, utilizing the IPEC-J2 porcine intestinal epithelial cell line and porcine-origin ETEC strains (Erume *et al.,* 2008). In contrast to the strains tested by Johnson *et al.* (2009), those used in the present study included both LT^- and STb^- deletion mutants, and the LT^- mutants had not demonstrated reduced intestinal colonization in gnotobiotic piglets (Erume *et al.,* 2008). Hence, we tested the effects of LT in adherence with these strains prior to conducting experiments with glucose. In addition, we tested whether other sugars (sucrose or fructose) had an effect on LT production and secretion. We found that growth of *E. coli* in media containing glucose at a concentration optimal for LT expression enhances bacterial adherence to intestinal epithelial cells through promotion of LT production.

Materials and Methods

Bacterial strains and culture conditions. The following strains were used in this study: 2534-86, a wild-type (WT) porcine-origin F4ac$^+$, LT$^+$, STb$^+$ ETEC (Moxley *et al.,*1998); WAM2317, a spontaneous nalidixic-acid resistant mutant of 2534-86 (Moxley *et al.,*1998); MUN297 (STb$^-$ mutant of WAM2317); MUN298 (MUN297 complemented with STb); MUN299 (LT$^-$ mutant of WAM2317); MUN300 (LT$^-$ STb$^-$ mutant of WAM2317); and MUN301 (MUN300 complemented with LT; Erume *et al.,* 2008). DH5α (K-12 *E. coli* laboratory strain), and G58-1 (WT non-toxigenic *E. coli* strain of porcine origin (Chen *et al.,* 1985) were also used in this study.

With the exception of experiments testing the effects of glucose and other sugars on LT secretion, strains were grown in Casamino Acid Yeast Extract (CAYE) medium containing Bacto Casamino Acids and Difco Yeast Extract at a final glucose concentration of 0.25% and pH of 8.5 (Mundell *et al.,*1979). Starter cultures were grown in 3.0 ml of CAYE in 15 ml tubes at 37°C and 225 rpm for 18 h. For adherence and cAMP assays, the starter culture was diluted 1:100 in 3 ml of CAYE in 15 ml tubes and grown for 2 h at 37°C and 225 rpm.

For experiments involving the effects of different sugars on growth, pH, LT production and secretion, strains were grown in CAYE media containing glucose, sucrose or fructose at 0%, 0.25%, 0.5%, 1%, or 2% at pH 8.5. For each strain and medium, a 150-µl aliquot of starter culture was inoculated into 15 ml of the same medium in a 125-ml flask and grown at 37°C and 225 rpm for 6 h.

IPEC-J2 cell culture conditions and bacterial adherence assays. IPEC-J2 porcine small intestinal epithelial cells (Schierack *et al.,* 2006), obtained from Dr.

Thomas Burkey, University of Nebraska-Lincoln, were cultured using methods previously described (Johnson *et al.,* 2009). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)-F12 medium supplemented with 5% fetal bovine serum (Sigma), 1% insulin-selenium-transferrin mix (Sigma), epidermal growth factor (5 ng/ml) (Invitrogen), 1% penicillin-streptomycin mix (Gibco) and were maintained in a humidified incubator in an atmosphere of 5% CO_2 at 37°C. The DMEM-F12 medium supplemented with serum and other growth factors as described above contained 28 to 33 μ*M* (0.000504%-0.000594%) glucose as determined by a glucose oxidase assay (Amplex Red Glucose/Glucose Oxidase assay, Invitrogen).

IPEC-J2 cells were seeded in 24-well plates at a concentration of 10^5 cells per well and grown to >90% confluence before inoculation. Bacterial inocula were prepared from 2-h cultures washed 3 times with PBS and seeded at a multiplicity of infection of 100:1. Plates were incubated for 1 or 2 h in an atmosphere of 5% CO_2 at 37°C and washed 3 times with 2 ml PBS per well (5 min per wash) to remove non-adherent bacteria. Plates were then treated with 0.25% trypsin (250 μl per well) at 5% CO_2, 37°C for 5 min. The contents of each well was collected, centrifuged (2500 × *g*, 5 min), re-suspended in 1 ml PBS, serially diluted, plated on LB, and incubated for 24 h at 37°C. The final CFU was measured by subtracting the CFU from control wells of the same 24-well plate lacking any IPEC-J2 cells.

To test for the effects of endogenous LT expressed by the bacteria on adherence in isogenic porcine-origin ETEC strains that had not demonstrably shown an effect of LT on colonization (Erume *et al.,* 2008), inocula were prepared and directly applied to the IPEC-J2 cells, as described above. To test the effect of exogenous LT on adherence on

these strains, LT (List Biological Laboratories) at a concentration of 1, 10, or 100 ng/ml per well was added (pre-incubation) 1 h prior to bacterial inoculation. To test the potential of GM1 ganglioside to mitigate the effects of LT on adherence, LT at a concentration of 10 ng/ml or 100 ng/ml was incubated with the cells 1 h prior to their inoculation with bacteria, and 10 ng/ml or 100 ng/ml GM1 ganglioside (Sigma) was applied to the IPEC-J2 cells at the time of bacterial inoculation. Three independent experiments were performed for each assay.

Test of effects of different carbohydrates on LT production and secretion. To test the effects of different sugars on LT secretion, CAYE media containing glucose, sucrose or fructose at 0%, 0.25%, 0.5%, 1%, or 2% were prepared. Strains were cultured in different media as described above, with aliquots taken at 0, 2, 4, and 6 h post-inoculation (PI). These samples were centrifuged (2500 × *g*, 10 min), and supernatants taken to analyze for LT concentrations by GM1-ELISA as previously described (Erume *et al.,* 2013). Three independent experiments were performed for each assay.

Tests of effects of glucose on bacterial adherence and cAMP concentrations in IPEC-J2 cells. IPEC-J2 media (50 ml) were incubated with 500 µl of glucose oxidase solution with an activity of 500-2500 U/ml (MP Biomedicals) for 1.5 h at room temperature to remove glucose naturally present in the media, followed by 1 h incubation in a 65°C water bath to denature glucose oxidase. After establishing the absence of glucose by an Amplex Red Glucose/Glucose Oxidase assay (Invitrogen), glucose was added to make IPEC-J2 media with 0%, 0.25%, 0.5%, 1% and 2% glucose concentrations. IPEC-J2 cells were added to media with the varying glucose concentrations followed by bacterial inoculation and then the cells were incubated,

treated, and plated in the same manner as in the bacterial adherence assays as described above, except that incubations occurred for 4 h.

To determine whether differences in glucose concentration, through their effects on the bacteria were associated with LT-mediated effects on host cells, cAMP levels of infected IPEC-J2 cells were determined. These experiments were performed using the same methods as for the experiments conducted for studying the effect of different glucose levels on adherence to IPEC-J2 cells except that instead of treating the cells with 0.25% trypsin, cells were lysed using cell lysis buffer (#9803) included in a cAMP assay kit (Cell Signaling Technology, Cyclic AMP XP Assay Kit #4339S) and tested for cAMP concentrations using this kit following the manufacturer's instructions. Three independent experiments were performed for all assays.

Statistical analyses. SAS Version 9.4 (Cary, NC) software was used to analyze the data for bacterial adherence assays using the least squares method with PROC GLM, with significant differences ($P < 0.05$) calculated by Tukey's method. Results for glucose effect on bacterial adherence and cAMP assay were analyzed by unpaired Student's *t*-tests with values of $P < 0.05$ considered significant.

Results

Confirmation that endogenously expressed LT increases adherence of porcine ETEC strains to IPEC-J2 cells. It was necessary to confirm that LT promoted the adherence of *E. coli* on IPEC-J2 cells as shown previously (Johnson *et al.,*2009) since our studies involved a different set of isogenic strains, and ones in which the production of LT had not resulted in an increase in intestinal colonization of gnotobiotic piglets

(Erume *et al.,*2008). In addition, our experiments included isogenic STb$^-$ mutants and STb$^+$ complemented mutant strains; the latter had reduced adherence in ligated jejunal loops in weaned pigs in association with fluid accumulation (Erume *et al.,* 2013). At 1 h PI, 2534-86 (WT LT$^+$ STb$^+$) had significantly greater adherence than non-isogenic LT$^-$ STb$^-$ controls, G58-1 and DH5α (P<0.05; Fig. 1). At 2 h PI, 2534-86 and MUN297 (LT$^+$ STb$^-$) had significantly greater adherence than LT$^-$ strains and MUN298 (LT$^+$ STb-complemented mutant; P<0.05; Fig. 1). These results confirmed the conclusions of Johnson *et al.* (2009) that endogenously expressed LT promotes adherence of porcine-origin ETEC to porcine intestinal epithelium.

Demonstration of dose-dependent increase by exogenous LT and inhibition by GM1 ganglioside of adherence of ETEC to IPEC-J2 cells. To confirm the results of Johnson *et al.* (2009) that adherence of LT$^-$ porcine *E. coli* strains could be increased by exogenously applied LT, IPEC-J2 cells were incubated with 100 ng/ml of LT, either 1 h prior to or at the time of inoculation. Addition of LT at either time-point did not affect the adherence level of 2534-86 (Fig. 2A). However, the adherence levels of MUN299 (LT$^-$ STb$^+$) Fig. 2B), MUN300 (LT$^-$ STb$^-$; Fig. 2C) and G58-1 (LT$^-$ STb$^-$; Fig. 2D) were significantly increased (P< 0.05) when the IPEC-J2 cells were pre-incubated with exogenous LT. Only the adherence of MUN300 was significantly increased (P<0.05) when IPEC-J2 cells were co-incubated with exogenous LT. Pre-incubation with 10 and 100 but not 1 ng/ml LT resulted in a significant increase in adherence, demonstrating that the effect was dose-dependent (Fig. 3). Addition of GM1 at 100 but not 10 ng/ml at the time of inoculation significantly reduced adherence of 2534-86 and MUN301 (LT$^+$ STb$^-$; Fig. 4). The adherence of MUN300 was not reduced by the addition of 10 or 100 ng/ml

GM1 (Fig. 4). These results confirmed the findings of Johnson *et al.* (2009) that exogenous LT increases the adherence of LT$^-$ *E. coli* and extended these results by demonstrating that the pro-adherent effects of LT are dose-dependent. In addition, dose-dependent inhibition of adherence by GM1 ganglioside was demonstrated.

Effect of different concentrations of glucose and other sugars on LT production. In studies involving H10407 (Mundell *et al.,* 1976) and other human-origin strains (Hegde *et al.,* 2009), the optimal glucose concentration for LT production in CAYE medium was 0.25%. Kunkel and Robertson (1979) reported that LT levels increase as the pH is raised in 0.5 U intervals from 6.0 to 10.0, with LT primarily remaining cell-bound at a pH lower than 7.0 and most LT released between pH 7.5 and 8.0. To determine the effects of glucose concentration on secreted LT by 2534-86, this strain was grown in CAYE medium containing 0, 0.25, 0.5, 1.0 or 2.0% glucose, supernatants were sampled at 0, 2, 4 and 6 h of culture, and growth and pH were monitored. The LT concentrations in the culture supernatants increased with time in all glucose concentrations; however, consistent with human-origin strains, the medium containing 0.25% glucose yielded the highest LT concentration at 4 and 6 h of culture (P<0.05; Fig. 5). No significant differences in cell growth were detected in media containing 0.25 to 2.0% glucose (Fig. 6A); however, the pH of media containing 0.5 to 2.0% glucose was significantly lower than that of media containing 0 and 0.25% glucose at 6 h of culture (Fig. 6B). These results were consistent with previous reports (Gilligan & Robertson, 1979; Kunkel & Robertson, 1979) that glucose at a concentration of 0.25% yielded maximal LT production, and decreases in the pH from 8.0 to 7.0 were associated with a significant decrease in the LT concentration in the culture supernatant (Fig. 5).

