Epidemiology, molecular characterization and toxin regulation of enterotoxigenic *Escherichia coli* (ETEC) isolated from children with diarrhoea.

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To my parents: Jairo and Isabel

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Acute diarrhoeal diseases are among the major causes of morbidity and mortality in children under 5 years of age in developing countries. Knowledge of the epidemiology of such diseases and the causative agents is important for development of vaccines and other interventions. Interactions with the host expose diarrhoeal pathogens to different environmental conditions such as different pH, osmolarity and nutrients at the site of infection and may be important for the virulence of microbial pathogens. In this thesis we have studied the epidemiology of diarrhoea associated with infections by diarrhoeagenic *Escherichia coli* (DEC) with emphasis on enterotoxigenic *Escherichia coli* (ETEC), as well as the role of host environmental factors in the regulation of the ETEC enterotoxins.

We studied the prevalence, seasonality, antibiotic resistance and severity of disease of diarrhoeas caused by DEC in children aged less than five years in two areas in Bolivia over a period of four years (2007-2010). We showed that enteroaggregative *E. coli* EAEC (11.2%), ETEC (6.6%) and enteropathogenic *E. coli* EPEC (5.8%) were the most prevalent DEC pathogens isolated from children, with a peak in children <2 years, and that these categories were significantly associated with disease. No difference in the severity of the disease was found between EAEC, ETEC and EPEC and antibiotic resistance was found in high frequency among the DEC strains isolated.

Subsequently, we performed a molecular characterization of the enterotoxin profile, colonization factors (CFs), putative virulence genes as well as the severity of disease of all ETEC strains isolated from diarrhoeal cases. Strains expressing heat-labile toxin (LT) or heat-stable toxin (STh) alone were isolated in 40% of the children, respectively; the remaining ETEC isolates produced both toxins. The most common CFs were CFA/I and CS14, which were mainly associated with STh strains whereas LT-only strains were significantly more often CF negative. Severity of disease was not related to the toxin or CF profile of the strains. Presence of the suggested ETEC virulence genes (*clyaA, EatA, tia, tibC, leoA* and *East-1*) was not associated with disease.

To study host factors that may influence expression and secretion of the two toxins LT and STh, clinical ETEC isolates were cultured under various conditions *in vitro*. LT and STh were shown to be differentially regulated by certain environmental factors, *i.e.* different carbon sources (glycerol, glucose, and amino acids), and osmolarity. Secretion of ST was down-regulated by glucose as carbon source under certain conditions but up-regulated by casamino acids and the osmoprotectant sucrose; LT was only secreted in complex media and up-regulated in the presence of glucose.

We also investigated the impact of external pH, which is known to fluctuate in the gastrointestinal tract, and the activity of the cyclic AMP receptor protein (CRP), which is regulated in response to glucose, on the regulation of the production and secretion of LT. The study was performed by constructing a *crp* mutant in an ETEC strain and subsequent analysis of the wild-type and mutant strains after growth in media buffered to pH 5, 7 and 9. We demonstrated that CRP is a repressor of LT transcription and production but a positive regulator of LT secretion. LT production and secretion increased at neutral to alkaline pH compared to acidic pH which was inhibiting secretion. An important finding was that at pH 9 the transcriptional negative regulation of the *eltAB* promoter was abolished and secretion was favored, resulting in maximal production and secretion of LT. We propose that ETEC is exposed to an environment characterized by low glucose levels and alkaline pH close to the epithelium in the small intestine and that this may be a signal for toxin release.

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ORIGINAL PAPERS

This thesis is based on the following papers, which are referred to in the text by the roman numerals (I-IV):

I. **Gonzales L.**, Joffre E., Rivera R., Sjöling Å., Svennerholm AM. and Iñiguez V.
Prevalence, seasonality and severity of disease caused by pathogenic *Escherichia coli* in children with diarrhoea in Bolivia.
Submitted for publication

II. **Gonzales L**., Sanchez S., Zambrana S., Iniguez V., Wiklund G., Svennerholm AM. and Sjöling Å.
Molecular characterization of enterotoxigenic *Escherichia coli* ETEC isolated from children with diarrhoea during a four year period (2007-2010) in Bolivia.
J Clin Microbiol. 51(4):1219-1225 *2013*

III. **Gonzales L**., Nicklasson M. and Sjöling Å.
Influence of environmental factors on the production and secretion of the heat stable (ST) and heat labile (LT) toxins of enterotoxigenic *Escherichia coli* (ETEC).
Manuscript

IV. **Gonzales L.**, Bagher-Ali Z., Nygren E., Wang Z., Karlsson S., Zhu B., Quiding-Järbrink, M. and Sjöling Å.
Alkaline pH is a signal for optimal production and secretion of LT in enterotoxigenic *Escherichia coli* (ETEC).
Submitted for publication

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ABBREVIATIONS

AMP	Ampicillin
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
cDNA	complementary DNA
CHL	Chloramphenicol
CF	Colonization factor
CIP	Ciprofloxacin
CRP	cAMP receptor protein
CT	Cholera toxin
CTX	Cefotaxime
DEC	Diarrhoeagenic *E. Coli*
DNA	Deoxyribonucleic acid
ETEC	Enterotoxigenic *E. coli*
EPEC	Enteropathogenic *E. coli*
EAEC	Enteroaggregative *E. coli*
EHEC	Enterohemorragic *E. coli*
EIEC	Enteroinvasive *E. coli*
Elisa	Enzyme-linked immunoabsorbent assay
FOX	Cefoxitin
GM1	Monosialotetrahexosylganglioside; receptor for CT and LT
ICDDR,B	International Center for Diarrhoeal Disease Research
LB	Luria Broth
LBK	Luria-Broth potassium modified media
LT	Heat-labile enterotoxin
mRNA	Messenger RNA
M9	Minimal media
NAL	Nalidixic acid
OD_{600}	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
q-PCR	Real-time PCR
SAM	Amoxicillin-sulbactam
STX	Sulfamethoxazole-trimethoprim
STh	Human Heat-stabile enterotoxin
STp	Porcine Heat-stabile enterotoxin
SUMI	Universal Mother and Child Insurance System
TET	Tetracycline
WHO	World Health Organization

INTRODUCTION

Diarrhoeas are a leading cause of mortality among children under 5 years of age around the world (Black *et al.*, 2010). The burden of diarrhoeal disease disproportionately affects young children in low- and middle-income countries who have higher incidence rates due to inadequate water and sanitation and nutritional risk factors, such as suboptimal breastfeeding and zinc and vitamin A deficiency (Lamberti *et al.*, 2011, Patel *et al.*, 2010). According to the World Health Organization (WHO), diarrhoea accounts for approximately one fifth of all deaths with around 1.9 million childhood deaths every year (Boschi-Pinto *et al.*, 2008). In developing countries children might suffer from 2-12 episodes of diarrhoea per year, usually with highest frequency during the first two years of life (Qadri *et al.*, 2000a). The enteric pathogens that cause most of severe acute diarrhoea are rotavirus, diarrheogenic *Escherichia coli* (DEC), *Vibrio cholerae*, *Shigella spp*., *Salmonella spp.*, and *Campylobacter jejuni* (Petri *et al.*, 2008).

Many bacteria have been identified in playing a role in the onset of diarrhoea by producing and delivering toxins to the target tissue. Some can damage the intestinal epithelial cells as they are cytotoxic and/or hemolytic. Others are cytotonic thus producing their effects without killing the cells (Dubreuil, 2012). The main ways by which these microorganisms cause diarrhoea include: a decrease in intestinal surface with resulting decrease in adsorption, mucosal destruction resulting in a change in mucosal osmotic permeability, and a change in fluid and electrolyte homeostasis due to toxin induced activation of ion channels (Morris & Estes, 2001). These pathogens colonize the intestinal mucosa, multiply and affect target cells causing large fluid losses, up to several liters a day.

Diarrheogenic E. coli (DEC)

Escherichia coli (E. coli) is one of the most important bacterial species in the human intestinal tract. In healthy humans, these bacteria are harmless commensals and live in a symbiotic relationship contributing to the welfare of the host. However, a small proportion of strains are pathogenic causing a wide variety of enteric diseases in humans and animals (Nataro & Kaper, 1998). The difference between them depends on the presence of acquired genetic material encoding virulence factors that are acquired and maintained either in plasmids, transposons or as pathogenicity islands (PAIs) on the bacterial chromosome. The various pathogenic subtypes possess additional genetic material that encodes specific virulence factors that determine the nature of the disease (Dubreuil, 2012). Five different groups of pathogenic *E. coli* exist that harbour various virulence factors which enable them to cause diarrhoeal disease: enteroaggregative *E. coli* (EAEC), enteropathogenic *E.coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), and enteroinvasive *E. coli* (EIEC) (Nataro & Kaper, 1998).

Enteroaggregative *E. coli* (EAEC)

EAEC can colonize the mucosa of both the small and large intestine and can lead to mild inflammation in the colon (Nataro *et al.*, 1998). EAEC is characterized by its aggregative adherent manner to intestinal tissues in association with a thick mucus layer. Adherence is followed by mucosal toxicity (Okeke & Nataro, 2001). Adhesion is mediated by the genes present in the pAA virulence plasmid (also known as CVD 432) which encode four variants of the aggregative

adherence fimbriae (AAFs) but express only one type per strain, AAF/I, AAF/II, AAF/III or Had (Weintraub, 2007). Expression of AAF genes requires the transcriptional activator AggR. AggR also regulates expression of the antiaggregation protein (Aap) or dispersin, which promotes dispersion of EAEC across the intestinal mucosa, (Huang *et al.*, 2006). Biofilms formed by EAEC on the surface of enterocytes are encased in a thick mucus layer. EAEC is also able to penetrate the mucus layer through the mucolytic activity of Pic or cause mucosal damage by secreting cytotoxins such as the plasmid-encoded toxin (Pet) (Harrington *et al*., 2006, Weintraub, 2007) and can express the *Shigella* enterotoxin 1 (ShET1) and the heat stable toxin EAST-1 (Harrington *et al*., 2006). Biofilm formation includes the presence of plasmid-borne and chromosomal genes, which in many cases can be AggR-regulated (Croxen & Finlay, 2010). However, specific virulence factors for the detection of EAEC have not been established since EAEC presents a very variable virulence profile. Hence the pAA (CVD 432) plasmid is often used for identification of EAEC (Figure 1).

Enteropathogenic *E. coli* (EPEC)

EPEC are characterized by the ability to produce attaching and effacing (A/E) lesions (Nataro *et al*., 1998). The attaching bacteria efface the microvilli and rearrange host cell actin to form distinct pedestals beneath the site of attachment (Croxen & Finlay, 2010). All of the genetic elements required for the A/E lesion are encoded on a 35 kb PAI known as the locus of enterocyte effacement (LEE). LEE contains genes encoding the outer-membrane protein (intimin), a type III secretion machinery, T3SS (Esc and Sep), chaperones (Ces proteins), translocators (EspA, EspB, and EspD), effector proteins (EspF, EspG, EspH, Map and EspZ), the translocated intimin receptor (Tir), and regulatory proteins (Ochoa & Contreras, 2011). Intimate attachment is mediated through interactions of the bacterial intimin and Tir._EPEC uses the T3SS, which forms a needle like structure that penetrates the host cell membrane to rapidly translocate Tir into the cytoplasm of the host cell. Tir is then displayed on the surface of the host cell and acts as a receptor for intimin (Kenny, 2002, Kenny *et al.*, 1997). Tir-Intimin interaction triggers signaling cascades in the host cell including phosphorylation of a host phospholipase and the recruitment of actin beneath the adherent bacteria (Dean *et al.*, 2005, Kenny, 2002). EPEC has a large repertoire of effectors that are translocated into host cells by the T3SS and subvert host cell processes causing, for example, cytoskeletal rearrangements and immune modulation, as well as contributing to diarrhoea (Croxen & Finlay, 2010). Actin polymerization is thought to enhance pathogenicity not only by disturbing the cytoskeleton of the target cells but also leading to changes in cell shape, motility and signaling (Ochoa & Contreras, 2011) (Figure 2).

EPEC are classified into typical and atypical strains based on the presence of the plasmid *E. coli* adherence factor (EAF) (Trabulsi *et al.*, 2002). This plasmid encodes the type IV bundle-forming pilus (BFP), a rope-like fimbriae that interacts with other EPEC bacteria to form microcolonies for localized adherence, and with *N*-acetyl-lactosamine-containing receptors on host cell surfaces (Hyland *et al.*, 2008, Saldana *et al.*, 2009). Typical EPEC strains (bfp^+) produce the localized adherence phenotype associated with the production of BFP whereas atypical EPEC (bfp^-) display localized-like, diffuse, or aggregative adherence patterns (Scaletsky *et al.*, 2010, Trabulsi *et al*., 2002) (Figure 2).

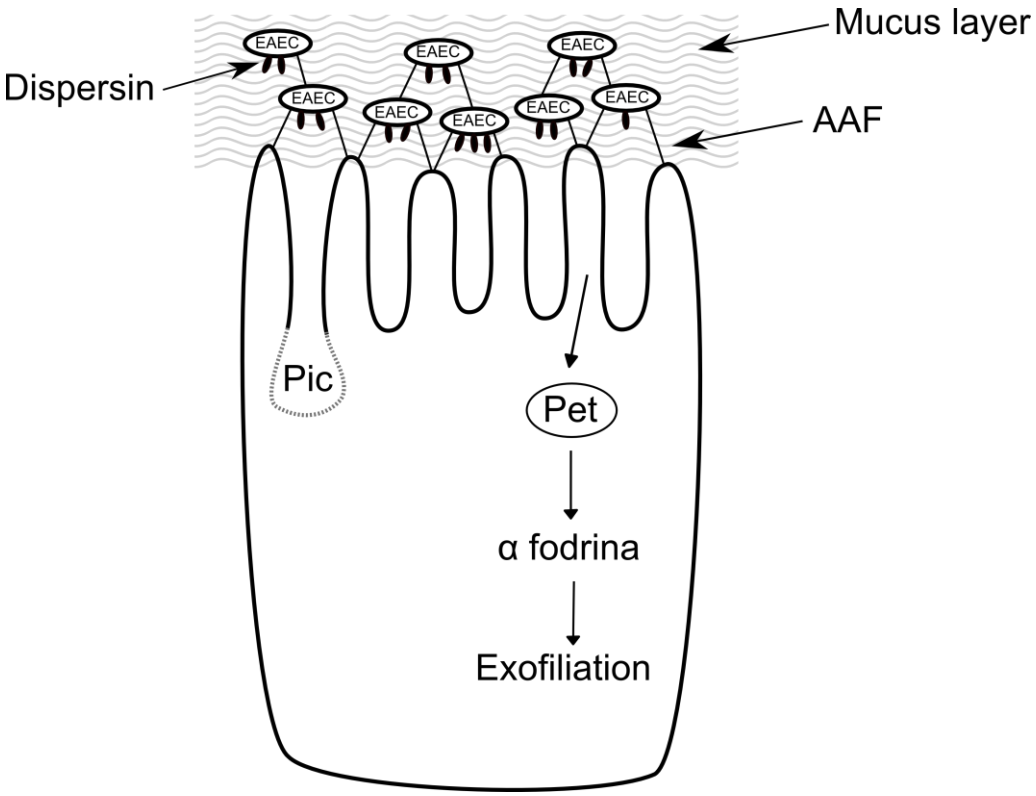

Figure 1. Pathogenesis mechanism of enteroaggregative *E. coli* (EAEC)

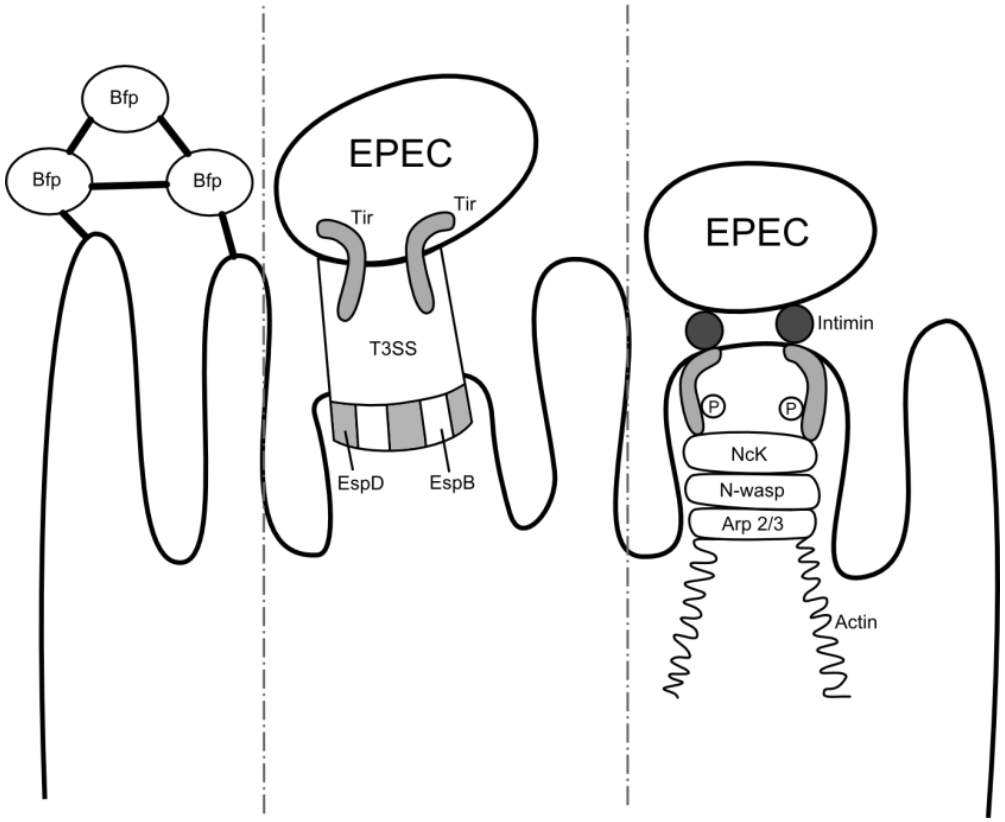

Figure 2. Pathogenesis mechanism of enteropathogenic *E. coli* (EPEC)

Enterohemorragic *E. coli* (EHEC)

Enterohemorrhagic *Escherichia coli* (EHEC) are responsible for major outbreaks of bloody diarrhoea and hemolytic uremic syndrome (HUS) throughout the world. EHEC has two major virulence strategies: production of Shiga toxin (Stx) and formation of attaching and effacing (AE) lesions on enterocytes (Nataro & Kaper, 1998). Stx inhibits protein synthesis and induces apoptosis. Stx is divided in two subgroups, Stx1 and Stx2. Stx is an AB_5 toxin consisting of a pentamer of the B subunit that is non-covalently bound to an enzymatically active A subunit. The Stx receptors are the globotriaosylceramides (Gb3s) found on Paneth cells in the human intestinal mucosa and the surface of kidney epithelial cells (Tarr *et al.*, 2005). Upon receptor binding, Stx is endocytosed by the eukaryotic cell, bypasses the late endocytic pathway and undergo retrograde transport from the trans-Golgi network to the endoplasmic reticulum where it encounters its target (Farfan & Torres, 2012). Intimate attachment of EHEC to host cells occurs through interactions between intimin and Tir, similarly to EPEC, however, *E. coli* common pilus (ECP) and the hemorrhagic coli pilus (HCP) are also involved in EHEC attachment. The mechanism of pedestal formation by EHEC is slightly different from that used by EPEC. Tir is not phosphorylated by the host cell, and pedestal formation is Nck-independent (DeVinney *et al.*, 1999). In addition, EHEC also carries the LEE plasmid also present in EPEC, but injects around twice as many effectors into host cells. Multiple environmental cues regulate the expression of Stx, including temperature, growth phase, antibiotics, reactive oxygen species (ROS), and quorum sensing (Pacheco & Sperandio, 2012) (Figure 3).

Enteroinvasive *E. coli* (EIEC)

Enteroinvasive *E. coli* (EIEC) has a similar pathogenic mechanism as *Shigella*, which causes an epithelial invasion of the large bowel leading to inflammation and ulceration of the mucosa (Croxen & Finlay, 2010). The T3SS required for invasion, cell survival and apoptosis of macrophages is harbored in a 220 kb plasmid (Ogawa *et al.*, 2008). Infection begins when bacteria pass through microfold cells (M cells) by transcytosis to reach the underlying submucosa. Access to the submucosa is also caused by the disruption of tight junctions and the damage produced by inflammation (Ogawa *et al*., 2008). EIEC are taken up into resident macrophages, escape from the phagosome, activate a caspase1-dependent inflammasome and are released from macrophages (Schroeder & Hilbi, 2008). When EIEC are released from dead macrophages into the submucosa, they invade the basolateral side of colonocytes with the aid of effectors that are secreted by the T3SS (Schroeder & Hilbi, 2008). Effectors such as IpaC, activate SRC kinases at the site of bacterial contact to recruit the ARP2/3 complex and cause actin polymerization and ruffle formation for bacterial entry. Some effectors are also involved in the escape from phagosomes (IpaB, IpaC, IpaD and IpaH) and in the destabilization of actin and microtubules that leads to invasion into a phagosome (IpgD, IpaA and VirA) (Ogawa *et al*., 2008, Schroeder & Hilbi, 2008). Once the bacteria are free in the epithelial cell cytoplasm, EIEC promote their survival by using effectors to further subvert host cell processes. IpaB prevents intestinal epithelial cell turnover, OspE prevents epithelial cell detachment and IpgD prevents apoptosis. These three mechanisms prevent cell death and sloughing, providing a replicative niche for EIEC to maintain an infection (Ogawa *et al*., 2008, Schroeder & Hilbi, 2008).

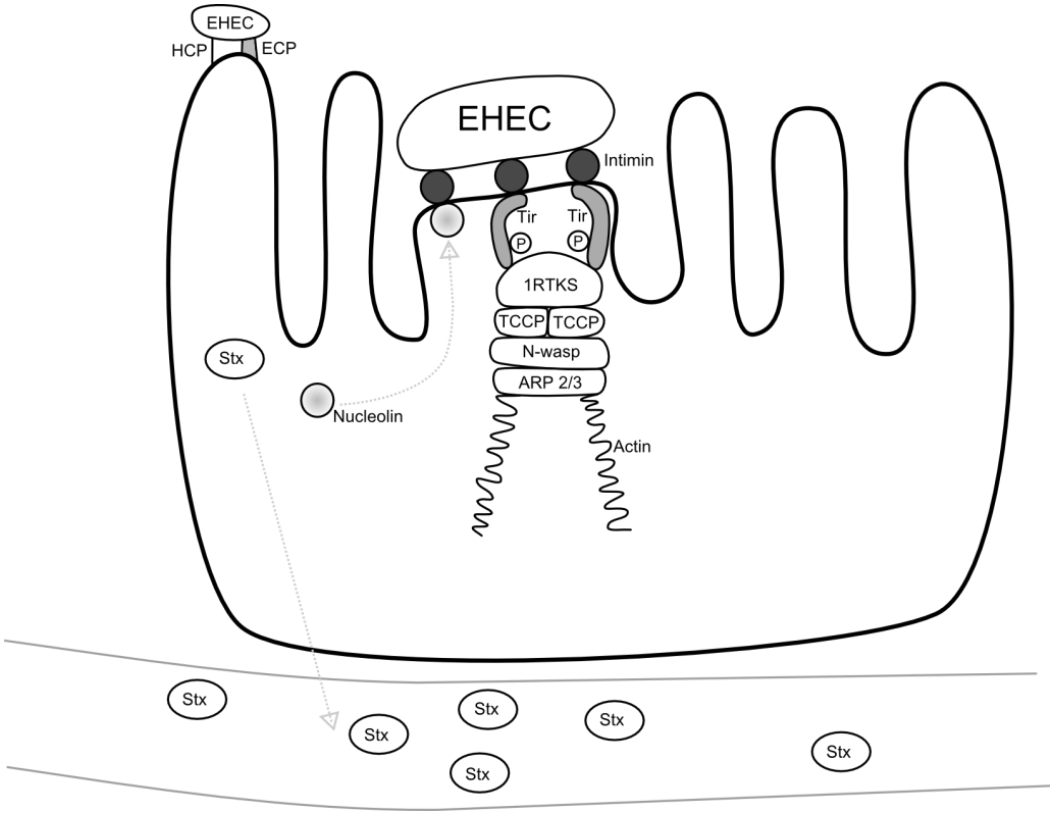

Figure 3. Pathogenesis mechanism of enterohemorragic *E. coli* (EHEC)

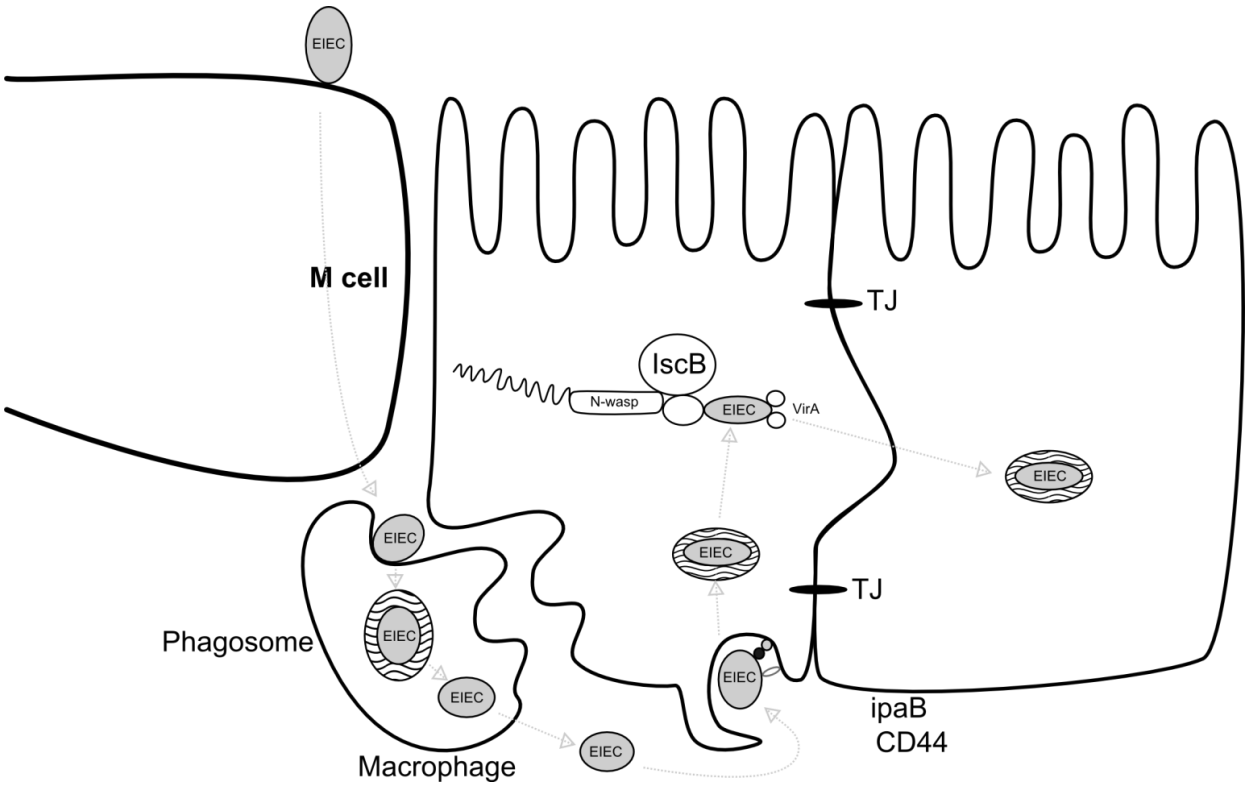

Figure 4. Pathogenesis mechanism of enteroinvasive *E. coli* (EIEC)

To persist inside colonocytes, EIEC must also evade innate immune responses. In addition, due to the lack of a flagella, movement in the host cytosol and cell-to-cell dissemination are mediated by VirG that localize to a single pole of the bacteria and initiate the recruitment and activation of the actin polymerization (N-WASP and ARP2/3 complex), that results in the growth of the actin filaments that push the bacteria through the cell (Schroeder & Hilbi, 2008) (Figure 4).

Enterotoxigenic *E. coli* (ETEC)

Human ETEC infections are contracted by consumption or use of contaminated food and water and presents as a sudden onset of secretory diarrhoea that is usually self-limiting but can lead to dehydration due to loss of fluid and electrolytes (Qadri *et al*., 2005). This pathogen is particularly common in the developing world where an estimated 650 million cases of ETEC infection occur each year, resulting in approximately 800 000 deaths mostly in young children. Additionally, it poses a significant problem for travelers and military personnel visiting countries where ETEC is endemic (WHO, 1999; Qadri *et al*., 2005). In addition, bovine and porcine ETEC also has important financial implications for the farming industry where it is a major pathogen of cattle and neonatal and postweaning piglets (Turner *et al.*, 2006).

Specific virulence factors such as enterotoxins and colonization factors differentiate ETEC from other categories of diarrhoeagenic *E. coli*. The ETEC pathogenesis suggests that the organism colonize the small intestine by virtue of colonization factors, followed by the elaboration of heat-stabile (ST) and/or heat-labile enterotoxin (LT). These virulence factors allow the organisms to readily colonize the small intestine and thus cause diarrhoea (Qadri *et al.*, 2005). In addition, ETEC belongs to a heterogeneous family of lactose-fermenting E. coli, belonging to a wide variety of O antigenic types. There are clearly preferred combinations of serotypes, CFs, and toxin profiles in ETEC (Qadri *et al*., 2005).