Hegde *et al.* (2009) reported that osmotic stress (induced by NaCl supplementation to CAYE medium) reduces LT production. To test whether strain 2534-86 grown in media containing glucose at concentrations higher than 0.25% was under osmotic stress that may have affected LT production, we repeated the experiments with sucrose and fructose in place of glucose. The levels of LT produced with different concentrations of sucrose or fructose were several folds lower than that in the glucose counterpart (Fig. 7). Furthermore, neither 0.25% sucrose nor fructose yielded a higher LT concentration compared to other concentrations of the same sugar, in contrast to what had been seen with glucose. Growth curves in CAYE medium containing different concentrations of sucrose and fructose revealed minor reductions in growth rate (mainly sucrose) and pH decline compared to their glucose counterparts (Fig. 6C-F). Collectively, these results tended to rule out osmotic stress as the cause of suboptimal LT production at higher glucose concentrations. Since glucose and fructose share the same molecular weight the osmotic pressures exerted by them are identical. In media containing glucose and fructose at 0.25, 0.5, 1.0 and 2.0% the osmotic pressures calculated by the Van't Hoff equation were 0.34, 0.68, 1.36 and 2.71 atm, respectively; in the case of sucrose, they were 0.18, 0.36, 0.71, and 1.43 atm. Therefore, the lower LT production occurring at glucose concentrations higher than 0.25% was not the result of differences in osmotic pressure.

Effect of glucose on ETEC adherence to IPEC-J2 cells. To determine whether the glucose concentration of the cell culture media has an effect on adherence of ETEC to porcine epithelial cells through induction of increased LT production and secretion, IPEC-J2 cells were inoculated with WAM2317 (spontaneous Nal^R mutant of 2534-86),

MUN300, or MUN301 in cell culture media (DMEM-F12) containing a final glucose concentration of 0, 0.25, 0.5, 1.0 or 2.0%, and incubated for 4 h. Only media containing 0.25% glucose resulted in an increased adherence level, and this was limited to WAM2317 and MUN301 (Fig. 8). MUN300 did not show any difference in adherence level at any glucose concentration. To determine whether the increased adherence level was associated with the toxigenic effects of LT, the same experiment was performed and the infected IPEC-J2 cells were lysed to assess the intracellular cAMP levels. Only IPEC-J2 cells infected in the presence of 0.25% glucose showed higher cAMP levels for WAM2317 and MUN301 (Fig. 9). MUN300 did not show any increase in the cAMP levels at any glucose concentration.

Discussion

This study demonstrated that exposure of LT^+ ETEC bacteria to glucose at a concentration optimal for LT production and secretion significantly increases bacterial adherence to intestinal epithelial cells through the effects of LT. This hypothesis had been raised by other investigators (Mudrak & Kuehn, 2010), but not tested. Our work also supports the conclusions of Johnson *et al.* (2009) that both endogenously expressed and exogenously applied LT enhance the adherence of *E. coli* to intestinal epithelial cells. A recent study by Fekete *et al.* (2013) using other porcine strains also found that LT expression enhanced adherence. However, in contrast to these studies, our experiments included isogenic STb^- deletion and complemented mutants, and strains in which expression of LT did not significantly increase intestinal colonization of gnotobiotic piglets (Erume *et al.,* 2008). The lack of a detectable increase in colonization raised the

question whether LT expression or complementation would increase their capacity for epithelial adherence. The present study confirmed that LT expression increases the epithelial cell adherence capacity of these strains. We hypothesize that the susceptibility of some litters of F4ac (K88) receptor-positive piglets to colonization by F4ac ETEC may be so great as to mask the pro-adherence effects of LT.

The present study confirmed the important role of glucose in LT production and control of pH in LT secretion or release from the bacterial cell. Delivery of LT to the host epithelial cells requires that the toxin first be secreted from the bacterium, a process known to require a type II secretion system (T2SS) (Tauschek *et al.,* 2002). In order to deliver LT to the host cell, the bacterium localizes the T2SS and LT secretion in polarized fashion with transfer of the preformed toxin at the site of contact of the bacterium with the host cell surface (Dorsey *et al.,* 2006). LT also has been shown to be delivered to host epithelial cells via outer membrane vesicles, to which it has coated (Horstman & Kuehn, 2000; Horstman & Kuehn, 2002; Kesty *et al.,* 2004).

The molecular mechanisms by which LT promotes bacterial adherence are not fully understood, but evidently involve the effects of A subunit-mediated intoxication and bacterial sensing of host cell-derived cAMP. Johnson *et al.* (2009) demonstrated that an inhibitor of protein kinase A abrogated LT's ability to promote subsequent bacterial adherence, and that increased adherence was not due to changes in the surface expression of the host receptor for F4ac fimbrial adhesin. Through the testing of LT A-subunit mutants, the authors further demonstrated that ADP-ribosylation activity was necessary to effect changes in bacterial adherence. Pre-treatment of WT non-enterotoxigenic strain G58-1 with cAMP significantly increased its capacity to adhere to IPEC-J2 cells. De

novo bacterial protein synthesis appeared to be required, as treatment of 2534-86 with tetracycline reduced the ability of cAMP to promote subsequent adherence. Incubation of 2534-86 but not 2534-86Δ*eltAB* with supernatants of IPEC-J2 cells intoxicated with LT increased expression of FaeG, the major F4ac fimbrial subunit; however, IPEC-J2 cells intoxicated with LT did not increase expression of the F4ac fimbrial receptor. It is possible that LT intoxication may induce expression of a receptor for a heretofore unrecognized adhesin.

Fekete *et al.* (2013) reported that the pro-adherence effects of LT are due to a combination of enzymatic and non-enzymatic properties. These conclusions were based on experiments in which adherence was unaltered by cycloheximide treatment which prevented host cell protein synthesis. Pretreatment of IPEC-J2 cells with LT promoted adherence of negatively charged latex beads (a surrogate for bacteria which carry a negative charge), and this effect was inhibited by cycloheximide, suggesting LT may induce a change in epithelial cell membrane potential. These authors concluded that LT may enhance ETEC adherence by promoting an association between the LT B-subunit and epithelial cells, and by altering the surface charge of the host plasma membrane. Collectively, this study and the one by Johnson *et al.* (2009) suggest that different molecular mechanisms may be involved in the LT-induced promotion of bacterial adherence.

ETEC is an important cause of neonatal and post-weaning diarrhea in swine, and diet has long been recognized to have a significant effect on enteric colibacillosis in recently-, and especially, early-weaned pigs. High starch (*e.g.*, cereal meal) and low indigestible fiber intake increase and low starch and high indigestible fiber intake reduce

ETEC-mediated diarrheal disease in weanling pigs with ETEC colonizing the proximal small intestine (Smith & Halls, 1968). Replacing 5% of ground corn with glucose in the feed of recently weaned baby pigs further increases the incidence of diarrhea (Bayley & Carlson, 1970). Feeds containing viscous soluble fiber (given to promote development of the large intestine) stimulate proliferation of ETEC in newly-weaned pigs, an effect thought to result from a decreased rate of passage of digesta through the small intestine, and interference with digestion and absorption of nutrients (McDonald *et al.,* 2001, Hopwood *et al.,* 2002). To our knowledge, the only hypothesis that has been put forth for the exacerbation of ETEC-mediated disease by excess glucose in the intestine is that it promotes increased bacterial growth.

ETEC isolated from cases of post-weaning colibacillosis in swine almost exclusively express F4 or F18 fimbria, with 100 or 39.5% of these isolates, respectively, producing LT (Frydendahl *et al.,* 2002). Porcine ETEC that express F4ac fimbria in particular, effectively colonize the duodenum as well as more distal portions of the small intestine (Hohmann & Wilson, 1975). The present study demonstrates that glucose, at an optimal concentration, promotes bacterial adherence to the intestinal epithelium through the increased production of LT. We propose that exposure of LT^+ ETEC to glucose in conjunction with bicarbonate buffering in the duodenum (Argenzio, 2004), promotes the expression and secretion of LT. LT, through induction of cAMP release by intoxicated host cells, may induce expression of F4ac fimbria by ETEC on the host cell surface (Johnson *et al.,* 2009) and also enhance ETEC adherence to the intestinal epithelium prior to fimbrial expression through alteration of the host cell membrane potential (Fekete *et al.,* 2013), thereby initiating colonization in the proximal small intestine.

Conclusion

Exposure of enterotoxigenic *Escherichia coli* (ETEC) to glucose at 0.25% resulted in significantly increased adherence of these bacteria to enterocytes by inducing production and secretion of heat-labile enterotoxin (LT). These results establish the physiological relevance of the effects of glucose on LT production, and provide an additional hypothesis beyond that of increased bacterial growth to explain how dietary glucose may increase the severity of ETEC infections.

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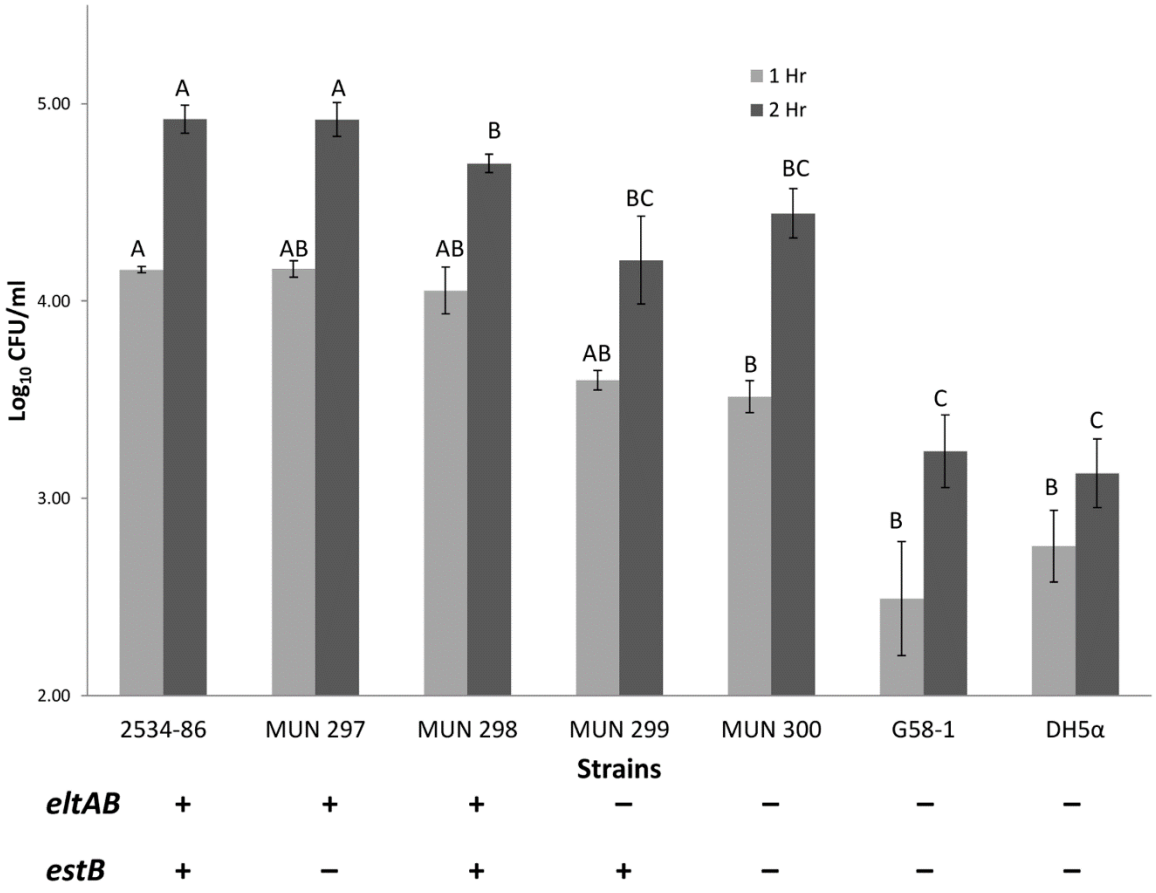

Figure 1. Effects of endogenous LT on adherence of isogenic porcine-origin ETEC strains to porcine intestinal epithelial (IPEC-J2) cells. Strains were cultured in Casamino Acid Yeast Extract (CAYE) medium containing 0.25% glucose, final pH 8.5, conditions which supported the production and secretion of LT. IPEC-J2 cells were inoculated at a multiplicity of infection of 100:1 in DMEM-F12 cell culture media. Adherence of $F4ac^+$ ETEC strains was compared with that of non-fimbriated LT^- control strains G58-1 and DH5α after 1 and 2 h of incubation. *eltAB* and *estB* denote presence (+) or absence (-) of genes for LT and STb production, respectively. Means with different letter designations indicate adherence levels that are significantly different ($P<0.05$) within the same incubation time group.

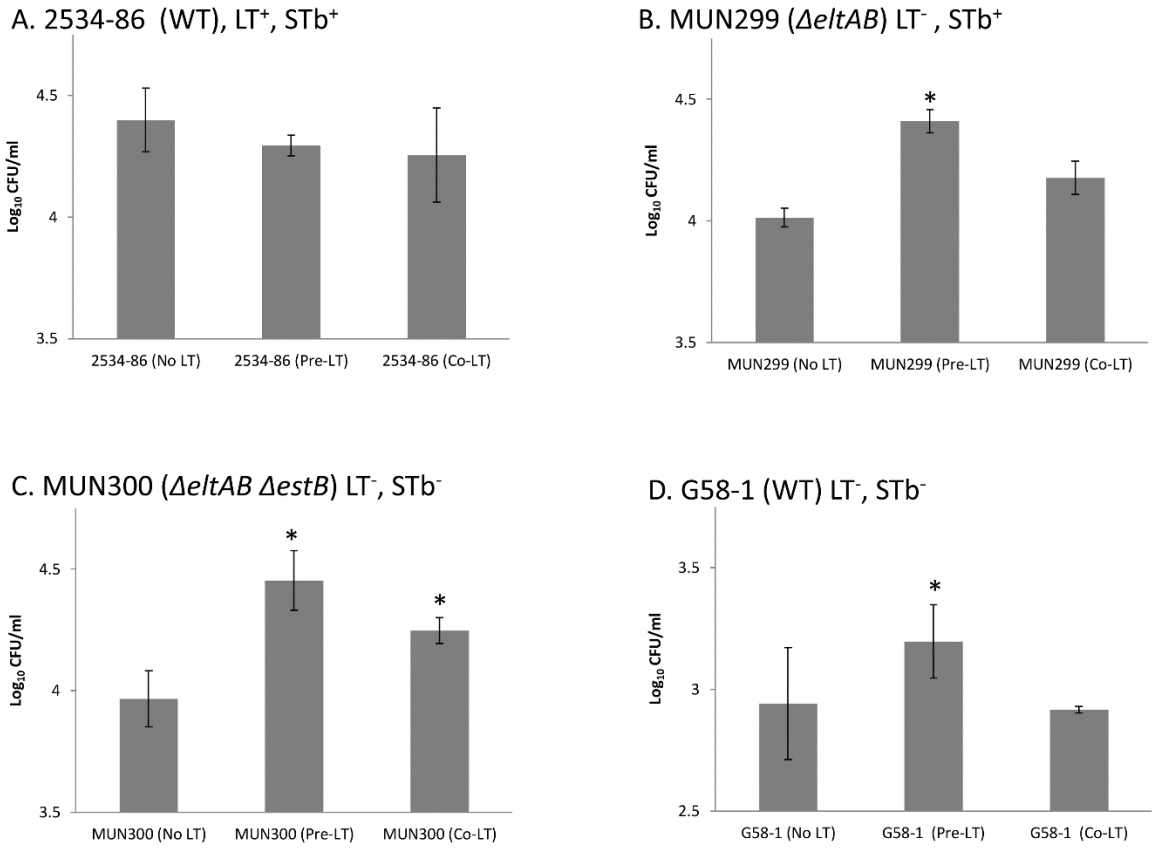

Figure 2. Effects of exogenous LT on adherence of isogenic porcine-origin ETEC strains to porcine epithelial (IPEC-J2) cells. A. Wild-type LT^+ STb^+ strain 2534-86. B. LT^- (Δ*eltAB*) strain MUN299. C. LT^- STb^- (Δ*eltAB* Δ*estB*) strain MUN300. D. Wild-type non-enterotoxigenic strain G58-1. IPEC-J2 cells were incubated 1 h prior to inoculation (Pre-LT) or co-incubated at the time of inoculation (Co-LT) with 100 ng/ml LT. Asterisk (*) denotes adherence levels of a given LT treatment were significantly different (P<0.05) from that of the non-LT-treated control (No LT).

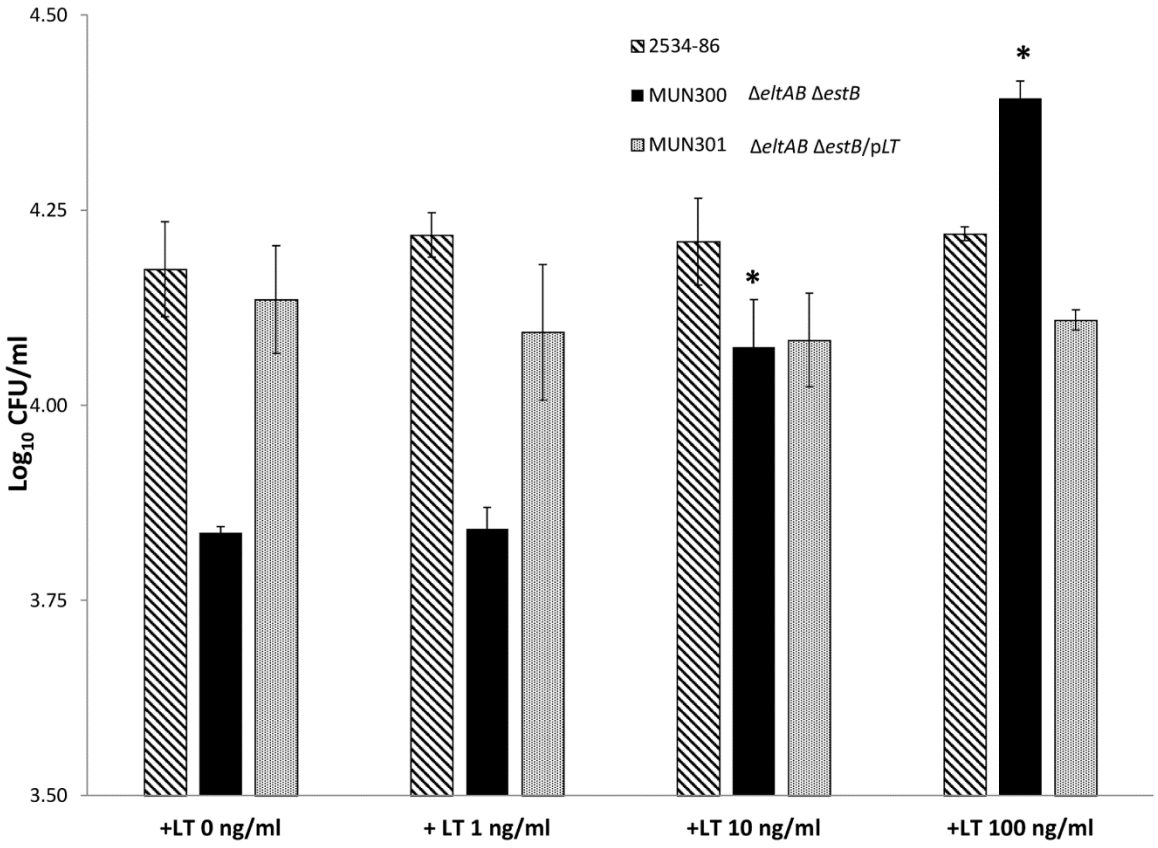

Figure 3. Dose-dependent effect of exogenous LT on the adherence of porcine-origin ETEC strains to porcine epithelial (IPEC-J2) cells. IPEC-J2 cells were pre-treated with 1, 10 or 100 ng/ml of exogenous LT 1 h prior to inoculation with wild-type LT^+ STb^+ strain 2534-86, LT^- STb^- (Δ*eltAB* Δ*estB*) MUN300, or LT^+ complemented (Δ*eltAB* Δ*estB/*p*eltAB*) strain MUN301. Asterisk (*) denotes a level of adherence significantly different (P<0.05) from that of the same strain without exogenous LT.

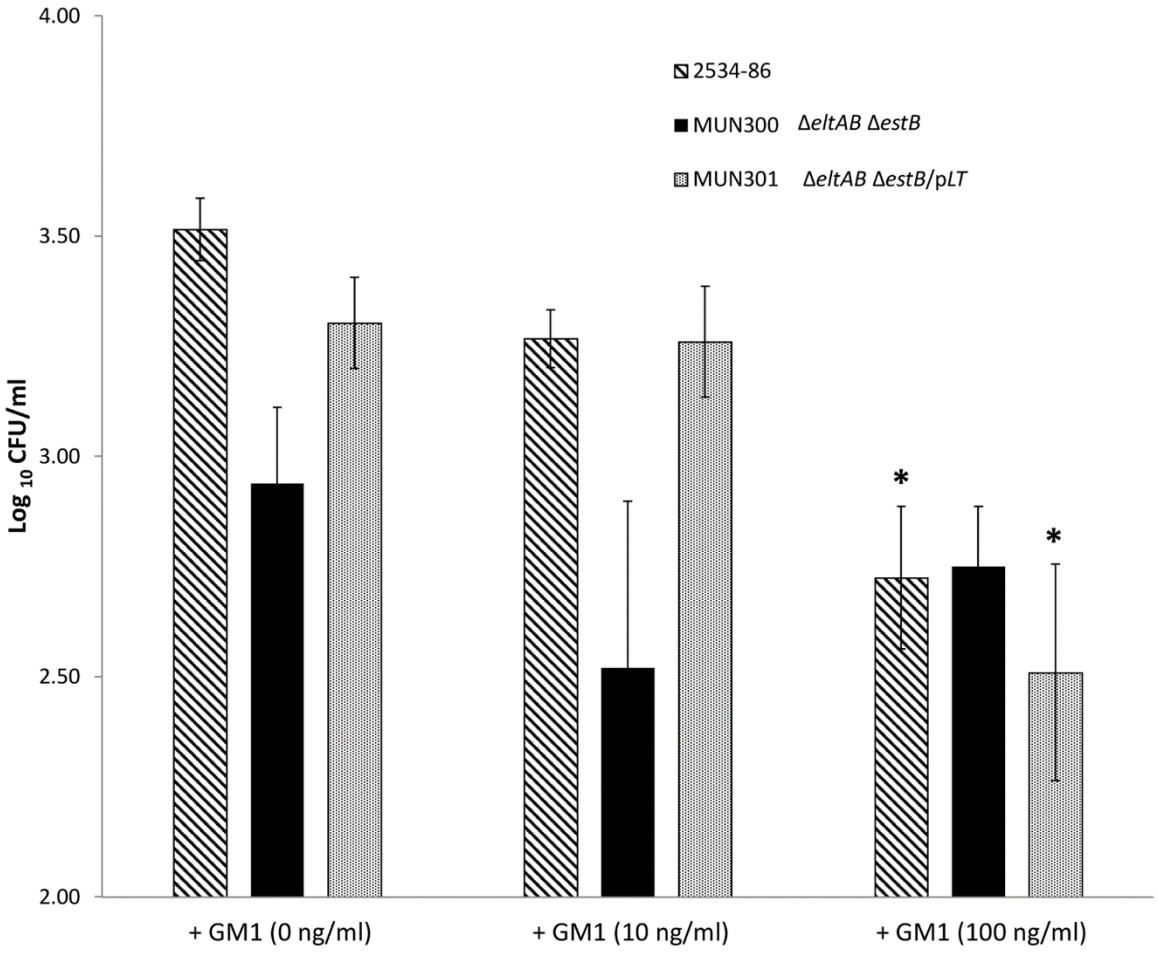

Figure 4. Inhibitory dose-response of exogenous GM1 ganglioside on the adherence of porcine-origin ETEC strains to porcine epithelial (IPEC-J2) cells. IPEC-J2 cells were treated with 10 or 100 ng/ml of GM1 at the time of inoculation with wild-type LT^+ STb^+ strain 2534-86, LT^- STb^- (Δ*eltAB* Δ*estB*) MUN300, or LT^+ complemented (Δ*eltAB* Δ*estB/*p*eltAB*) strain MUN301. Asterisk (*) denotes a level of adherence significantly different (P<0.05) from that of the same strain without exogenous GM1.