ETEC Colonization factors (CFs)

Colonization factors are proteinaceous surface polymers that facilitate adherence to the intestinal mucosa (Evans *et al.*, 1975). At least 25 different CFs have been described to date in human ETEC strains and most are plasmid-encoded. Human ETEC CFs are designated as coli surface antigens (CS) and a number corresponding to the chronological order of identification, with the exception of CFA/I (Gaastra & Svennerholm, 1996). CFs are also classified according to their structural morphology in fimbrial, fibrillar, helical or nonfimbrial CFs (Gaastra & Svennerholm, 1996). CFs can been subdivided into families based on their antigenic and genetic relationships, for instance the CFA/I, CFA/II and CFA/IV groups, which represent the most prevalent CFs worldwide (Gaastra & Svennerholm, 1996, Torres *et al.*, 2005).

The genes for the structural subunits and the transport and assembly proteins required for the biogenesis of CFs are usually encoded in operons. These DNA fragments have a lower GC content and codon usage, and are generally flanked by insertion sequences and transposons (Gaastra & Svennerholm, 1996, Wolf, 1997). The majority of the CFs are synthesized via the chaperone-usher-

dependent pathway. In general, these CFs are encoded in a four-gene operon consisting of a periplasmic chaperone, a major fimbrial subunit, an outer membrane usher protein and a minor subunit. The minor subunit is located at the tip of the fimbriae, with the N-terminal half of the protein responsible for binding to the host cell receptor (Anantha *et al.*, 2004). However, longus and CFA/III are both synthesized as Type IV pili in a process analogous to the Type II secretion pathway (Peabody *et al.*, 2003). In both cases, the production of CFs is energetically expensive and is therefore tightly regulated by the bacterium. Once the CFs are expressed and located on the cell surface, the adhesive moiety can interact with the cognate receptor on the host cell surface. The precise receptors for most of the CFs have not been identified, however, many are thought to bind to glycoprotein conjugates on the surface of host cells (Jansson *et al.*, 2006).

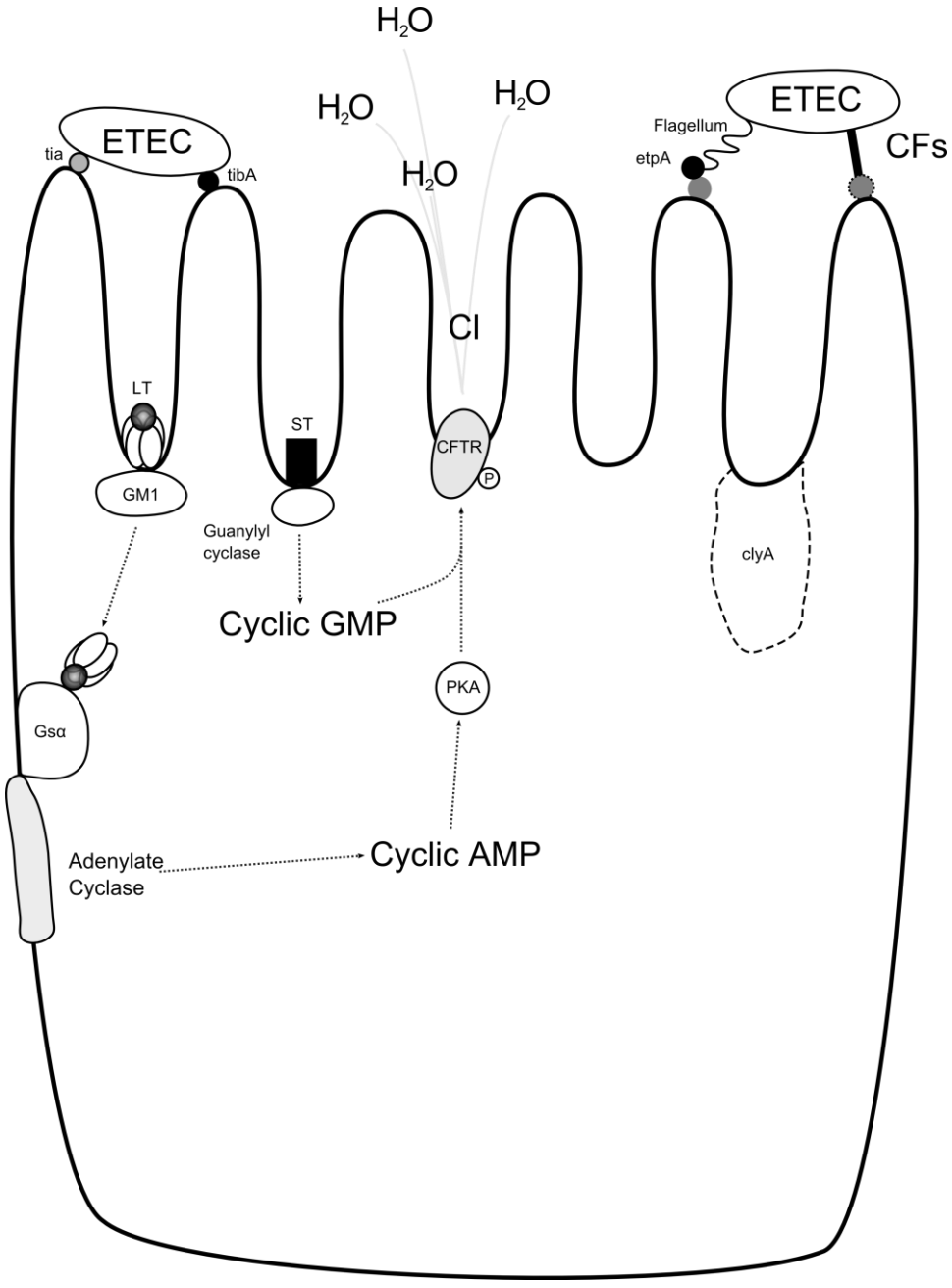

Figure 5. Pathogenesis mechanism of enterotoxigenic *E. coli* (ETEC)

Heat stable enterotoxin (ST)

Heat-stable toxins are small cysteine-rich peptides secreted by ETEC, which are non-immunogenic in its natural form (Fleckenstein *et al.*, 2010). ETEC can produce two distinct heat-stable toxins, STa/STI and STb/STII, which are unrelated structurally, functionally, and immunologically, and have different targets and different mechanisms of action (Nair & Takeda, 1998). STb is only produced by porcine and bovine ETEC and has no implication for disease in humans. The STa toxins consist of two subtypes STh and STp that have been categorized according to the host from which the specific ETEC strain harboring the toxin was initially isolated. STh was produced by an ETEC strain infecting humans while STp was initially identified from a strain that infected pigs. However STp have now been isolated from strains of both bovine and human origin (Lin *et al.*, 2010) and STp is clearly able to cause disease in humans (Bolin *et al.*, 2006).

Both STh and STp are plasmid encoded, often flanked by transposons and synthesized as a pre–pro-peptide of 72 amino acids that are processed during export to produce the mature active toxins of 18 or 19 amino acids (Moseley *et al.*, 1983). The presequence is a signal peptide that directs translocation of the pre-pro-polypeptide across the inner membrane, mediated by the Sec machinery. The Sec-dependent signal sequence is removed during translocation across the inner membrane, releasing the propeptide into the periplasmic space where disulphide bond isomerase, DsbA, catalyses the formation of three disulphide bonds in the C-terminus of the propeptide before it is exported through the TolC outer membrane protein transporter. The proregion is removed to release the small mature toxin. The disulphide bond formation in the mature portion of these toxins confers their heat stability (Dubreuil, 2012, Lin *et al.*, 2010) (Figure 6).

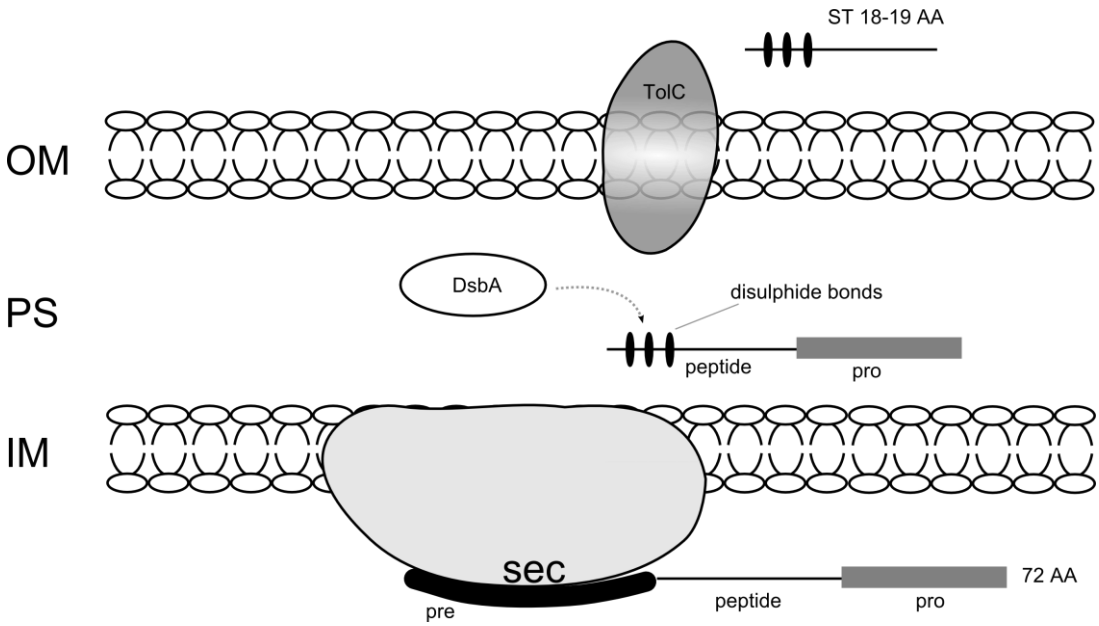

Figure 6. Schema of the assembly and delivery of heat-stabile enterotoxin (STh)

Heat labile enterotoxin (LT)

LT is a heterohexameric molecule composed of a pentameric B subunit and a single A subunit. LT is structurally and functionally similar to cholera toxin (CT) (Sanchez & Holmgren, 2005). The A subunit consists of two domains linked by a disulfide bridge. The B subunits of LT bind with high affinity to cellular GM-1 ganglioside and possibly other membrane receptors such as AB blood antigens and LPS (Mudrak & Kuehn, 2010a). Upon binding, the B pentamer directs endocytosis of the catalytic A-subunit, which enters the cytoplasm by retrograde transport via the endoplasmic reticulum complex. Once in the cytoplasm, the A subunit mediates ADP-ribosylation of the Gsα signaling protein, which leads to constitutive activation of adenylate cyclase and production of 3´,5´-cyclic AMP (cAMP). Intracellular increase of cAMP leads to activation of the cAMP-dependent protein kinase A (PKA), which phosphorylates the R domain of the cystic fibrosis transmembrane conductance regulator chloride channel (CFTR) on the enterocyte membrane (Fleckenstein *et al*., 2010, Mudrak & Kuehn, 2010a). These events induce chloride and water efflux into the intestinal lumen leading to significant volumes of watery diarrhoea (Sears & Kaper, 1996) (Figure 5).

LT is encoded by a two-gene operon, with the gene for LTA (*eltA*) overlapping with the start of the gene for LTB (*eltB*) by four nucleotides forming together the *eltAB* operon (Yamamoto *et al*., 1982). In ETEC, *eltAB* is found on extrachromosomal virulence plasmids *e.g.* pEnt. *eltAB* is flanked by approximately 250 base pairs of conserved sequence, often followed by partial or intact insertion sequence (IS) elements (Schlor *et al*., 2000). The conserved region upstream of *eltA* contains a strong consensus promoter, and the region downstream of *eltB* contains a probable transcriptional terminator, indicating that these genes are transcribed as a single message (Schlor *et al*., 2000)

The transcriptional regulation of *eltAB* is not well known. Different growth conditions have consistently shown to induce LT production, however, it is important to note that the levels of production of LT vary among different strains (Lasaro *et al*., 2006). Therefore, the regulation of LT production may occur differently in individual ETEC isolates (Mudrak & Kuehn, 2010a). A model of the regulation of *eltAB* during infection has been suggested. In a natural environment, low osmolarity and H-NS repress the *eltAB* promoter, inhibiting LT production. Upon the entry into the host intestine, free glucose and body temperature 37°C de-repress the *eltAB* operon by inhibiting CRP and H-NS producing high levels of LT. When ETEC reaches the ileum, it adheres to intestinal cells and begins to multiply. In the ileum intestinal absorption decreases the levels of glucose and releases high amounts of cAMP resulting in a CRP repression and therefore in lower levels of LT. Finally, in the large intestine CRP and fatty acids fully repress LT (Mudrak & Kuehn, 2010a) (Figure 7). As discussed in the Results section this model is refined in the present thesis (Paper IV).

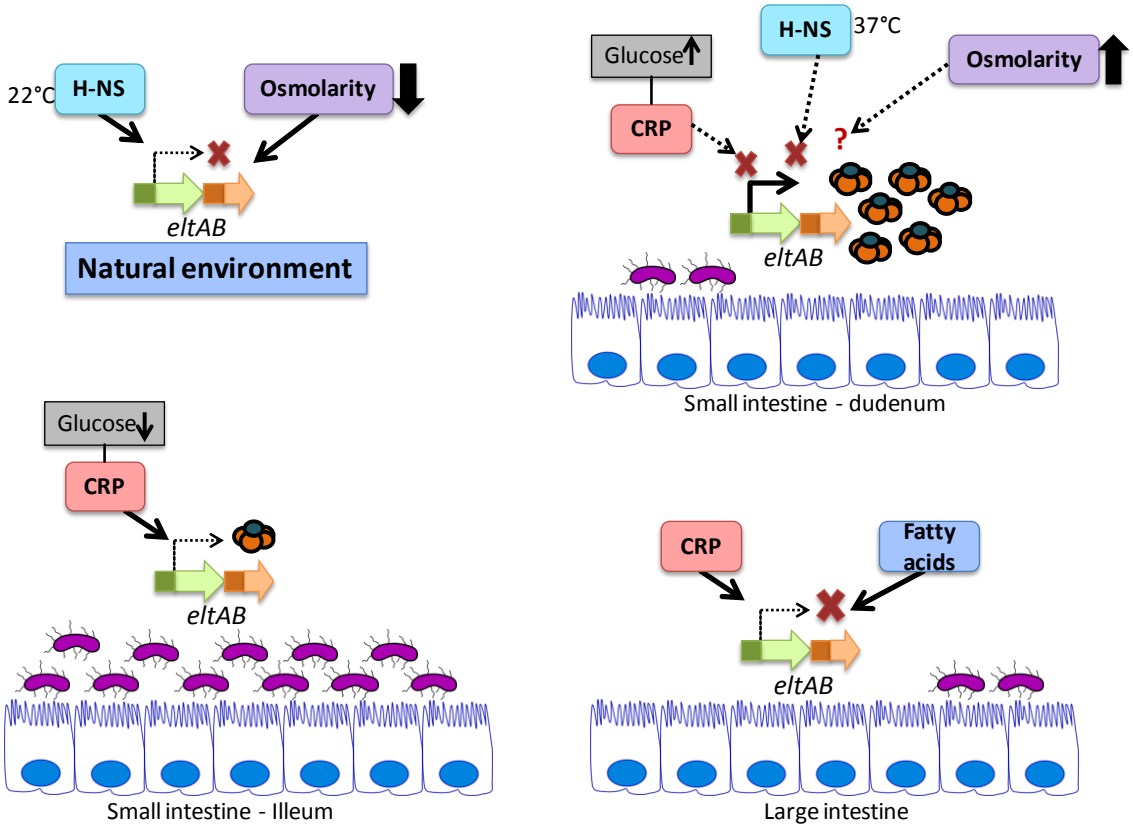

Figure 7. A model of the regulation of *eltAB* (LT toxin) during infection

Putative virulence genes

LeoA. LeoA is a GTP-binding protein encoded on a pathogenicity island in the prototype H10407 strain that has been described to modulate LT secretion (Brown & Hardwidge, 2007, Fleckenstein *et al.*, 2000). The role of LeoA in the secretion of LT is certainly not an universal one since only few strains have been reported to carry the *leoA* gene (Turner *et al.*, 2006). It is possible that certain strains use LeoA to provide additional energy for the export of LT (Mudrak & Kuehn, 2010a).