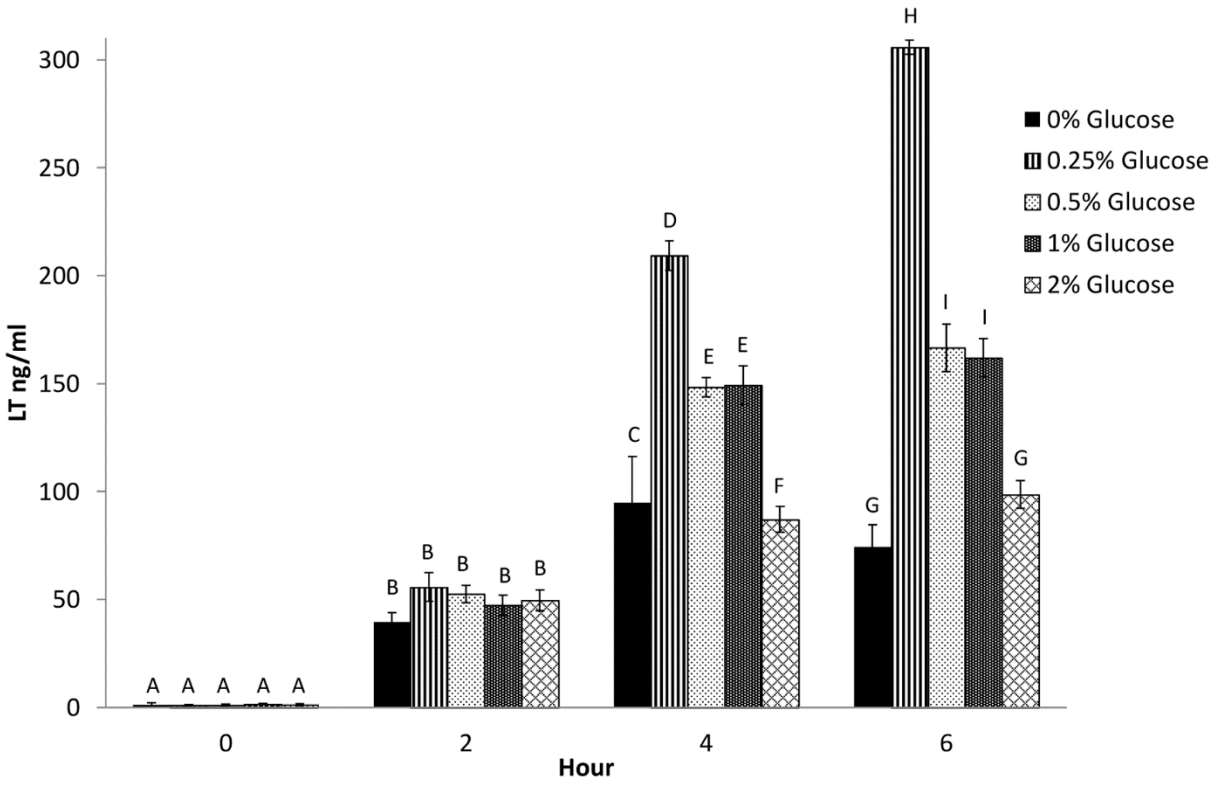

Figure 5. Effect of glucose concentration of the media on LT production by wild-type LT^+ STb^+ strain 2534-86. CAYE media with glucose concentrations of 0, 0.25, 0.5, 1 and 2 % adjusted to pH 8.5 were inoculated with an overnight (18 h) culture of 2534-86 and LT concentrations in the culture supernatants were measured at 0, 2, 4 and 6 h of culture by GM1-ELISA. Separate Tukey's tests for multiple means comparisons of LT secreted for different glucose concentrations at each time point were conducted. Different letter designations denote mean LT concentrations that are significantly different within a sampling time point (P<0.05).

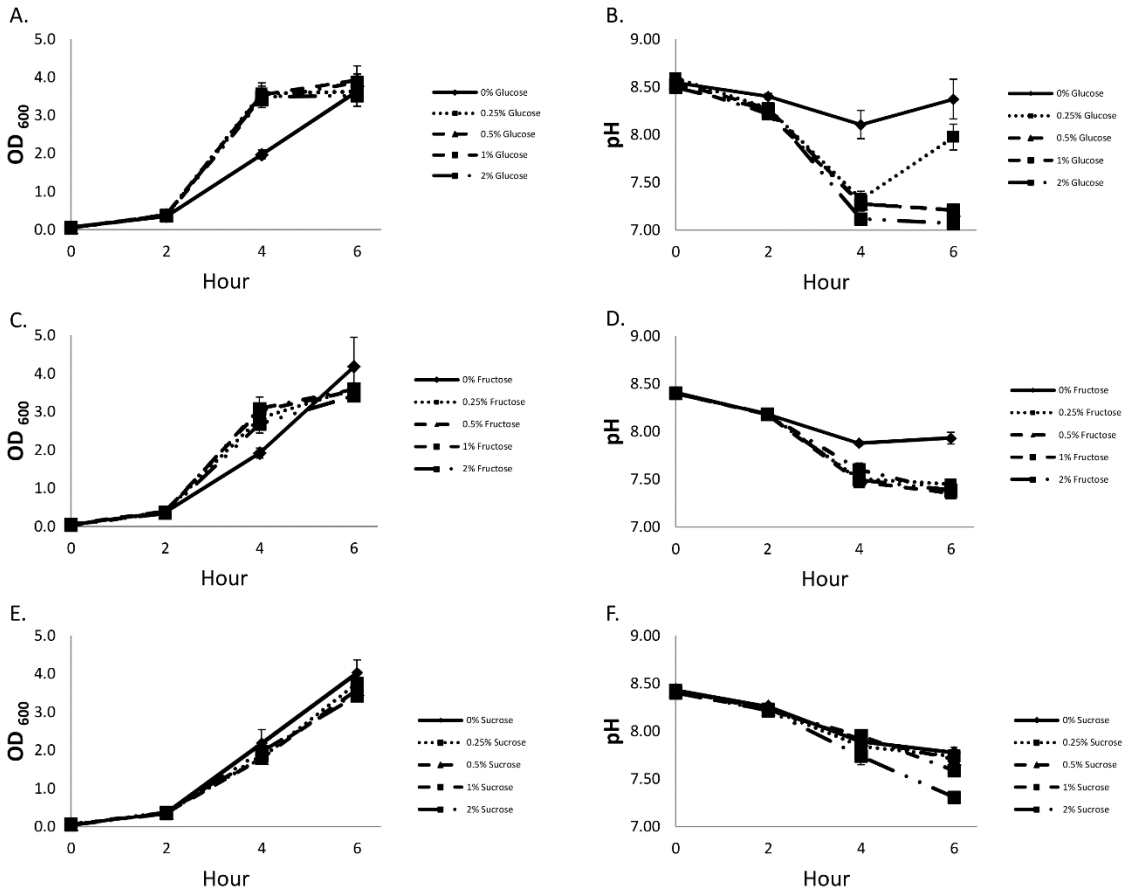

Figure 6. Growth and pH of wild-type LT^+ STb^+ strain 2534-86 in CAYE medium containing glucose (A, B), fructose (C, D), or sucrose (E, F) at concentrations of 0, 0.25, 0.5, 1 and 2%. CAYE media, pH 8.5, containing different carbohydrate sources were inoculated with an overnight (18 h) culture of 2534-86, and incubated at 37°C and 225 rpm for 6 h. Growth (OD_{600}) and pH were measured on samples collected at 0, 2, 4 and 6 h of culture.

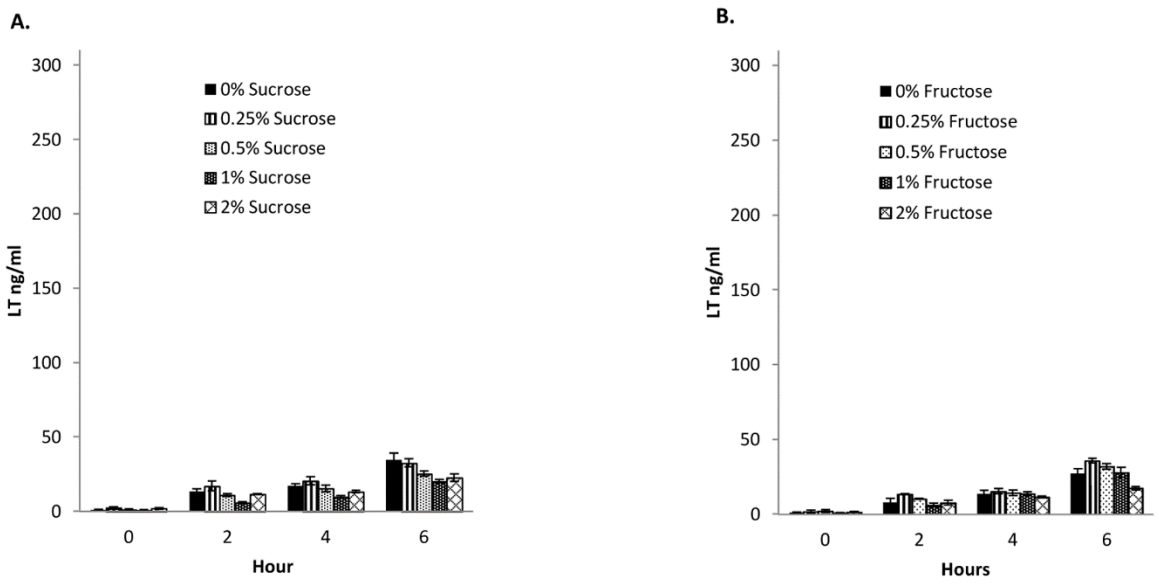

Figure 7. Effect of sucrose (A) and fructose (B) in the culture medium on LT production and secretion by wild-type LT^+ STb^+ strain 2534-86. CAYE media with fructose or sucrose at concentrations of 0, 0.25, 0.5, 1 and 2% were inoculated with an overnight (18 h) culture of 2534-86 and LT concentrations in samples of culture supernatant collected at 0, 2, 4 and 6 h of culture were determined by GM1-ELISA.

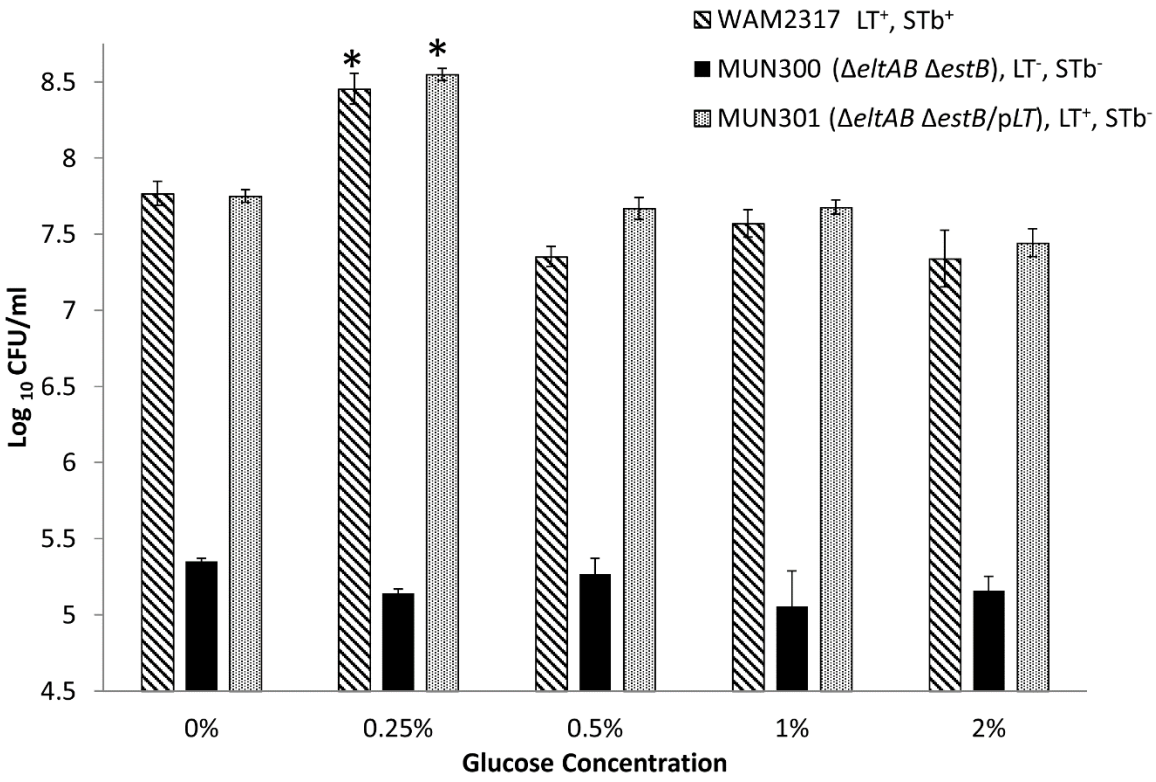

Figure 8. Effect of glucose in the IPEC-J2 cell culture media on adherence of porcine ETEC strains to porcine epithelial (IPEC-J2) cells. IPEC-J2 cells in the presence of DMEM-F12 media containing 0, 0.25, 0.5, 1 and 2% glucose were inoculated with a 2 h culture of strain WAM2137 (spontaneous nalidixic acid-resistant mutant derivative of wild-type LT^+ STb^+ 2534-86), MUN300 [LT^- STb^- (Δ*eltAB* Δ*estB*) derivative of WAM2317], or MUN301 (LT^+ complemented derivative of strain MUN300) at a multiplicity of infection of 100:1 and incubated for 4 h. Asterisk (*) denotes a level of adherence significantly different (P<0.05) from that of the same strain in IPEC-J2 cell culture media containing other glucose concentrations.