East-1. EAST1, the enteroaggregative heat-stable toxin, shares structural similarity to STI peptides, and also leads to increases in cGMP (Savarino *et al.*, 1993). East-1 has been identified in a variety of enteric pathogens including ETEC (Savarino *et al.*, 1996). East-1 resides in a mobile element and has functionally active enterotoxin activity. It is possible that East-1 provokes elevated levels of cGMP generating functional redundancy of the ST toxins (Fleckenstein *et al.*, 2010)

TibA. Tib is a chromosomally encoded locus associated with nonfimbrial adherence of ETEC to human cells (Elsinghorst & Kopecko, 1992). This locus consists of four genes, *tibDBCA*, organized in a single operon. TibA is an autotransporter protein with homology to several autotransporter adhesins from other mucosal pathogens. TibA is synthesized as a 100 kD precursor protein, preTibA, that is glycosylated through the action of TibC, a putative glycosyltransferase. Only the glycosylated form enables the bacteria to bind to a specific receptor on epithelial cells and invade (Lindenthal & Elsinghorst, 1999, Lindenthal & Elsinghorst, 2001). In addition to adherence to mammalian cells,

TibA promotes aggregation of a bacterial population and the formation of a biofilm and this occurs independent of TibA glycosylation (Sherlock *et al.*, 2005).

Tia. Tia, is a 25 kD outer membrane protein encoded on a large pathogenicity island inserted in the selC tRNA gene of H10407 (Fleckenstein *et al.*, 1996). Tia acts as an invasin as well as an adhesion interacting with host cell surface proteoglycans, promoting adherence and epithelial cell invasion (Fleckenstein *et al.*, 2002, Mammarappallil & Elsinghorst, 2000). However, tia has show to promote adhesion *in vitro* and to have significant homology with known virulence proteins suggesting that tia might promote adherence in ETEC (Fleckenstein *et al*., 2010).

ClyA. ClyA was first identified in *E. coli* K-12 but has now been detected in a range of *E. coli* clinical isolates including ETEC strains (Ludwig *et al.*, 2004). ClyA has been described as a pore forming enterotoxin. Expression of ClyA is negatively regulated by H-NS and positively regulated by SlyA, FNR and CRP. ClyA traverses the inner and outer membranes by an unknown mechanism and is associated extracellularly with outer membrane vesicles. The protein has been shown to interact with cholesterol moieties in the eukaryotic cell membrane and to oligomerize to form pores in the lipid bilayer, thereby inducing cytotoxicity (Turner et al., 2006).

EatA. EatA is a serine protease autotransporter encoded on the pCS1 plamid of *E. coli* H10407 and in multiple clinical ETEC isolates (Patel *et al.*, 2004). EatA has been suggested to play a role in ETEC virulence by damaging the epithelial cell surface and by having mucinase activity or cleaving proteoglycans on the host cell surface. EatA has been associated with accelerated virulence in a rabbit ileal loop model (Patel et al., 2004). Recent studies have shown that eatA plays a significant role in modulating adherence of ETEC by degrading the EtpA adhesion, accelerating delivery of the LT toxin to target epithelial cells (Roy *et al.*, 2011).

EtpA. EtpA is encoded by a two-partner secretion (TPS) locus, initially discovered in the model ETEC strain H10407. EtpA is a secreted glycoprotein that functions as an adhesin. Studies suggest that EtpA functions as a molecular bridge, binding both the host cell receptor and to the tips of ETEC flagella, where it interacts with highly conserved regions of flagellin proteins (Roy *et al.*, 2009). EtpA and its interactions with flagellin are required for optimal adhesion of H10407 *in vitro* and intestinal colonization in murin models *in vivo* (Roy et al., 2008)

Type 2 secretion system (T2SS)

LT is secreted through the outer membrane by a two-step process. In the first step, N-terminal signal peptides of the subunits are cleaved during sec-dependent transport across the inner membrane to the periplasm where the monomers assemble into a holotoxin (Fleckenstein *et al*., 2010). After assembly the holotoxin is secreted across the outer membrane through a complex type II secretion apparatus (T2SS) (Tauschek *et al.*, 2002) (Figure 8)

The T2SS is multiprotein complex consisting of 12-15 components that spans the inner and outer membrane, allowing for the controlled release of certain folded proteins that have been directed to the periplasm through the Sec or Tat secretion machinery (Mudrak & Kuehn, 2010b). From the periplasm, folded proteins are transported through a pore formed by the GspD complex located in the

outer membrane. The GspD protein is an outer membrane transporter, termed the secretin, which function as the outer membrane pore through which proteins or macromolecular complexes are translocated (Strozen *et al.*, 2012). In some systems, localization of the secretin in the outer membrane requires the function of a small lipoprotein that serves as a pilotin to direct the secretin to the outer membrane and protect the multimer from degradation (Strozen *et al*., 2012). The genes that encode the T2SS proteins are arranged in a major operon composed of genes *gspC* to *gspO* and, in some cases, a minor operon composed of *gspA* and *gspB* or an independently encoded *gspS* (Sandkvist, 2001).

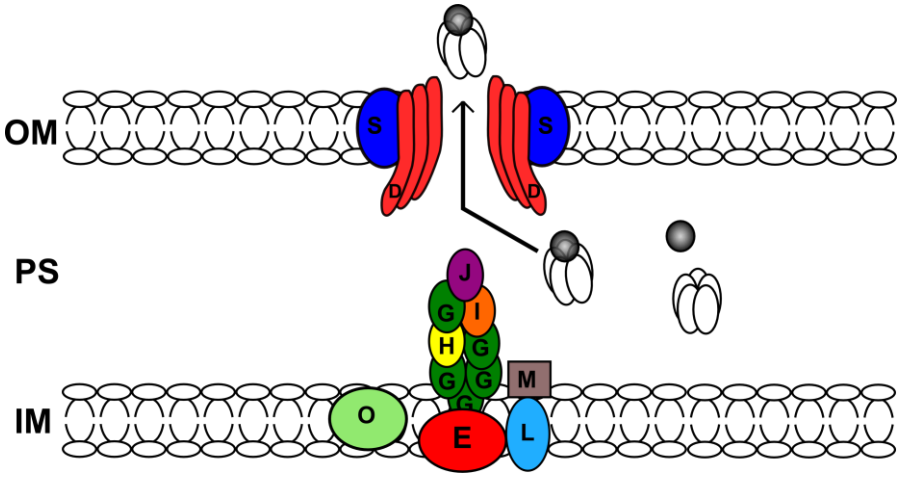

Figure 8. Schema of type 2 secretion system in ETEC.
OM: outer membrane protein, IM: inner membrane and PS: periplasmic space

The T2SS in ETEC, was initially identified in H10407 ETEC when the operon *gspC-M* coding for a functional type II secretion system was described (Tauschek *et al*., 2002). The genes encoding the T2SS are also regulated by H-NS, indicating that it can be turn on under the same conditions that favor LT expression (Yang *et al.*, 2007, Yang *et al.*, 2005). Expression of a functional T2SS is required for secretion of LT into culture supernatant (Horstman & Kuehn, 2000). Two T2SSs capable of secreting the LT toxin have been described in H10407 ETEC. The systems have been designated as alpha ($T2SS_{\alpha}$) and beta ($T2SS_{\beta}$), each of which is variably present within the genome of *E. coli* pathotypes (Strozen et al., 2012). The $T2SS_{\alpha}$ requires specific environmental conditions during intestinal colonization for its induction, it has been shown to secrete LT toxin when it has been expressed in a *hns E. coli* k-12 strain. In contrast the $T2SS_{\beta}$ operon is atypical in comparison to T2SS operons of other species by including three genes (*yghJ*, *pppA*, and *yghG*) upstream of *gspC*. *YghJ* is not required for assembly or function of $T2SS_{\beta}$ and has been considered to be a substrate of the $T2SS_{\beta}$; *pppA* likely encodes the $T2SS_{\beta}$ prepilin peptidase that is required for assembly of the pseudopilus. The *yghG* encodes a novel outer membrane lipoprotein that functions as a pilotin, required for localization of GspD to the outer membrane, and therefore, for secretion of the LT toxin (Strozen *et al*., 2012).

TRANSCRIPTIONAL REGULATION

Transcription mechanisms in bacteria

RNA polymerase is the central component in transcriptional regulation in bacteria and responsible for all transcription (Borukhov & Nudler, 2008). The core enzyme consists of 5 subunits named β,β′, α, α and ω. The active site of the polymerase is formed by the large β and β′ subunits that mediate the binding of both template DNA and the RNA product during transcription. Two identical α subunits are present, the larger amino-terminal domain (αNTD) dimerizes and is responsible for the assembly of the β and β′ subunits whereas the smaller carboxy-terminal domain (αCTD) is a DNA-binding module that has an important role at certain promoters. The small ω subunit has no direct role in transcription, but seems to function as a chaperone to assist the folding of the β′ subunit. To start the transcription at a particular promoter, the RNA polymerase core enzyme must recruit a σ subunit to crease the fully active RNA polymerase holoenzyme. The σ subunit has three main functions: to ensure the recognition of specific promoter sequences; to position the RNA polymerase holoenzyme at a target promoter; and to facilitate unwinding of the DNA duplex near the transcript start site (Borukhov & Nudler, 2008, Browning & Busby, 2004, Saecker *et al.*, 2011) (Figure 9).

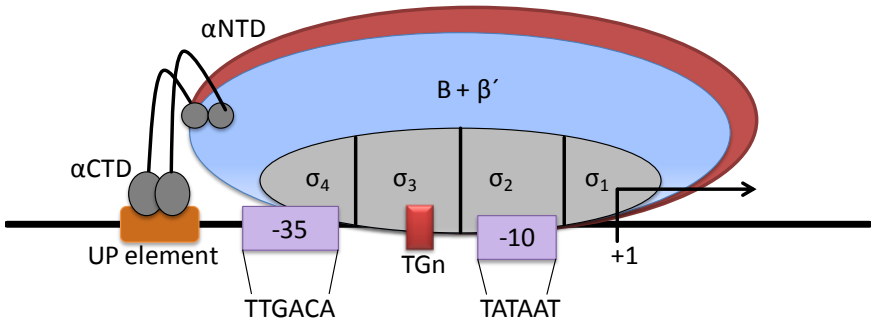

Figure 9. Schema of the RNA polymerase

Promoters control the transcription of all genes. Transcription initiation requires the interaction of RNA polymerase with promoter DNA and the formation of an open complex, in which the duplex DNA around the transcript start-point is unwound (Borukhov & Nudler, 2008). Synthesis of the DNA template-directed RNA chain then begins, with the formation of the first phosphodiester bond between the initiating and adjacent nucleoside triphosphates. After this initiation phase, RNA polymerase is moved into the elongation complex, which is responsible for RNA-chain extension (Browning & Busby, 2004, Saecker et al., 2011). The main step in initiation is promoter recognition by RNA polymerase. Four different elements have been identified: the –10 hexamer and the –35 hexamer, which are located 10 and 35 base pairs (bp) upstream from the transcript start site, respectively. Promoter –10 elements are recognized by domain 2 of the RNA polymerase σ subunit and the –35 elements are recognized by domain 4 of the RNA polymerase σ subunit. The two other important promoter elements are the extended –10 element and the UP element. The extended –10 element is located immediately upstream of the –10 hexamer that is recognized by domain 3 of the RNA polymerase σ subunit, and the UP element is located upstream of the promoter –35 hexamer that is recognized by the C-terminal domains of the RNA polymerase α subunits (Browning & Busby, 2004). After initial binding of RNA polymerase, the DNA strands from approximately

position –10 to position +2, just downstream of the transcript start-point, are unwound to form a 'bubble', and to generate the open complex in a process known as isomerization (Borukhov *et al.*, 2005) (Figure 10).

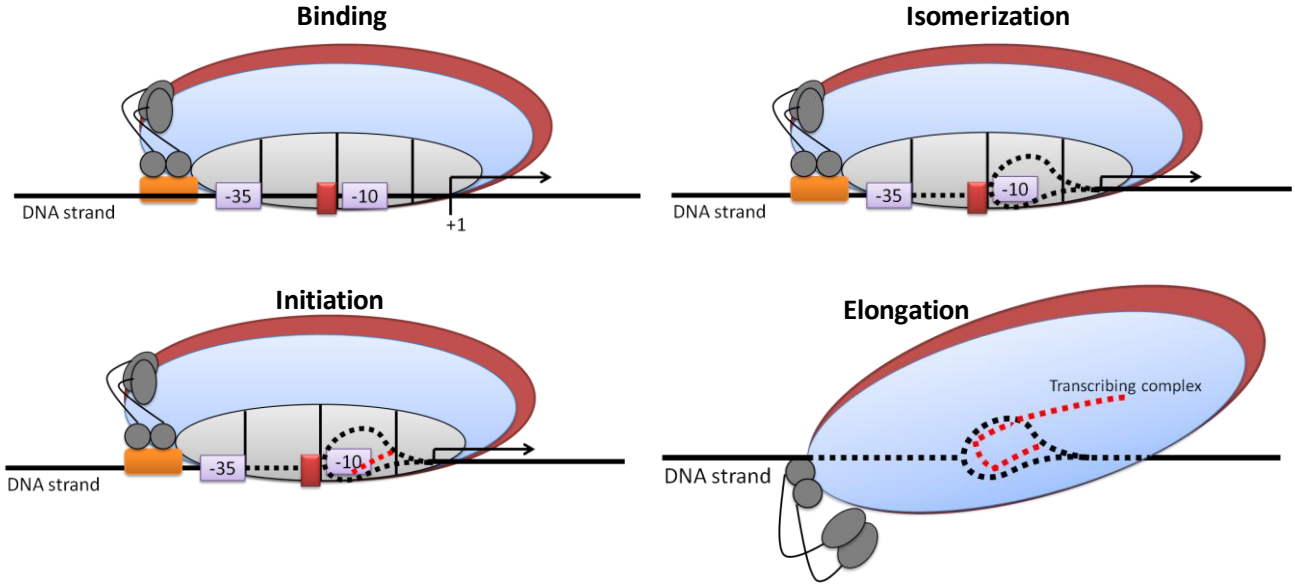

Figure 10. The pathway of transcriptional initiation and the interaction of RNA polymerase with the promoter.