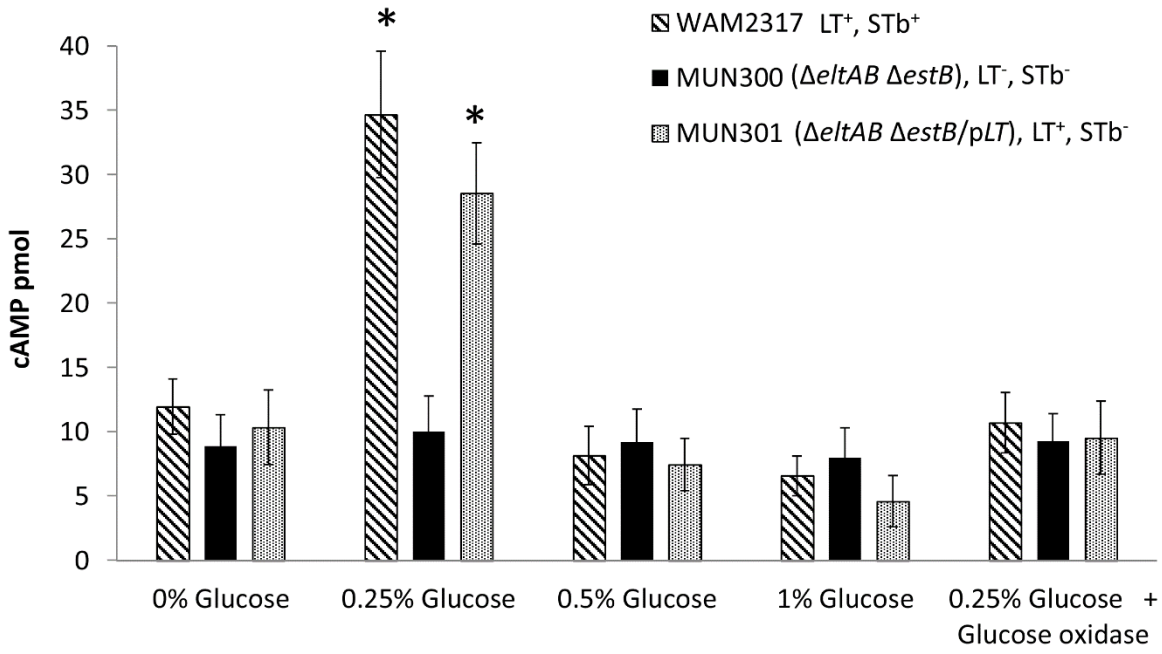

Figure 9. Effect of glucose in the IPEC-J2 cell culture media on cAMP levels of IPEC-J2 cells infected with porcine ETEC strains. IPEC-J2 cells in the presence of DMEM-F12 media containing 0, 0.25, 0.5, 1% glucose and 0.25% glucose + glucose oxidase were inoculated with a 2 h culture of the strains at a multiplicity of infection of 100:1 and incubated for 4 h. Strains included WAM2137 (spontaneous nalidixic acid-resistant mutant derivative of wild-type LT^+ STb^+ 2534-86), MUN300 [LT^- STb^- (Δ*eltAB* Δ*estB*) derivative of WAM2317], or MUN301 (LT^+ complemented derivative of strain MUN300). Asterisk (*) denotes a level of adherence significantly different ($P<0.05$) from that of the same strain grown in other glucose concentrations.

CHAPTER 4

PRELIMINARY EXPERIMENTS TESTING THE EFFECTS OF GLUCOSE ON TRANSCRIPTION OF ENTEROTOXIN GENES IN PORCINE-ORIGIN ENTEROTOXIGENIC *ESCHERICHIA COLI* LUX-REPORTER STRAINS

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Introduction

Escherichia coli heat-labile enterotoxin (LT) and heat-stable enterotoxin-b (STb) are virulence factors of major importance in porcine-origin enterotoxigenic *E. coli* (ETEC) strains (Berberov *et al.*, 2004; Erume *et al.*, 2008, 2013; Wijemanne *et al.*, 2015). Exposure of ETEC strains to glucose at a final concentration of 0.25% in the media and an alkaline pH (8.5-9.0) results in production of LT and STb that are maximal and minimal, respectively (Busque *et al.*, 1995; Hegde *et al.*, 2009; Wijemanne & Moxley, 2014). Based on the results of different studies, these responses may be explained by the STb promoter being subject to catabolite repression and that of LT to catabolite de-repression (Busque *et al.*, 1995; Bodero & Munson, 2009; Munson, 2013; Haycocks *et al.*, 2015). However, no studies have been done to demonstrate whether LT and STb in an individual porcine ETEC strain are differentially regulated at the transcriptional level in response to glucose concentration. Demonstration of this effect would add credence to the hypothesis that the effects are physiological. Preliminary experiments describing the use of Lux reporter strains to test the hypothesis that the two toxins are differentially regulated by glucose at the transcriptional level are described herein.

Materials and Methods

Strains used in the study. Lux reporter strains, MT65, MT68 and MT69 were constructed by Dr. George Munson, University of Miami, using previously described methods (Haldimann & Wanner, 2001). The LT and STb promoters (*eltAp* and *estBp*, respectively) from porcine-origin ETEC strain 2534-86 were cloned into the promoter site of the *Photorhabdus luminescens lux* operon (*luxCDABE*) contained within

conditional-replication, integration and modular (CRIM) plasmids having temperature-inducible *att* sites. Individual plasmids containing promoterless-, *eltAp*-, or *estBp*- *lux* operon fusions were integrated at att_{BHK022} by site-specific recombination into the chromosome of 2534-86 recipient strains. This resulted in the construction of strains MT68 (*eltAp*::*luxCDABE*), MT69 (*estBp*::*luxCDABE*), and MT65 (promoterless *luxCDABE*). MT65, MT68 and MT69 were then used as parents for the construction of Δ*crp* and Δ*crp* (*crp*) complemented strains MUN324, MUN328, MUN330, MUN334, MUN335 and MUN336. Detailed descriptions of these strains are listed in Table 1.

Construction and validation of Δ*crp* and Δ*crp* (*crp*) complemented strains. Site-directed mutagenesis was performed using the bacteriophage lambda recombinase system (λ-Red) as previously described (Datsenko & Wanner, 2000). To construct strains MUN324, MUN328 and MUN330, *crp* in MT65, MT68 and MT69, respectively, was disrupted using linear PCR products generated by primers targeting the kanamycin-resistance (Km^R) cassette with flippase recognition target (FRT) sites from the pKD13 template, flanked by 50-nucleotide homologies of either the upstream or downstream region of the *crp* gene. The 70-mer oligonucleotide primers used were ckF (5'- GGCG TTATCTGGCTCTGGAGAAAGCTTATAACAGAGGATAACCGCGCATGATTC CGGGGATCCGTCGACC-3') and ckR (5'-CTACCAGGTAACGCGCCACTCCGA CGGGATTAACGAGTGCCGTAAACGACTGTAGGCTGGAGCTGCTTCG-3'). The 1,403-bp PCR product was digested with modification-dependent restriction enzyme *Dpn*I and purified with GeneJet PCR purification kit (Thermo Scientific) according to manufacturer instructions. Transformant strains carrying the Red helper plasmid (pKD46) were made electrocompetent and electroporated with the purified PCR product by

standard methods (Sambrook & Russell, 2001). Electroporated cells were spread-plated onto LB agar containing Km (50 μg/ml) to select for Km^R transformants. To confirm the insertion of the Km^R cassette as well as the two junction sites, PCR was done using primers, K1FA (AGCATATTTCGGCAATCCAG), K1RC (TGTCTGTTGTGCCCAGT CAT), K2FD (CGTTGGCTACCCGTGATATT) and K2RB (GCGTTTGTCGAAGTGC ATAG). To construct *crp* complemented strains MUN334, MUN335 and MUN336, Δ*crp* strains MUN324, MUN328 and MUN336, respectively, were cured of pKD46 by incubating at 42°C, followed by electroporation with plasmid pSE186, carrying *crp* and the ampicillin-resistance (Amp^R) marker. Amp^R transformants were selected on LB agar containing Amp (100 μg/ml) and the presence of *crp* was confirmed by PCR with primers K1FA and K2RB.

Luminescence assays. Casamino Acids-yeast extract (CAYE) medium (Mundell *et al.*, 1976; Ristaino *et al.*, 1983) at a final glucose concentration of 0.25%, adjusted to a final pH of 8.5, was used to grow overnight cultures (18 h) of strains MT65, MT68, MT69, MUN324, MUN328, MUN330, MUN334, MUN335 and MUN336 with a flask-to-volume ratio of 8.3:1, and incubated at 37°C, 225 rpm. An overnight culture of each strain was used to inoculate CAYE media at a final glucose concentration of 0, 0.25, 0.5 or 1 % in a flask-to-volume ratio of 8.3:1, and these cultures were incubated at 37°C, 225 rpm. Aliquots were taken at 0, 2, 4 and 6 h post-inoculation to assess luminescence (490nm) and optical density (600 nm). The Relative Light Unit (RLU) measurements were calculated by dividing the luminescence by the OD readings.

Growth curves for Δ*crp* strains and wild-type strain 2534-86. Overnight cultures of MUN324, MUN328, MUN330, and 2534-86 were grown in CAYE medium

containing glucose at a final concentration of 0.25% and a flask-to-volume ratio of 8.3:1, and incubated at 37°C, 225 rpm. Overnight cultures of each of the strains were used to inoculate CAYE media with 0.25% glucose at a flask-to-volume ratio of 8.3:1, and cultures were incubated at 37°C, 225 rpm. Samples were taken at 0, 2, 4 and 6 h post inoculation. Samples were used to measure the OD_{600} by a spectrophotometer, and the colony-forming units (CFU)/ ml were determined by serial 10-fold dilution in phosphate-buffered saline (pH 7.4, 0.1 M; PBS) and plating on LB agar.

Results

Effect of glucose on enterotoxin transcription as assessed by Lux reporters. Since the glucose concentration of the culture medium has an effect on LT and STb production as well as ETEC adherence to IPEC-J2 cells, we sought to test whether these effects originated at the transcriptional level as currently hypothesized (Wijemanne & Moxley, 2014; Hegde *et al.*, 2009). To address this question, strains with *eltAp*, *estBp*, or no promoter fused to the *luxCDABE* operon of *P. luminescens*, MT68, MT69 and MT65, respectively, were constructed and strains were grown in CAYE medium containing different concentrations of glucose.

The luminescence signals emitted by MT65 were several-fold lower than that observed for MT68 and MT69, and did not vary in relation to the concentration of glucose or the time of incubation for the culture (Fig. 1). The highest RLU level for MT68 was observed at 6 h of culture for all media and glucose levels (Fig. 2). No relationship, negative or positive, was observed between the RLU and glucose concentration of the media for MT68. For MT69, the highest RLU was observed with 0%

glucose at 6 h post-inoculation, while the other media with higher glucose levels showed several-fold lower RLU at the same time point (Fig. 3). At time points before 6 h, this relationship was not observed. Furthermore, the RLU signal seen at 6 h with 0% glucose by MT69 was several-fold higher than that of the RLU signal seen with MT68 exposed to higher glucose levels.