Transcriptional regulation is believed to be mainly regulated at the initial binding of RNA polymerase to generate the closed complex, at isomerization when the open complex is formed or at the initial steps of RNA-chain synthesis (Browning & Busby, 2004). Microbial gene regulation should consider a short supply of RNA polymerase and σ factors that creates an intense competition between different promoters for RNA polymerase holoenzyme. Five distinct molecular mechanisms seem to ensure the prudent distribution of RNA polymerase between competing promoters. These involve promoter DNA sequences, σ factors, small ligands, transcription factors and the folded bacterial chromosome structure. Each mechanism participates in the fine-tuning of the level of expression of genes, (Browning & Busby, 2004).

The *E. coli* sigma factor 70 (σ70) regulates the majority of genes expressed. However, the *E. coli* genome also contains six other σ factors that accumulate in response to specific stresses and compete with σ70 for RNA polymerase. The activity of the σ70 factor can also be controlled by an anti-sigma factor, which sequesters it away from the RNA polymerase (Borukhov & Severinov, 2002, Gruber & Gross, 2003). Certain small ligands provide an alternative mechanism by which RNA polymerase can respond quickly and efficiently to the environment. Guanosine 3′,5′ bisphosphate (ppGpp) is the best example. ppGpp is synthesized when amino-acid availability is restricted to the extent that translation is also limited. ppGpp works by destabilizing open complexes at promoters that control synthesis of the machinery for translation (Barker *et al.*, 2001). In addition, the folded bacterial chromosome structure is characterized by the presence of nucleoid proteins that bind to DNA nonspecifically, or with weak specificity at specific sites that are distributed throughout the chromosome. The binding of these nucleoid proteins to DNA, and the resulting folding of the bacterial chromosome affect the distribution of RNA polymerase between promoters (Travers & Muskhelishvili, 2005).

Transcriptional factors

Transcription factors are proteins that bind to specific sequences on the DNA near their target genes, thus modulating transcription initiation (Seshasayee *et al.*, 2011). Transcription factors can activate or repress transcription of target genes typically in response to an environmental or cellular trigger (Balleza *et al.*, 2009). These factors may be global or local depending on the number of genes and range of cellular functions that they target (Balleza *et al*., 2009, Seshasayee *et al*., 2011). Transcription factors in *E. coli* are responsible for more than 61% of regulatory interactions in this bacterium. Global regulators exhibit pleitropic phenotypes and regulate several operons that belong to different functional groups (Perrenoud & Sauer, 2005). In *E. coli* only seven global regulators directly modulate the expression of about one-half of all genes: These global regulators include the catabolite-responsive CRP (cyclic AMP receptor protein); anaerobiosis regulators FNR (fumarate reductase) and ArcA (anaerobic respiratory control); the feast or famine LRP (leucine regulatory protein); and three other DNA structuring proteins FIS (factor for inversion), IHF (integration host factor) stimulation, and H-NS (heat-stable nucleoid-structural) (Martinez-Antonio & Collado-Vides, 2003). In addition *E. coli* contains more than 250 transcription factors that regulate certain operons or functions in the bacterial cell.

The activities of both global and local transcription factors may be regulated either at a post-transcriptional level via signal-sensing protein domains or at the level of their own expression (Seshasayee *et al*., 2011). Different mechanisms are used to achieve this. First, the DNA-binding affinity of transcription factors can be modulated by small ligands, whose concentrations fluctuate in response to nutrient availability or stress. Second, the activity of some transcription factors is modulated by covalent modification. Third, the concentration of some transcription factors in the cell controls their activity. Finally, a less common mechanism for regulating the effective concentration of a transcription factor is sequestration by a regulatory protein to which it binds (Browning & Busby, 2004).

Global regulators such as Lrp, CRP, Fis and H-NS modulate gene expression on a genome wide scale, in response to various stresses**. Crp** is the most abundant global transcription factor in *E. coli*. It is activated by the binding of the second messenger cyclic-AMP (cAMP) in response to glucose starvation and other stresses (Grainger *et al.*, 2005). **Lrp** was first identified as a regulator of branched amino acid transport. In *E. coli*, the transcription factor regulates genes involved in amino acid metabolism and transport, and non-metabolic functions such as pili biosynthesis (Chen *et al.*, 2001). **Fis** is a versatile DNA binding protein that can affect multiple processes including transcription. In *E. coli*, it is thought to be a major regulator of growth transitions. Fis is expressed in a growth phase dependent fashion, showing high expression during logarithmic growth. It activates more genes than it represses, though it represses several non-essential genes during exponential growth (Grainger *et al.*, 2008, Schneider *et al.*, 1997). **H-NS** is a global repressor of gene expression in enterobacteria. It is expressed throughout all the growth phases in *E. coli* and simultaneously affects DNA structure and transcription by forming DNA–HNS–DNA bridges and reinforcing supercoiled structures (Dorman, 2004, Dorman, 2007). H-NS binds to its DNA targets in a sequence-independent manner, but shows strong preference for regions of intrinsic curvature. In pathogenic bacteria, H-NS functions mainly as a negative regulator repressing transcription in response to

environmental changes such as temperature and osmolarity (Yang *et al.*, 2005). Among the other global regulator are the **ArcB/ArcA** two component signal transduction system that regulates gene expression in response to redox conditions and anaerobic conditions (Liu & De Wulf, 2004), **FNR** that regulates proteins that are involved in cellular adaptation to growth in anoxic environments (Unden *et al.*, 2002, Unden & Schirawski, 1997) and **IHF** which contributes to genome organization and the control of DNA transactions. IHF binds to a conserved sequence in DNA and it bends the DNA by angles of up to 180°. This DNA-bending activity is critical to the role it plays in several systems due to its ability to promote long-range interactions The influence of IHF on local DNA structure is critical to its contribution to transcription control. In some cases, it has been shown to enhance the formation of open complexes at promoters by transferring DNA twist from upstream regions of A+T-rich DNA to the promoter through its DNA-bending activity. The intracellular concentration of IHF is growth phase dependent and most studies agree that IHF concentration increases with the onset of the stationary phase (Mangan *et al.*, 2006).

Regulation of glucose in *E. coli*

Glucose is the most abundant sugar in nature, being present mostly in polymeric states as starch and cellulose. This sugar is the preferred carbon and energy source for *E. coli* (Saier, 1996). Specialized protein systems are present in *E. coli* to sense, select and transport glucose. Glucose is internalized and phosphorylated by the phosphoenolpyruvate: sugar phosphotransferase system (PTS). This system catalyzes group translocation, a process that couples transport of sugars to their phosphorylation (Tchieu *et al.*, 2001). It is composed of soluble non sugar-specific protein components, Enzyme I and the phosphohistidine carrier protein (HPr) which relay a phosphoryl group from the glycolytic intermediate, phosphoenolpyruvate (PEP), to any of the different sugar-specific enzyme II complexes. Glucose is imported by the II^{Glc} complex, composed of the soluble IIA^{Glc} enzyme and the integral membrane permease $IICB^{Glc}$ (Tchieu *et al.*, 2001).

The preferred nutritional status of glucose for *E. coli* is regulated by carbon catabolite repression (CCR). There is a repression and inhibition regulated by this sugar on gene expression and the activities of enzymes and transporters related to the consumption of other carbon sources (Saier, 1996). PTS plays a central role in CCR since it functions as a sensor of the presence of glucose in the external medium. When glucose is present in the medium and it is being transported by the PTS, the IIA^{Glc} protein is non-phosphorylated, and in this state, it binds to various non-PTS permeases inhibiting uptake of other carbon sources. This form of IIA^{Glc} also binds to the enzyme glycerol kinase, inhibiting its activity. When glucose is absent from the culture medium, IIA^{Glc} is mainly in its phosphorylated state. In this condition, IIA^{Glc}~P binds to the enzyme adenylate cyclase, which synthesizes cyclic AMP (cAMP), and as a consequence cAMP concentrations increase in the cell. Then cAMP binds to the cAMP receptor protein (CRP) and activated cAMP-CRP promotes the induction of catabolite-repressed genes, which typically consist of genes involved in metabolism of other carbon sources than glucose (Gutierrez-Rios *et al.*, 2007, Saier, 1996) (Figure 11).

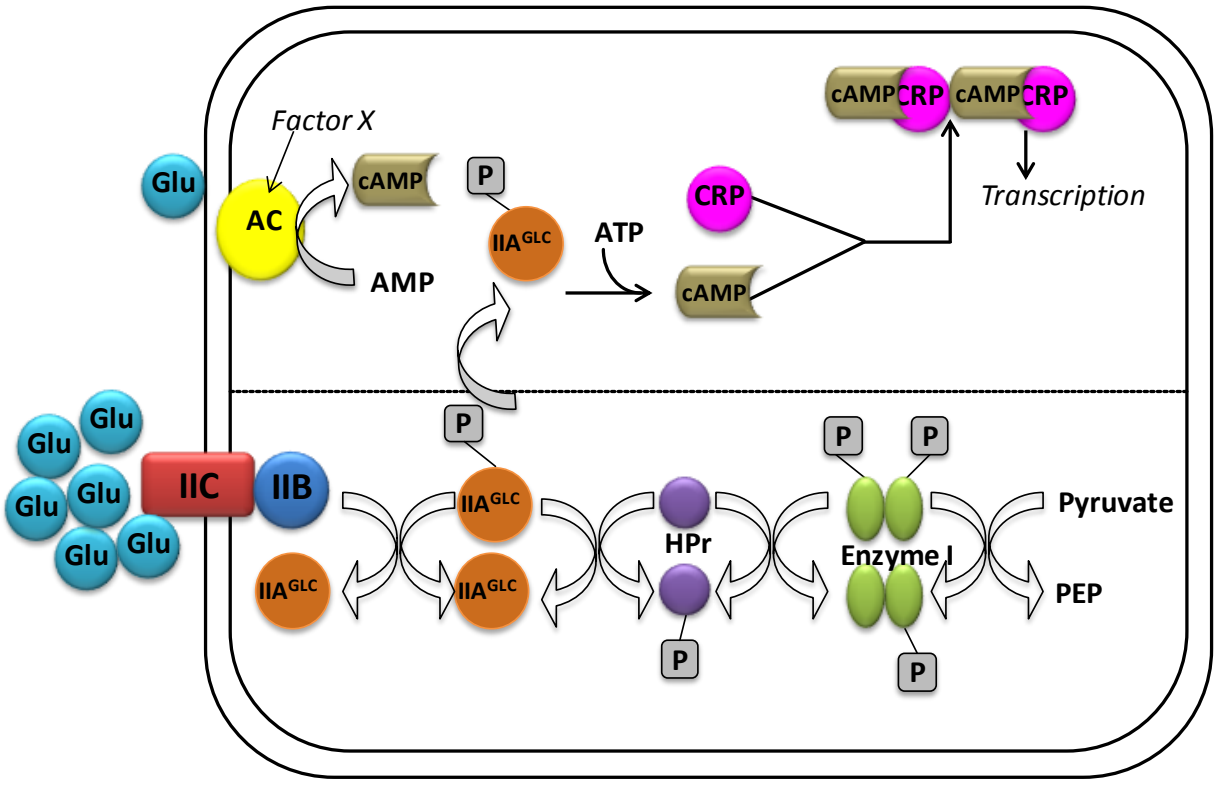

Figure 11. Model for carbon catabolite repression (CCR) mechanism in *Escherichia coli.*

Cyclic AMP receptor protein (CRP)

Cyclic AMP receptor protein (CRP) was the first purified, the first crystallized and is still the best studied transcription activator from *E. coli*. CRP is a sequence-specific DNA binding protein, which regulates transcription of a number of genes in response to the carbon source. CRP has a role in the activation of a number of the genes for utilization of carbon sources other than glucose (Fic *et al.*, 2009). The CRP regulon includes the genes encoding the transporters and the catabolic enzymes of non-glucose sugars (Shimada *et al.*, 2011). Expression of these genes is activated in the absence of glucose through functional conversion of CRP into an active form after interaction with cAMP which is synthesized by the membrane-bound adenylate cyclase. The *cyaA* gene encoding adenylate cyclase is activated in the absence of glucose. The intracellular level of cAMP is controlled in response to glucose availability (Pastan & Perlman, 1970). When complexed with its allosteric effector cAMP, CRP undergoes a conformational transition and binds to 22-bp target sites within or near promoters of catabolite-sensitive operons to activate their transcription (Hanamura & Aiba, 1991).

When CRP undergoes autoregulation, in the absence of CRP-cAMP, the promoter is the only functional promoter and RNA polymerase exclusively occupies it to transcribe the *crp* gene. As the concentration of CRP-cAMP increases, the binding to the CRP site increases (Figure 12a). The binding of CRP-cAMP to the CRP site allows RNA polymerase to bind predominantly at the divergent promoter inhibiting the transcription of CRP (Figure 12b). The occupancy of the divergent promoter by RNA polymerase excludes RNA polymerase occupancy of CRP binding site resulting in the inhibition of the *crp* transcription. The principal role of CRP-cAMP is to determine the binding mode between RNA polymerase and the overlapping promoters. Additionally, RNA polymerase bound at one promoter is acting as a direct repressor for the transcription from the other promoter (Hanamura & Aiba, 1991).

cAMP-CRP is a dual regulator, acting either as an activator or a repressor. These feature correlates with the position of DNA binding relative to the target promoter. The binding sites of activator CRP are mostly located upstream from the promoters while those of repressor CRP are mostly located downstream (Ishihama, 1993, Zou *et al.*, 1992). Most factors that downregulate promoter activity bind to targets that overlap or are downstream of the transcription start and -10 element and very few cases of repression from upstream locations have been reported. In addition, multiple DNA sites for CRP are common place at promoters. However, recent studies in *E. coli* have found that the upstream-bound CRP could either enhance or repress transcription, depending on its location. Analysis of the interactions between CRP and the C-terminal domains of the RNA polymerase subunits at some of these promoters reported that the upstream-bound CRP interacts with these domains irrespective of whether it up or downregulates promoter activity. Hence, disruption of this interaction can lead to either down- or upregulation, depending on its location (Lee & Busby, 2012). Interestingly, the interaction of LT toxin and CRP in ETEC has been reported to happen at the upstream region of *eltAB* operon (Bodero & Munson, 2009).