A previous study had shown that in some human ETEC strains, LT and STa were inversely regulated at the transcriptional level by cAMP concentrations. In this context, cAMP binds the Crp protein and forms a complex that binds to the promoter of the enterotoxin, thereby either repressing or promoting transcription (Bodero & Munson, 2009). In our study, Δ*crp* mutant derivatives with enterotoxin promoters fused to the *luxCDABE* operon were constructed to test whether glucose concentration was important for enterotoxin regulation in porcine ETEC. Three Δ*crp* mutants were made: MUN324 (promoterless *luxCDABE*), MUN328 (*eltAp*::*luxCDABE*) and MUN330 (*estBp*::*luxCDABE*). These three strains were complemented with *crp* by transforming with the *crp*+ plasmid pSE186 to make MUN334, MUN335 and MUN336 respectively. When the Δ*crp* mutants were grown in different glucose concentrations, neither MUN328 (Fig. 5) nor MUN330 (Fig. 6) showed any correlation of RLU with the levels of glucose in the medium, while promoterless MUN324 (Fig. 4) showed RLU at several folds lower than both at each time point. This is in contrast to the RLU levels observed with *crp* complemented strains MUN335 (Fig. 9) and MUN336 (Fig. 10) which showed a positive and negative relationship, respectively, with the glucose concentration in the medium. Starting at 4 h post- inoculation, LT promoter fusion strain MUN335 showed high RLU levels with increased glucose concentrations, while the STb promoter fusion strain

MUN336 showed the highest RLU levels with a 0% glucose concentration which decreased steadily with increased glucose amounts. Furthermore, the absolute RLU levels of MUN336 at 0% glucose were several folds higher than that of the levels observed for MUN335 at 1% glucose. The RLU levels observed for MUN334 did not show any relationship with glucose concentration of the medium, and were several-fold lower than that of both MUN335 and MUN336 (Fig. 8).

Growth curves of Δ*crp* mutant strains. All three Δ*crp* mutant strains, MUN324, MUN328, and MUN330, were grown in CAYE medium containing 0.25% final glucose concentration, alongside the wild-type (WT) parent strain 2534-86, to assess their growth patterns. At the time points measured, all three mutant strains showed greatly reduced optical density levels (Fig. 6A). Viable cell counts of these time points showed that the CFU of the three mutant strains were at least 10-fold lower than those of 2534-86 at each time point (Fig. 6B).

Discussion

A model whereby LT and STa genes in ETEC strains are differentially regulated at the transcriptional level was proposed by Bodero and Munson (2009). In this model, an increased glucose concentration reduces the intracellular cAMP concentration which results in dissociation of the cAMP-CRP complex bound to the LT promoter, resulting in de-repression and increased transcription. Alternatively, in the presence of lower glucose concentrations, the cAMP-CRP complex binds to the STa promoter and activates transcription. Therefore, glucose concentration has opposing effects on transcription of the two enterotoxins. However, a recent study (Haycocks *et al.*, 2015) cast doubt on

whether both genes are directly affected by glucose concentrations. These authors proposed that STa is directly regulated while LT is indirectly regulated by glucose, via the interaction with CRP. In the case of LT regulation, it was proposed that interaction between CRP and H-NS is essential in order to repress *eltAB*. In porcine ETEC strains, STb, rather than STa is more commonly found in combination with LT, as the two genes are linked on the same plasmid (Gyles *et al.*, 1974; Smith, 1984). Interestingly, STb is unrelated to STa structurally, functionally and antigenically, suggesting the two genes provide a common function, which may be to provide the survival-related benefits of an enterotoxin under conditions opposite to that of LT. However, the question of whether the LT and STb regulation in response to glucose occurs exactly in the manner described by Bodero and Munson (2009) or that of Haycocks *et al.* (2015) has not yet been further addressed.

In our study, in contrast to the findings of Bodero and Munson (2009), an increase in glucose concentration did not yield a higher luminescent signal in the strain bearing the LT promoter (MT68), although the signal itself was several-fold higher than that of the promoterless control strain (MT65). In contrast, the strain bearing the STb promoter (MT69) showed a negative correlation with the glucose concentration, as described before for STa in human-origin ETEC strains in the studies of both Bodero and Munson (2009) and Haycocks *et al.* (2015). This relationship between STb and glucose was not seen when *crp* was deleted, suggesting that the control exerted over the STb promoter may be through its interaction as described for STa in the human-origin ETEC strains. Complementation of *crp* restored this relationship, but the luminescent signal was less than that observed for MT69. Furthermore, the luminescent signal produced by both

MT69 and MUN336 at 0% glucose were higher than the signal produced by MT68 and MUN335 at higher glucose concentrations. Therefore it is possible that in porcine ETEC strains a lack of glucose results in a more profound effect on the transcription of STb, while the presence of glucose exerts a lesser effect on LT. If the effects of glucose on transcription of STa and STb are the same, this scenario would be more consistent with the model proposed by Haycocks *et al.* (2015) whereby STa is directly activated by CRP, while indirect control is exerted over LT through the interaction between CRP and H-NS. The very low luminescence detected with Δ*crp* strains may be attributed to the low growth levels of these strains compared to the WT strain 2534-86.

Table 1. Description of the strains used.

Strain	**Origin**	**Characteristics**	**Antibiotic pattern**
2534-86	WT ETEC[a] strain	[b]LT, STb, *crp*+	
MT65	2534-86	LT, STb, *crp*+	[c]Amp^R, St^R, Sp^R
MT68	2534-86	LT, STb, *crp*+	Amp^R, St^R, Sp^R
MT69	2534-86	LT, STb, *crp*+	Amp^R, St^R, Sp^R
MUN324	MT65	LT, STb, Δ*crp*	Amp^S, St^R, Sp^R , Kan^R
MUN328	MT68	LT, STb, Δ*crp*	Amp^S, St^R, Sp^R , Kan^R
MUN330	MT69	LT, STb, Δ*crp*	Amp^S, St^R, Sp^R , Kan^R
MUN334	MUN324	LT, STb, Δ*crp::crp*	Amp^R, St^R, Sp^R , Kan^R
MUN335	MUN328	LT, STb, Δ*crp::crp*	Amp^R, St^R, Sp^R , Kan^R
MUN336	MUN330	LT, STb, Δ*crp::crp*	Amp^R, St^R, Sp^R , Kan^R

[a]WT, wild-type; ETEC, enterotoxigenic *Escherichia coli*.

[b]LT, heat-labile enterotoxin; STb, heat-stable enterotoxin-b; *crp*, structural gene for cAMP-receptor protein.

[c]Amp^R, ampicillin-resistance; St^R, streptomycin-resistance; Sp^R, spectinomycin-resistance; Kan^R, kanamycin-resistance.

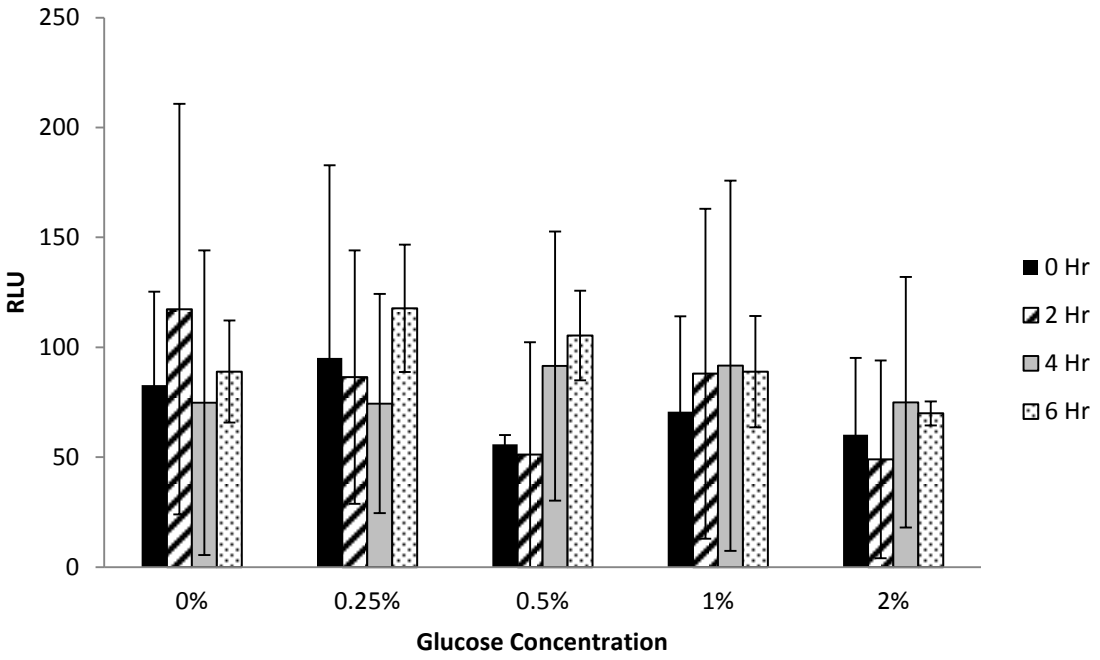

Figure 1. Effect of glucose concentration on luminescence emitted by strain MT65 (promoterless *luxCDABE*). CAYE medium containing glucose at a final concentration of 0, 0.25, 0.5, 1 or 2%, adjusted to pH 8.5, was inoculated with an overnight (18 h) culture of MT65 and luminescence and absorbance in the culture was measured at 0, 2, 4 and 6 h of culture. Relative light units (RLU) were calculated by normalizing the luminescence reading at 490nm to the absorbance reading at 600 nm.

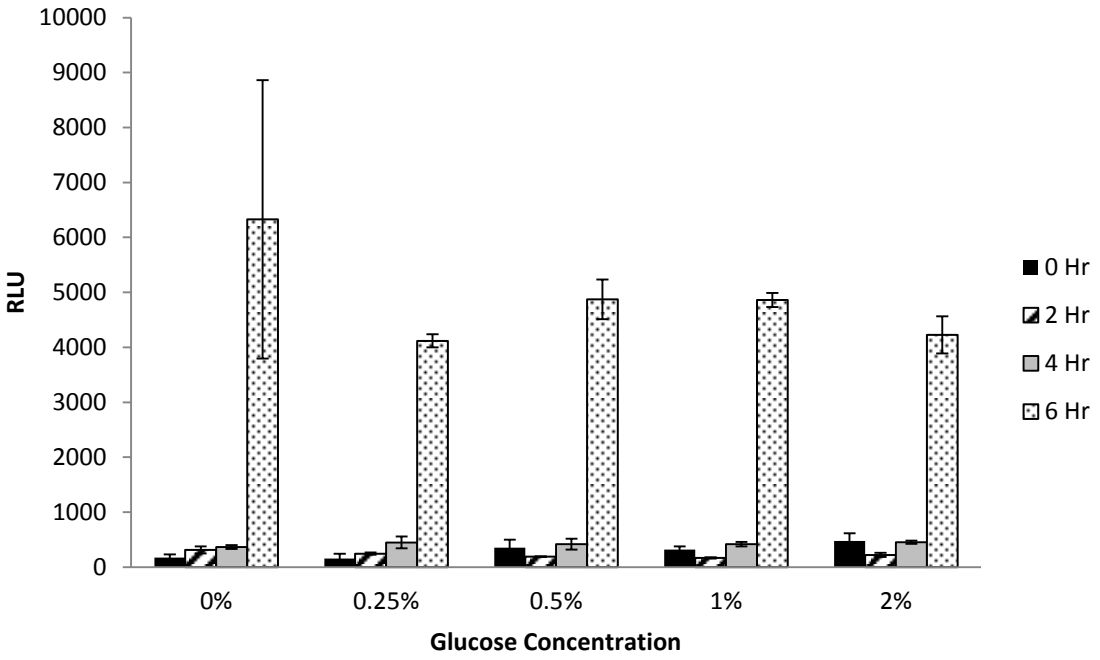

Figure 2. Effect of glucose concentration on luminescence emitted by strain MT68 (*eltAp::luxCDABE*). CAYE medium containing glucose at a final concentration of 0, 0.25, 0.5, 1 or 2%, adjusted to pH 8.5, was inoculated with an overnight (18 h) culture of MT68. Luminescence and absorbance were measured at 0, 2, 4 and 6 h of culture. Relative light units (RLU) were calculated by normalizing the luminescence reading at 490 nm to the absorbance reading at 600 nm.