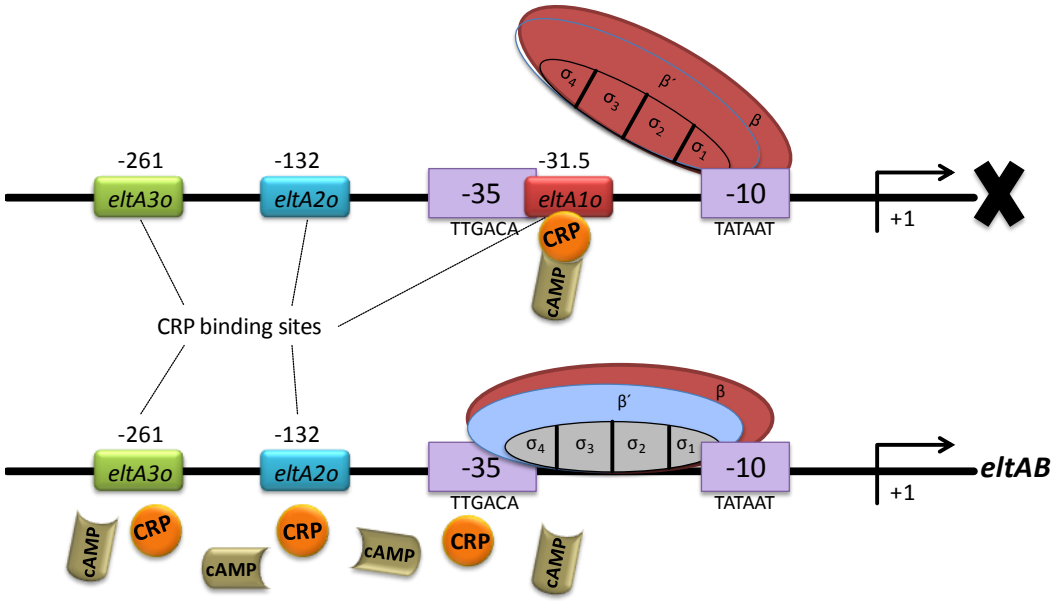

Figure 12. Schema of the transcriptional regulation of LT by CRP.

Transcriptional activation by CRP

CRP was the first transcription regulator that was identified to interact directly with the promoter-bound RNA polymerase for function (Busby & Ebright, 1999). CRP activates the promoter by interacting with the C-terminal domain of the RNA polymerase α subunit (αCTD) (Lee *et al.*, 2012). Four different activation forms have been described for CRP. The Class I activation is characterized by an upstream-bound CRP that interacts with the αCTD domain of the RNA polymerase (Figure 13a) (Lee *et al.*, 2012). In the Class II activation, CRP binds to a target that overlaps the promoter −35 element, where αCTD is dispensable. Two additional activating regions are present, the AR2 which interacts with a target on the surface of the αNTD and the AR3 which interacts with σ Domain 4. The AR1-αCTD interaction promotes RNA polymerase recruitment, whereas the AR2-αNTD interaction promotes transition to the open complex, both subunits of the CRP dimer make activation interactions with RNA polymerase. Hence, CRP can easily be converted from activating by interaction with one part of RNA polymerase (αNTD) to activating by interaction with another part (σ Domain 4) (Figure 13b) (Lee *et al.*, 2012).

The Class III and IV activations describe promoters in which two transcription factors make independent contacts with polymerase. Many CRP-regulated promoters carry two or more DNA sites for CRP. At some promoters, full activation can be achieved with just one bound CRP and thus the other target sites are redundant. In other cases, two bound CRPs are essential for optimal activation, and at many of these promoters, the two sites have different binding affinities for CRP (Lee *et al.*, 2012). In the Class III activation, there is activation by tandem-bound CRP dimers where the downstream CRP is in a Class II position (Figure 13c) (Lee *et al.*, 2012), whereas in the Class IV, the downstream CRP is in a Class I position (Figure 13d). In these cases, each bound CRP makes an independent activatory contact with one of the two RNA polymerase αCTDs. This mechanism works because, at simple CRP-dependent promoters, there is always a spare αCTD available for interaction with a correctly positioned second CRP (83). In class III and IV activation both αCTDs are essential for activation (Lee *et al.*, 2012).

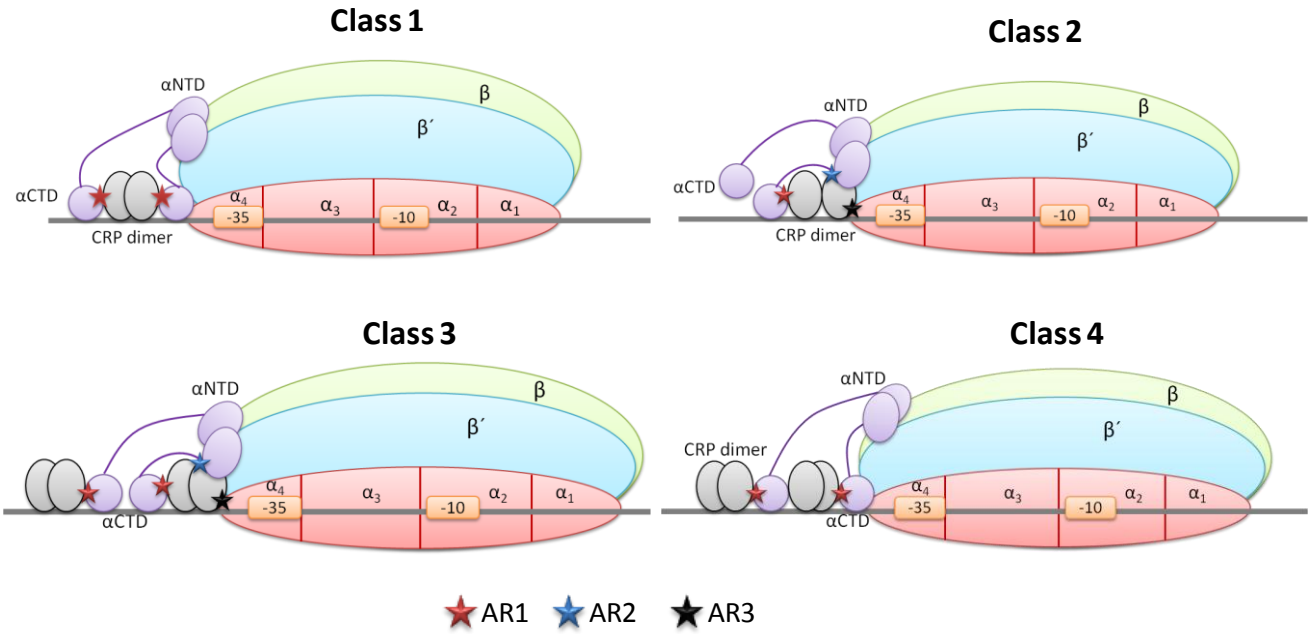

Figure 13. Activation of transcription by the cyclic AMP receptor protein (CRP)

Regulatory roles of CRP.

The most important regulatory role of CRP is the control of central metabolism (Fic et al., 2009). CRP plays a key regulatory role in the whole processes from the selective transport of carbon sources, the glycolysis-gluconeogenesis switching to the metabolisms downstream of glycolysis, including tricarboxylic acid (TCA) cycle, pyruvate dehydrogenase (PDH) pathway and aerobic respiration (Lemuth *et al.*, 2008, Nanchen *et al.*, 2008, Perrenoud & Sauer, 2005). Around 30 of 40 transcription units of carbon transporters are under the control of CRP (Shimada *et al.*, 2011). Therefore, the major regulatory roles of CRP are the sorting of transport of carbon sources by controlling the level of substrate-specific transporters within the membranes (Lemuth *et al.*, 2008, Nanchen *et al.*, 2008, Perrenoud & Sauer, 2005). In addition, CRP regulates a number of genes encoding transcription factors, so far 70 transcription factors have been identified among the whole set of 349 CRP targets. This includes specific regulators of carbon metabolism, regulators of nitrogen metabolism, regulators for response to external stresses, the global transcription factors FIS and the stationary phase-specific sigma RpoS (Shimada *et al.*, 2011).

Studies of CRP on the regulation of the LT toxin in ETEC have shown that CRP acts as a negative regulator of the production of the toxin (Bodero & Munson, 2009). LT promoter is repressed by CRP when it occupies a site centered at -31.5, relative to the transcription start site, occluding RNA polymerase from the promoter and therefore repressing the transcription of *eltA* gene encoding LT toxin. Three binding sites have been reported for CRP in the LT gene, *eltA1o* at -31.5, *eltA2o* at -132 and *eltA3o* at -261; and start site of 55 bp located upstream of the *eltA* initiating codon. CRP prevents RNA polymerase from forming an open complex at *eltA* polymerase binding site by the occupancy of the binding site which prevents RNA polymerase from recognizing the -35 hexamer and, subsequently, the formation of an open complex. However, the formation of the open complex is prevented by CRP when cAMP is included (Bodero & Munson, 2009).

ENVIRONMENTAL FACTORS

All food-borne pathogens, including *E. coli*, are exposed to multiple stresses to which they must respond to survive. These potentially harmful conditions include heat, low pH, osmotic shock, bile, shift to microaerobiosis or anaerobiosis, interaction with gut microflora, and cationic antimicrobial peptides (Ramos-Morales, 2012). The ability to sense these conditions and respond by turning on a set of appropriate genes is an essential feature that enables enteric bacteria to survive in different environments.

pH

Bacteria must maintain a cytoplasmic pH that is compatible with optimal functional and structural integrity of the cytoplasmic proteins that support growth. Most bacteria grow over a broad range of external pH values, from 5.5–9.0, and maintain a cytoplasmic pH that lies within the narrow range of pH 7.2–7.8. To maintain pH homeostasis, bacteria are able to acidify or alkalinize the cytoplasm relative to the external milieu (Padan *et al.*, 2005). The first stress that bacteria find upon ingestion is the very acidic pH of the stomach of the host. Different bacterial species have developed mechanisms that are more or less efficient to promote survival during passage through the stomach. Acid survival is a virulence factor that can affect disease. For instance, the oral infective doses of pathogens i.e. *Vibrio cholerae* (10^6-10^9) and *Shigella flexneri* (10^2) correlate with their different levels of acid resistance (Ramos-Morales, 2012).

Acid survival systems in enteropathogens are divided into two general categories: the acid resistance (AR) mechanisms, which require nutrient supplementation for either induction or function and the acid tolerance responses (ATR), that can be induced and function in unsupplemented minimal medium (Lin *et al.*, 1995). Pathogenic and non-pathogenic strains of *E. coli* are remarkably resistant to extreme acid stress. Three acid-resistant systems, AR1, AR2 and AR3, have been characterized in *E. coli*, whose activity depends on the media used for growth and acid challenge (Foster, 2004). AR1 is active when bacteria are grown to stationary phase in LB medium, without glucose, buffered to pH 5.5, and requires the stationary phase sigma factor RpoS and CRP. The other two systems depend on the presence of specific amino acids in the media, glutamate for AR2 and arginine for AR3, and have similar simple mechanisms of action (Foster, 2004). These AR systems contribute to pH homeostasis so that, when the external pH is 2.5, the internal pH is maintained at 4.5. The strategy of resistance also includes a change in the electrical potential, from negative to positive, and a decrease in metabolic activity (Foster, 2004).

A shift to an alkaline environment, like a shift to an acid environment, is stressful for bacteria. *E. coli* responds to alkali with SOS and heat shock-like responses. Bacteria also presents alkali-tolerance and alkaliphilic mechanisms for alkaline pH homeostasis. A large number of adaptive strategies have been developed for alkaline pH homeostasis. The strategies include: increased metabolic acid production through amino acid deaminases and sugar fermentation which will reduce the cytosolic pH; increased ATP synthase that couples H^+ entry to ATP generation to maintain protons within the cytosol; changes in cell surface properties; and increased expression and activity of monovalent cation/proton antiporters. Among these strategies, monovalent cation/proton antiporters play an essential and dominant role in alkaline pH homeostasis in many bacteria (Padan *et al*., 2005).

Bile salts

Bile is a bactericidal agent found in the digestive system. One of the multitude bile components are the bile salts which provide protection against pathogenic bacteria and assist in the digestion of fatty acids (Merritt & Donaldson, 2009). Bile salts are secreted into the duodenum at estimated concentrations of 0.2–2% (Begley *et al.*, 2005). However, the concentration of individual bile salts *in vivo* is affected by the nutritional status of the host, e.g. the ingestion of a fatty meal will increase their concentration, and the concentration of bile in the intestinal lumen is lower in malnourished patients. Additionally, the bile level is likely to differ between the intestinal lumen and the mucosal layer (Begley *et al.*, 2005). Bile salts have an effect on the integrity of the bacterial membrane. Bacteria have developed mechanisms to cope with its toxic effect, such as the efflux pumps that remove bile salts from the cell preventing damage in the membrane as has been shown in *Vibrio cholerae* (Chatterjee *et al.*, 2004). In *E. coli*, bile salts have been found to be oxidative agents with the ability to induce the SOS response avoiding DNA damage (Merritt & Donaldson, 2009). However, bile salts can also induce the expression of virulence factors in *Campylobacter*, *Shigella*, *Salmonella*, *V. cholerae* and ETEC (Nicklasson *et al.*, 2012, Rivera-Amill *et al.*, 2001, Stensrud *et al.*, 2008, Torres *et al.*, 2007). For instance the bile acid glycocholate hydrate and sodium deoxycholate have been shown to induce expression of the ETEC colonization factor CS5 (Nicklasson *et al.*, 2012).

Osmolarity

E. coli maintains an osmotic pressure in the cytoplasm that is higher than the surrounding environment, resulting in a positive osmotic pressure on the cell wall. The turgor pressure generated is essential for the division and growth of the cells (Lucht & Bremer, 1994). When the extracellular osmotic pressure rises or falls, the transmembrane water fluxes concentrate or dilute the cytoplasm, disrupting the cell structure and the function. Bacteria respond by adjusting their internal solute concentration using several different regulatory pathways (Wood, 2006). In an osmotic upshifts event, bacteria respond with dehydration, adjustment of cytoplasmic solvent composition and rehydration, and cellular remodeling. In contrast, bacteria respond to osmotic downshifts with water uptake, extrusion of water and cosolvents, and cytoplasmic cosolvent reaccumulation and cellular remodeling (Wood, 1999). During osmoadaptation to high external osmolarity, high intracellular concentrations of K^+-glutamate are accumulated by increased K^+ uptake and synthesis of glutamate. Further, osmoprotective compounds such as trehalose, proline glycine, betaine and proline betaine are accumulated by either synthesis or uptake from the environment (Wood, 2006). After this process, the turgor is restored and the cells can grow in high osmolarity environments. Enteropathogens face increased osmolarity in the human gut and several virulence factors have been proposed to be regulated by external osmolarity (Hoffmann *et al.*, 2013, Vail *et al.*, 2012, Soni *et al.*, 2011).

AIMS OF THE THESIS

General aim:

To study the epidemiology and molecular characteristics of ETEC strains in children with diarrhoea in Bolivia and the influence of environmental, host and transcription factors associated with the expression of ETEC enterotoxins.

Specific aims:

- To determine the prevalence, seasonal distribution and antibiotic susceptibility of ETEC and other diarrhoeagenic *E coli* i.e. EAEC, EPEC, EHEC and EIEC in children less than 5 years hospitalized with diarrhoea in Bolivia and the association of such infections with age, sex and severity of disease.
- To characterize ETEC strains isolated during a 4 year study period with regards to virulence factors, *i.e.* toxins, colonization factors and putative virulence genes.
- To study the role of different host environmental factors present in the gastrointestinal tract *i.e.* bile, glucose, osmolarity and pH, on the production and secretion of ETEC LT and ST enterotoxins.
- To investigate the role of the global transcriptional regulator CRP on the regulation of LT production and secretion under different pH and osmolarity conditions.

KEY METHODOLOGY

Sample collection

The procedure for collection of stool specimens from children aged under 5 years, hospitalized with acute diarrhoea in the hospitals enrolled in the diarrhoea surveillance study in Bolivia during 2007-2010, is outlined below (Figure 14).

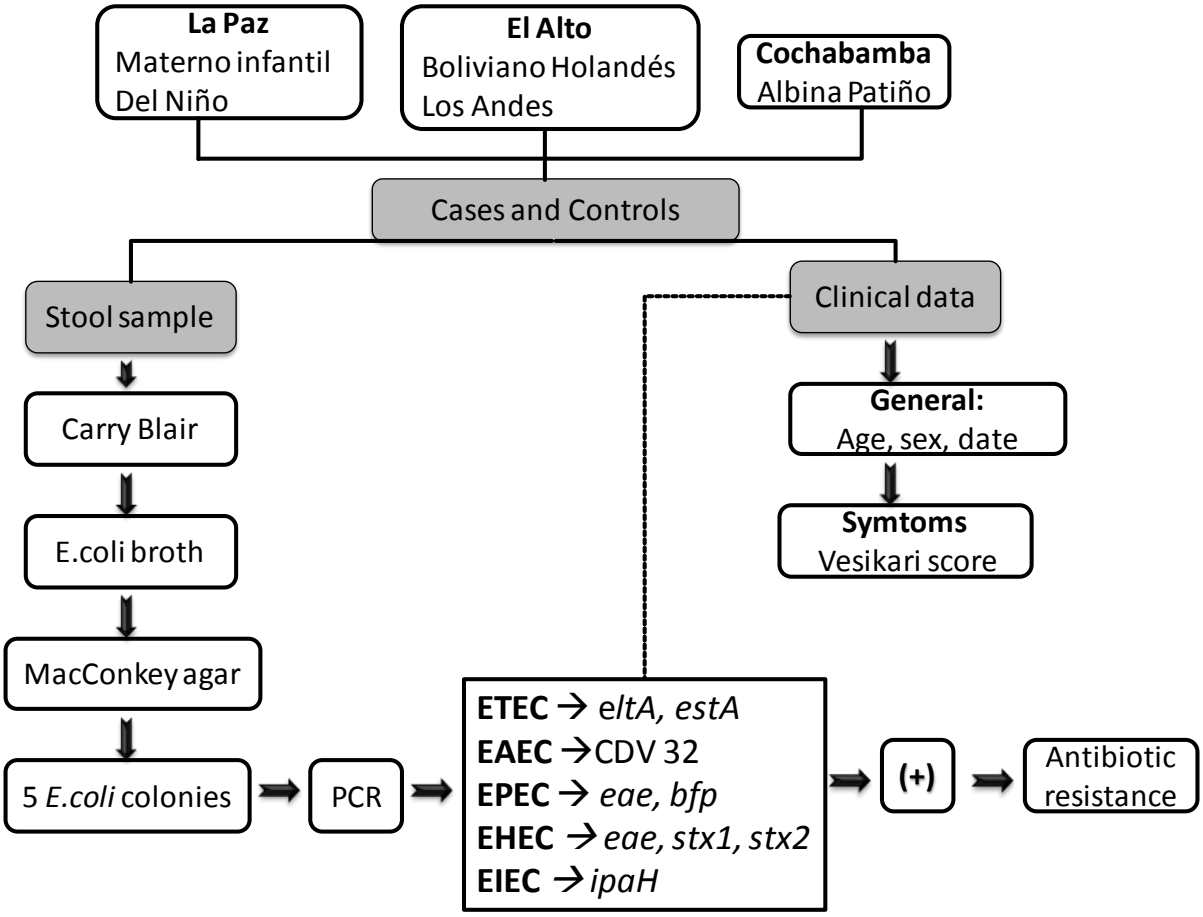

Figure 14. Collection and identification of DEC samples from children with diarrhoea in Bolivia. Stool samples from all children under 5 years of age hospitalized with acute diarrhoea and with fulfilled case criteria as well as from children who fulfilled the control criteria were collected at the 5 hospitals that were part of the surveillance program. Stool samples were collected in Carry blair media and transported to the lab where they were pre-grown in *E. coli* broth followed by culturing on MacConkey agar and subsequent isolation of lactose-fermenting bacteria resembling *E. coli*. For detection and isolation of DEC strains, DNA from 5 pooled colonies were tested by PCR for the different DEC categories (ETEC, EAEC, EPEC, EHEC and EIEC) using specific primers. For all positive cases PCR of single-colonies was performed to isolate the colony. Isolated colonies were stored at -70°C in LB supplemented with 20% glycerol. Antibiotic resistance was performed in all positive isolates. Simultaneously, general data as age, sex and date of sampling were collected. In addition, clinical information with the number of stool and vomits per day, days with diarrhoea, level of dehydration, metabolic acidosis, electrolytic disequilibrium and rehydration treatment were used to calculate the Vesikari score.

ETEC strains

The clinical ETEC strains used in this thesis were derived from diarrhoeal stool specimens collected during the DEC epidemiological study performed in Bolivia during 2007-2010 (paper II), from strains collected at the International Center for Diarrhoeal Disease Research (icddr,b) in Dhaka, Bangladesh (paper III and IV) or the laboratory clinical strain H10407 (paper III and IV). The details of the strains as well as the recombinant strains used in papers III and IV are listed below.

Table 1. Wild-type, mutant and recombinant strains included in Papers III and IV

Clinical strains					
Paper	**Strain**	**Origin**	**Toxin profile**	**Colonization factors**	**References**
III	p08	Bangladesh	LT/STh	CS5 CS6	*(Nicklasson et al., 2012)*
III	2527507	Bangladesh	LT/STh	CS5 CS6	*(Nicklasson et al., 2012)*
III	2533435	Bangladesh	LT/STh	CS5 CS6	*(Nicklasson et al., 2012)*
III	2545618	Bangladesh	LT/STh	CS5 CS6	*(Nicklasson et al., 2012)*
III, IV	E2863	Bangladesh	LT	CS6	*This study*
III, IV	H10407	Lab strain originally from Bangladesh	LT/STh/STp	CFA/I	*(Evans et al., 1977)*
Mutants and recombinant strains					
III, IV	2863ΔCRP		LT	CS6	*This study*
III, IV	H10407ΔCRP		LT/STh/STp	CFA/I	*This study*
IV	H10407ΔCRP rec		LT/STh/STp	CFA/I	*This study*

Culture media and conditions (Paper III and IV)

Bacterial strains were grown to exponential phase in LB media for 3 hours at 180 rpm, 37°C. At this point, bacterial culture densities were measured at OD_{600} (OD_{600} 0.8 ≡ 1 x 10^9 bacteria). For each experiment, the same amount of starting culture (10^7 bacteria per ml medium) was transferred into a different culture media with the respective growth requirements. LB (1% tryptone peptone, 0.5% yeast extract, 0.17 M NaCl), LBK (10 g Tryptone, 5 g yeast extract, 6.4 g KCl) and M9 defined minimal media (42 mM Na_2HPO_4, 22 mM KH_2PO_4, 8 mM NaCl, 19 mM NH_4Cl, 0.1 mM $CaCl_2$, 2 mM $MgSO_4$) were prepared according to standard methods. LB was supplemented with glucose 0.4 % w/v (III and IV); 171mM NaCl (III), 300 mM NaCl (III) and 600 mM sucrose (III); M9 was supplemented with glucose (0.2 % w/v), glycerol (0.2 % w/v) or casamino acids (1 % w/v) with or without addition of 0.15% bile salts (III) and LBK was adjusted to different pH using pH-appropriate sulfonate buffer at 100 mM. pH 5 was adjusted with 2-(N-morpholino) ethanesulfonic acid (MES), pH 7 with piperazine-N,N9-bis-(2-ethanesulfonic acid) (PIPES), and pH 9 with 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid (AMPSO) (IV). Microaerophilic conditions were performed by exposing the liquid culture to a gas mixture consisting of 10% CO2, 5% O2, and 85% N2 and seal the flask to avoid evaporation of the gas mixture (III). All culturing was performed at 37°C at 180 rpm.

Construction of the mutant and recombinant strains (Paper III and IV).

Mutagenesis of the *crp* gene was performed in the E2963 and H10407 strains. Mutagenesis of the ETEC strain E2863 (E2863ΔCRP*)* was performed by making an in-frame deletion that removed most of the codons, using a procedure described previously (Skorupski & Taylor, 1996, Vaitkevicius *et al.*, 2006, Valeru *et al.*, 2009), whereas *crp* deletion mutant of H10407 (H10407ΔCRP*)* was constructed by a modified form of *lambda red* recombination, as described previously (Datsenko & Wanner, 2000).

PHENOTYPIC METHODS

Antimicrobial drug susceptibility testing (Paper I)

Antimicrobial drug susceptibility was performed using the Disc diffusion method. Bacteria were grown in Mueller-Hinton agar and tested using antibiotic discs for cefotaxime (CTX), chloramphenicol (CHL), ampicillin (AMP), cefoxitin (FOX), amoxicillin-sulbactam (SAM), sulfamethoxazole-trimethoprim (STX), nalidixic acid (NAL), tetracycline (TET), and ciprofloxacin (CIP). Measurement of the zone diameters was performed and strains were characterized as susceptible, intermediately resistant or resistant according to NCCLS standards (NCCLS, 2002).

Detection of ETEC toxins by GM1 Elisa (Paper II)

ETEC strains that were positive for the toxin PCR detection in Bolivia were retested in the lab in Gothenburg for confirmation by ganglioside GM1 enzyme-linked immunoabsorbent assay (GM1-ELISA) as described before (Svennerholm & Wiklund, 1983, Svennerholm *et al.*, 1986).

Quantitative Elisa (Paper III and IV)

For the analysis of the production and secretion of LT and ST toxins, GM1 ELISA for the quantification of CTB/LTB was carried out in 3h, 5h and overnight cultures as previously described (Sjoling *et al.*, 2007). Cultures were centrifuged and LT and ST levels were estimated in the culture supernatant and the corresponding pellet resuspended in its respective media followed by sonic disruption to release bacteria-bound toxins. Determination of LT and ST concentration in the culture was determined using purified r-CTB (0.3µg/ml) and ST-ref 881108 (0.3nmol/ml), respectively. Regression analysis (R^2>0.98) was used to generate a standard curve used for determination of LT and ST concentrations in the tested samples. For the analysis total production was calculated by summing the LT concentrations in the pellet and supernatant, whereas the percentage of LT secretion was determined by the relation of LT concentration in the supernatant with respect to the total production of LT as is show in Figure 15.

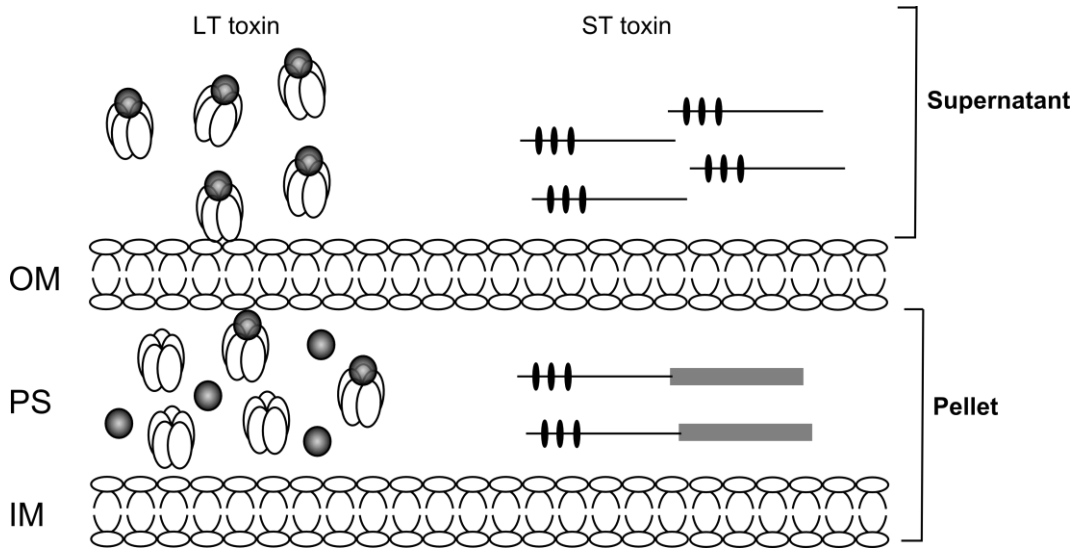

Figure 15. Schematic representation of total production and secretion of the LT and ST toxin. OM: outer membrane, PS: periplasmic space, IM: inner membrane.

Dot blot for the detection of ETEC colonization factors (Paper I and II)

All ETEC bacteria that were positive in the toxin identification were grown in colonization factor antigen (CFA) agar with and without bile salts at 37°C overnight and tested for the expression of CFA/I, CS1, CS2, CS3, CS4, CS5, CS6, CS7, CS8, CS12, CS14, CS17 and CS21 using specific monoclonal antibodies for the different CFs (Lopez-Vidal, 1997, Qadri *et al*., 2000a, Sjoling *et al*., 2007).

Detection of cAMP levels (Paper IV)

cAMP levels were measured in the pellet and supernatant of bacteria grown for 3h in LBK media only and adjusted to pH 5, 7 and 9 by the Direct cyclic AMP Enzyme-linked Immunosorbent Assay, ELISA (Enzo, life Sciences) according to the manufacturer's instructions.

Flow cytometry (Paper IV)

Membrane integrity of the bacteria was assessed using the Live-Dead BacLightTM (Molecular Probes Eugene, OR, USA) which consists of two nucleic acid stains, the green fluorescent SYTO 9 that is cell-permeable and can freely either live or dead bacteria; and the red fluorescent propidium iodide (PI) that can only enter membrane-comprised cells. Bacteria that underwent pH treatment were resuspended in sterile saline and stained with SYTO 9 and PI and incubated for 15 min in the dark at room temperature according to the manufacturer's instructions. Flow cytometric measurements were performed using a BD FACSCalibur 4-color dual laser flow cytometer. Data of live, compromised and dead bacteria were acquired and processed using Flow cell software (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

Infant mice model (Paper IV)

We measured the colonization potential of the wild-type ETEC strain (E2863) and the isogenic Δcrp-mutant either in competition experiments or side by side as described previously (Nygren *et al.*, 2009). The procedure followed is represented in the figure below (Figure 16).