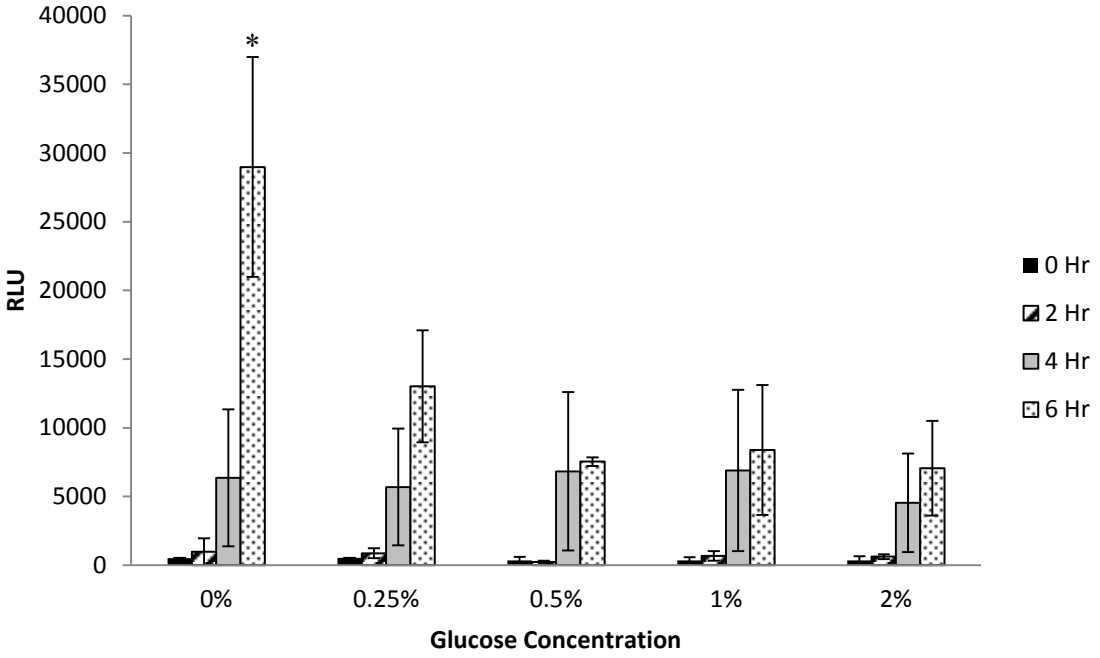

Figure 3. Effect of glucose concentration on luminescence emitted by strain MT69 (*estBp::luxCDABE*). CAYE medium containing glucose at a final concentration of 0, 0.25, 0.5, 1 or 2%, adjusted to pH 8.5, was inoculated with an overnight (18 h) culture of MT69. Luminescence and absorbance were measured at 0, 2, 4 and 6 h of culture. Relative light units (RLU) were calculated by normalizing the luminescence reading at 490nm to the absorbance reading at 600 nm. Asterisk (*) denotes luminescence level significantly different (P<0.05) from other glucose concentrations at the same time point.

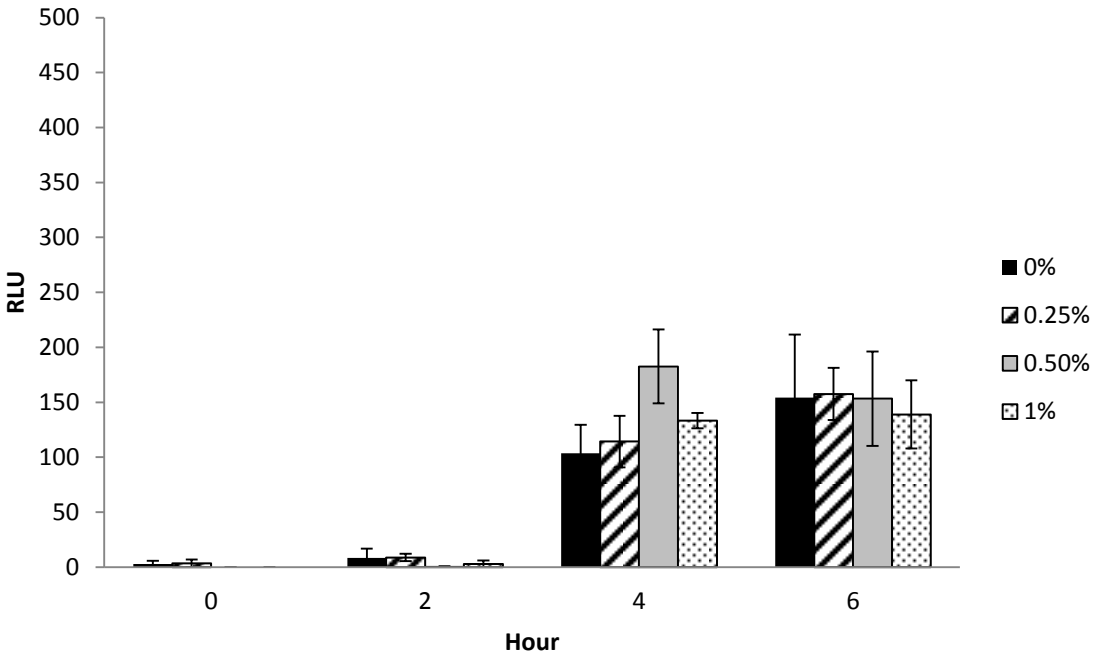

Figure 4. Effect of glucose concentration on luminescence emitted by Δ*crp* strain MUN324 (promoterless *luxCDABE*). CAYE medium containing glucose at a final concentration of 0, 0.25, 0.5, or 1%, adjusted to pH 8.5, was inoculated with an overnight (18 h) culture of MUN324 and luminescence and absorbance in the culture were measured at 0, 2, 4 and 6 h of culture. Relative light units (RLU) were calculated by normalizing the luminescence reading at 490 nm to the absorbance reading at 600 nm.

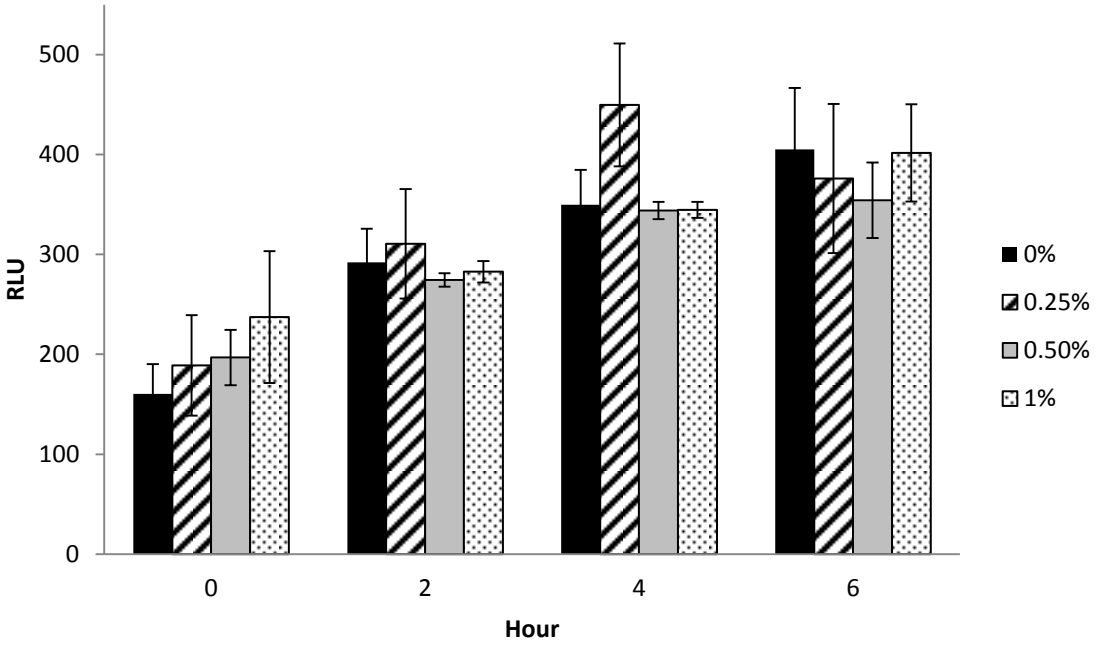

Figure 5. Effect of glucose on luminescence emitted by Δ*crp* strain MUN328 (*eltAp::luxCDABE*). CAYE medium containing glucose at a concentration of 0, 0.25, 0.5, or 1%, adjusted to pH 8.5, was inoculated with an overnight (18 h) culture of MUN328 and luminescence and absorbance in the culture were measured at 0, 2, 4 and 6 h of culture. Relative light units (RLU) were calculated by normalizing the luminescence reading at 490nm to the absorbance reading at 600 nm.

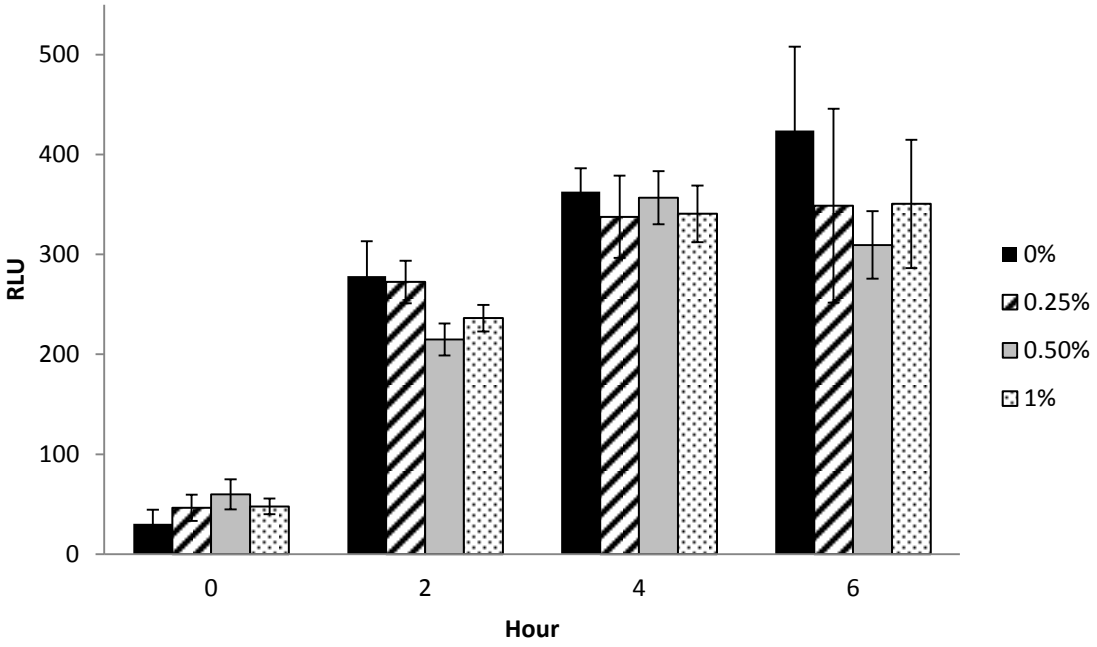

Figure 6. Effect of glucose on luminescence emitted by Δ*crp* strain MUN330 (*estBp::luxCDABE*). CAYE medium containing glucose at final concentrations of 0, 0.25, 0.5, 1 or 2%, adjusted to pH 8.5, was inoculated with an overnight (18 h) culture of MUN330 and luminescence and absorbance in the culture was measured at 0, 2, 4 and 6 h of culture. Relative light units (RLU) were calculated by normalizing the luminescence reading at 490nm to the absorbance reading at 600 nm.

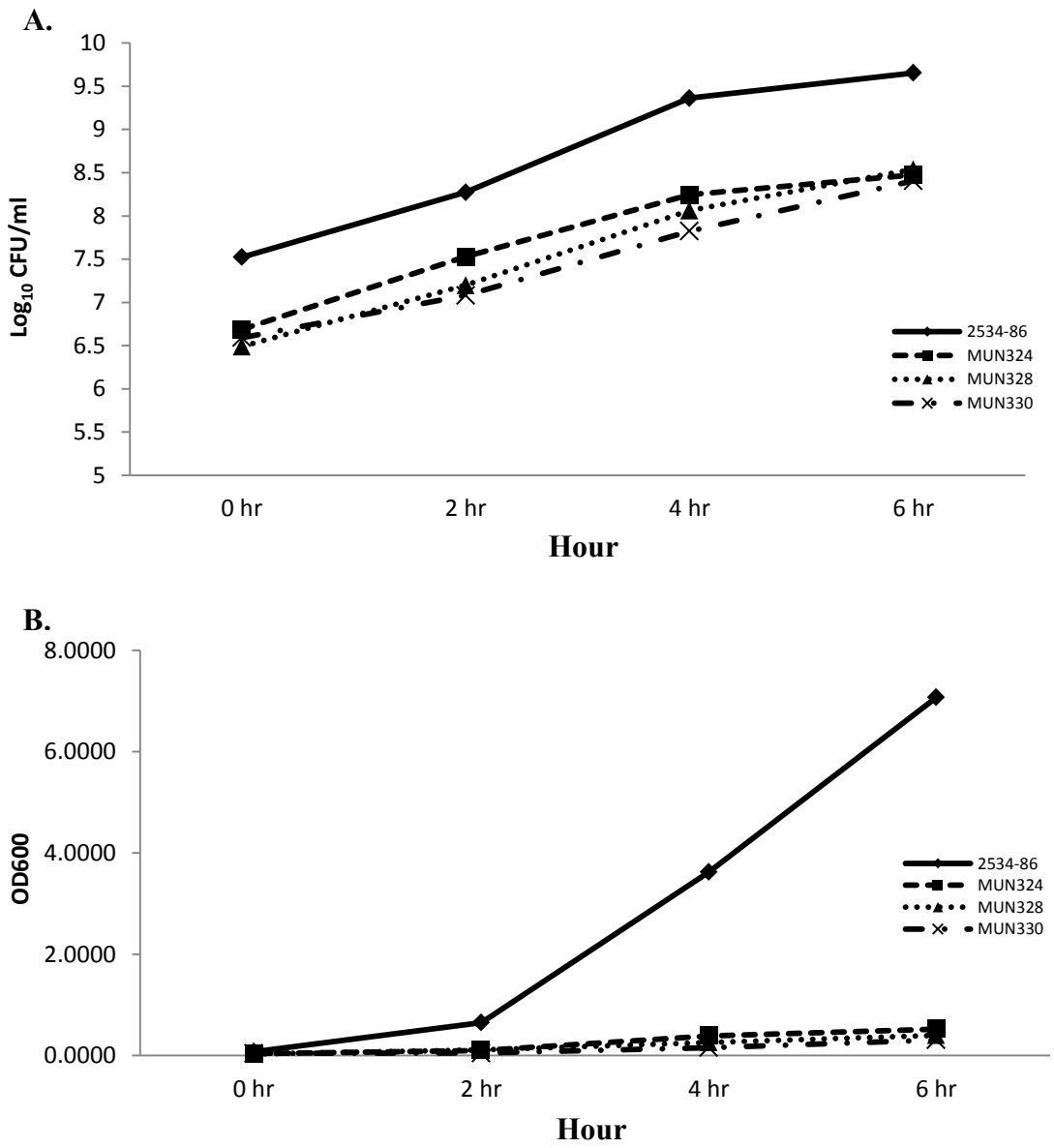

Figure 7. Growth curve comparison between Δ*crp* strains MUN324, MUN328, MUN330 and their WT parent strain 2534-86. CAYE medium containing glucose at a final concentration of 0.25 % glucose, adjusted to pH 8.5 was inoculated with an overnight (18 h) culture of one of these strains at a flask-to-volume ratio of 8.3:1, and incubated at 37°C and 225 rpm. Samples were obtained at 0, 2, 4 and 6 h of incubation, and the OD_{600} (A), and colony-forming units (CFU)/ml (B) were measured, the latter by serial 10-fold dilution and plating on LB agar.

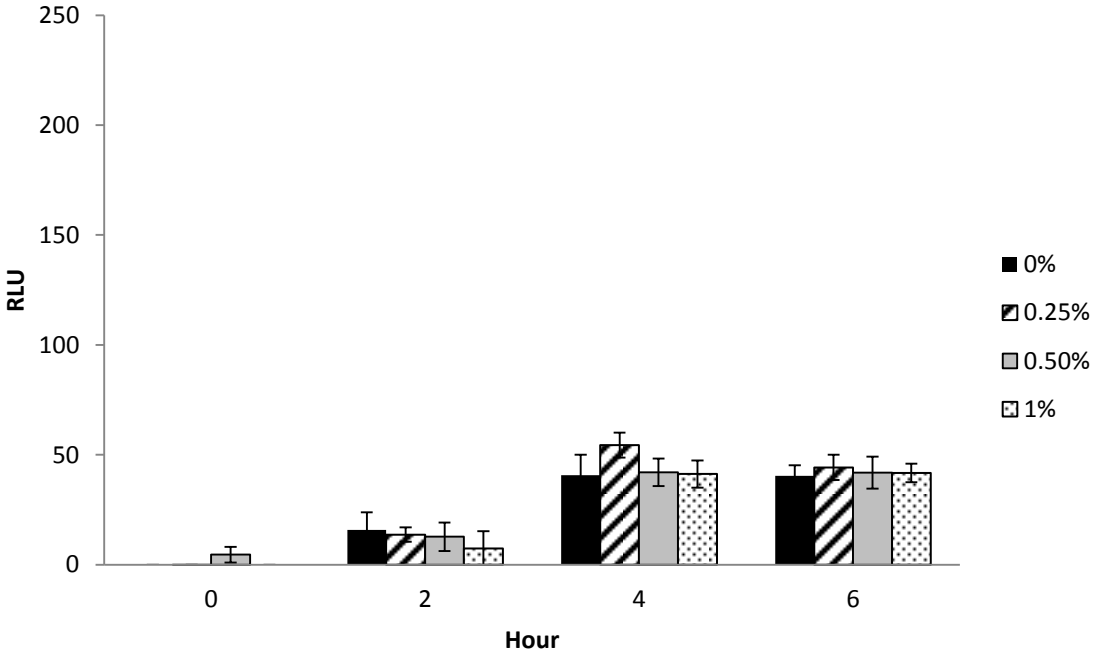

Figure 8. Effect of glucose on luminescence emitted by *crp* complemented strain MUN334 (promoterless *luxCDABE*). CAYE media with glucose concentrations of 0, 0.25, 0.5, and 1 % adjusted to pH 8.5 were inoculated with an overnight (18 h) culture of MUN334 and luminescence and absorbance in the culture was measured at 0, 2, 4 and 6 h of culture. Relative light units (RLU) were calculated by normalizing the luminescence reading at 490nm to the absorbance reading at 600 nm.

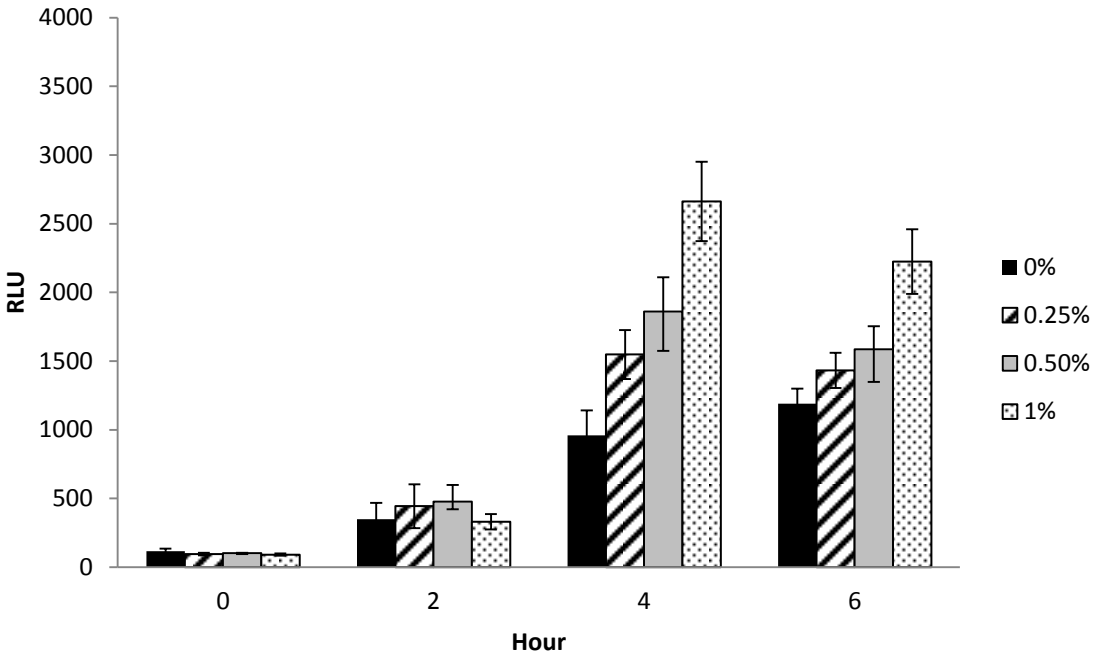

Figure 9. Effect of glucose on luminescence emitted by *crp* complemented strain MUN335 (*eltAp::luxCDABE*). CAYE media with glucose concentrations of 0, 0.25, 0.5, and 1 % adjusted to pH 8.5 were inoculated with an overnight (18 h) culture of MUN335 and luminescence and absorbance in the culture was measured at 0, 2, 4 and 6 h of culture. Relative light units (RLU) were calculated by normalizing the luminescence reading at 490nm to the absorbance reading at 600 nm.

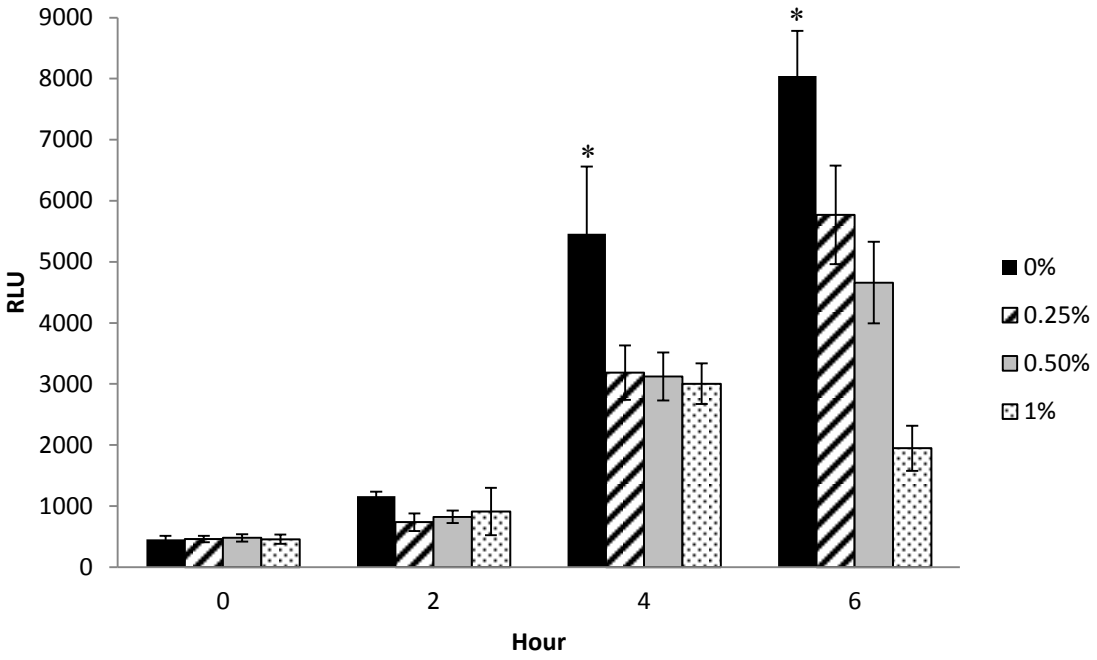

Figure 10. Effect of glucose on luminescence emitted by *crp* complemented strain MUN336 (*estBp::luxCDABE*). CAYE media with glucose concentrations of 0, 0.25, 0.5, 1 and 2 % adjusted to pH 8.5 were inoculated with an overnight (18 h) culture of MUN336 and luminescence and absorbance in the culture was measured at 0, 2, 4 and 6 h of culture. Relative light units (RLU) were calculated by normalizing the luminescence reading at 490nm to the absorbance reading at 600 nm. Asterisk (*) denotes luminescence level significantly different (P<0.05) from other glucose concentrations at the same time point.

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