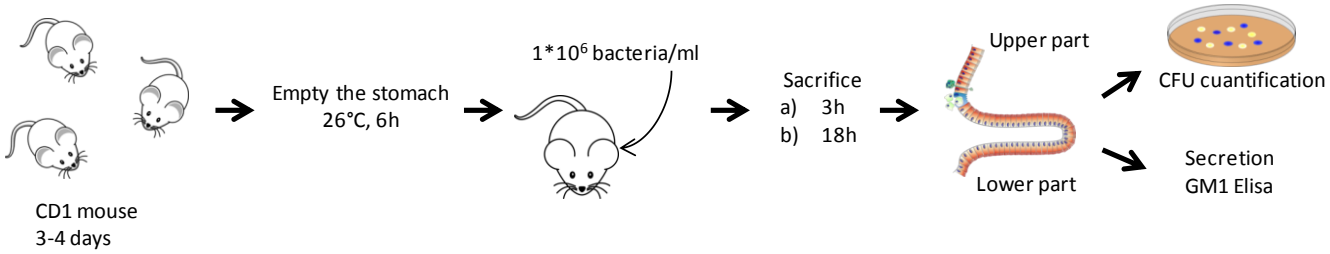

Figure 16. Schema of the infant mice methodology. Three to four day old CD1 mice (2.4-2.7 g) were removed from their mothers and kept at 26°C for approximately 6 hours prior to inoculation to allow emptying of stomach contents. Subsequently the pups were inoculated intragastrically with 10^6 bacteria/ml suspended in using a standard smooth-tipped hypodermic mouse feeding needle. Infected mice were kept on sterile tissue paper in plastic containers at 26°C. After 3 and 18 hours groups of mice were sacrificed. For colonization, potential experiments the entire small intestine excised and divided in two parts (upper and lower half) was collected and homogenized in 2 ml of PBS. Estimation of the pH the tissue was previously done. Suitable 10-fold serial dilutions of the resulting suspensions were plated onto blood plates for determination of CFUs. For secretion, detection the small intestinal tissue samples were collected directly into ice-cold PBS, cut open using surgical scissors, vortext to release the luminal content and centrifuged at 16000 G in a table top centrifuge to remove bacteria and debrie. Secreted LT toxin was measured by GM1-ELISA.

Membrane proteomic analysis (Thesis)

We used the LPI™ Hexalane FlowCell technique (Nanoxis Consulting AB, Sweden) to study the variation in the membrane proteins due to pH conditions or by regulation of CRP. The procedure of the technique is outlined below (Figure 17). The collected peptides were labeled using a tandem mass tag kit (TMT 6 plex kit, Pierce (Thermo Scientific) according to the protocol from the manufacturer and were analyzed by ESI-LC-MS/MS at the proteomics core facility at University of Gothenburg.

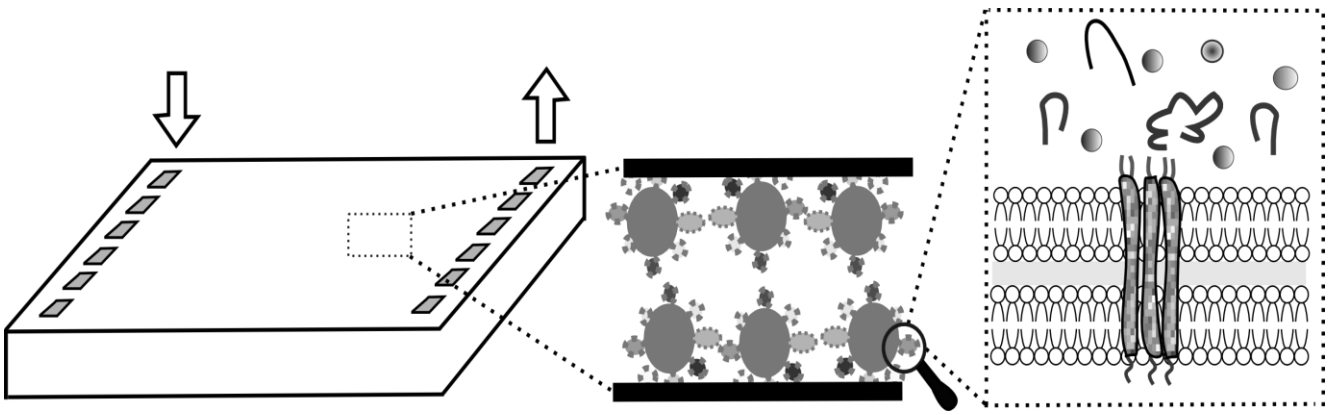

Figure 17. Schema of the Hexalane FlowCell technique. Bacteria were washed three times with PBS (4000g, 8 min) and resuspended 1 mL of PBS. The bacterial cell concentration was adjusted to an approximately OD_{600} of 0.8 (1×10^9 CFU/mL). An excess of bacteria was applied to the flow cell by adding 100 µL of the washed bacterial suspension to fill the LPI™ HexaLane FlowCell channel. Excess bacterial suspension was removed from the inlet and outlet ports. The immobilized bacteria were incubated for 2 h, at room temperature, to allow cell attachment, and the LPI™ HexaLane FlowCell channels were washed subsequently with 1.0 mL of Ambic (NH_4HCO_3, 20 mM, pH 8), using a syringe pump, with a flow rate of 50 µL/min. Enzymatic digestions of the bacteria were performed by injecting 100 µL of trypsin (20 µg/mL) into the LPI™ HexaLane FlowCell channels and incubating for 30 min at room temperature. The generated peptides were eluted by injecting 200 µl Ambic into the LPI™ HexaLane FlowCell channels at a flow rate of 50 µL/min and collected at the outlet ports, using a pipet.

GENOTYPIC METHODS

Quantification of the LT toxin by q-PCR (Paper IV)

Quantification of gene transcription of *eltA* was performed by real-time RT-PCR assays by absolute quantification using a PCR-product based standard curve. The figure below explains in detail the procedure of the technique (Figure 19). The standard curve was generated in ten-fold serial dilutions of a known amount of the *eltA* gene PCR product as described before (Lothigius *et al.*, 2008).

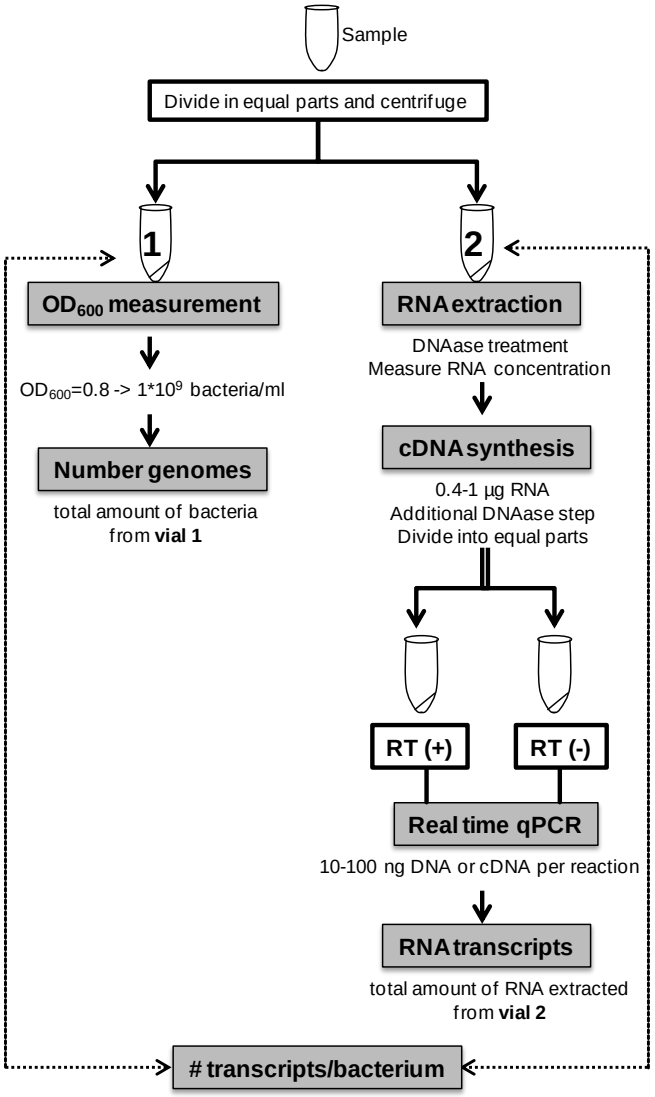

Figure 18. Schema of absolute quantification by gene expression analysis. A defined volume of a sample was divided into two equal parts for OD_{600} measurement and RNA extraction, respectively. Extracted RNA was DNase-treated twice and used for cDNA synthesis. cDNA synthesis was performed with the QuantiTect cDNA kit (Qiagen), with an additional DNAse step. To ensure complete removal of DNA a reverse transcriptase negative (-RT) control reaction was prepared in parallel with the cDNA synthesis. The pair-wise prepared cDNA samples and –RT controls, were analyzed by real-time PCR using SYBR® Green and 2 µl of the sample on an ABI 7500 (Applied Biosystems, Foster City, CA) employing the standard settings for absolute quantification. Absolute quantification was performed using a standard curve generated from the respective gene-specific PCR products. The absolute numbers in the original sample mass or volume were calculated based on the gene copy and transcript numbers in the 2 µl reaction, and considering the RNA extraction elution volumes and the volume used for cDNA synthesis. The level of transcription in each sample was obtained by dividing the number of transcripts in the original sample by the number of genomes and expressed as the number of transcripts per bacterium.

PCR for the detection of DEC categories (Paper I) and ETEC putative virulence genes (Paper II).

For the detection of EPEC (*eae* and *bfp*) and EHEC (*stx1* and *stx2*) a multiplex PCR was set up in our laboratory. Detection of ETEC toxins LT (*eltB*) and STh (*estA*) was performed by a multiplex PCR according to Sjöling *et al*., 2007 (Sjoling *et al.*, 2007). Detection of EAEC (*paa*) and EIEC (*ipaH*) was performed by single PCR. In addition, a multiplex PCR with two set was set up for the detection of ETEC putative virulence genes.

STATISTICS

In paper I and II, comparisons of frequencies were performed using Chi-square or Fisher's exact test using SPSS version 18 software. In paper III we used the paired Wilcoxon test and in paper IV Student´s t-test and non-parametrical non-paired Mann-Whitney test. Paper III and IV analysis were performed using the GraphPad Prism version 4.00 for Windows. In all analyses $p<0.05$ was considered significant.

RESULTS AND COMMENTS

This thesis was designed to study the epidemiology of DEC in Bolivia and to understand more specifically the role of different environmental host factors and the transcriptional regulator CRP in the regulation of ETEC toxins.

EPIDEMIOLOGICAL STUDIES

Prevalence of DEC in Bolivia

The first aim of this thesis was to determine the prevalence of diarrhoeagenic *E. coli*: ETEC, EAEC, EPEC, EHEC and EIEC in children <5 years of age in Bolivia. The studies were based on analysis of stool samples collected within the rotavirus surveillance program in Bolivia during 2007 to 2010. Samples were collected from two hospitals in La Paz: Materno Infantil and Del Niño, two hospitals in El Alto: Boliviano Holandes and Los Andes and the Albina Patiño Hospital in Cochabamba. In total 3943 stool samples from diarrhoea cases and 1026 from control children were analyzed. As many as 95% of all samples collected from children hospitalized with diarrhoea were analyzed. The collection of control samples was randomly performed due to difficulties in collecting samples from children matched in age with diarrhoea patients particularly from children <2 years during 2007-2010, which represented an important percentage of the case study population (Papers I and II).

In this study, all DEC categories were significantly more prevalent in children with diarrhoea than in children without diarrhoea ($p<0.001$) (Figure 20a). Among the five categories initially included in the study, we only tested EHEC and EIEC during the first year due to their very low prevalence (0.05% and 0.5%, respectively). We found that EAEC was the most prevalent DEC found in 11.8% of diarrhoea cases in children <5 years in Bolivia followed by ETEC (6.6%) and EPEC (5.2%) (Paper I).

We also analyzed differences of DEC in the prevalence between different cities in Bolivia to evaluate if there was a geographical difference on pathogen distribution. La Paz and Cochabamba display a striking variation in altitude and climate but usually share similar rain fall distribution. La Paz is a mountain city with a mean temperature of 13°C, whereas Cochabamba is a typical valley city with a mean temperature of 20°C. In despite of the differences on climate, we did not observe difference in the prevalence of ETEC and EAEC in the two cities, whereas EPEC was more prevalent in Cochabamba ($p<0.001$) (Figure 20b). When comparing geographical distribution, we considered La Paz and El Alto as one city. Thus, El Alto is a suburban city, almost merged with La Paz, where mainly low-income people live; because its proximity to La Paz, children seek medical attention in hospitals in La Paz (Paper I).

In general, there were no significant differences in prevalence of the different DECs, when comparing isolates from different hospitals, suggesting that the study population was homogenously distributed (Figure 20c). When comparing the prevalence of the different DEC categories during the different study years, we observed that in La Paz EAEC was the most prevalent category during all years whereas ETEC was the second most prevalent during 2007, 2009 and 2010 and EPEC during

2008 (Figure 20d). In Cochabamba, EAEC was the most prevalent followed by ETEC during all years (Figure 20e).

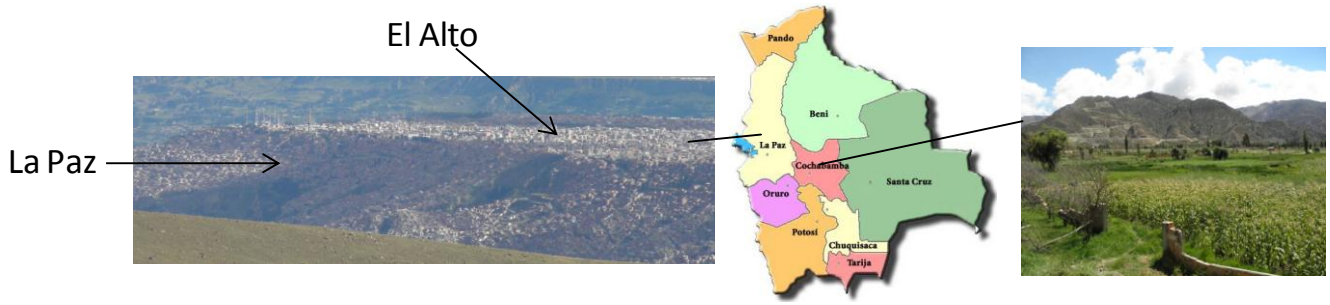

Figure 19. The geographical locations of the cities in Bolivia where the study was performed.

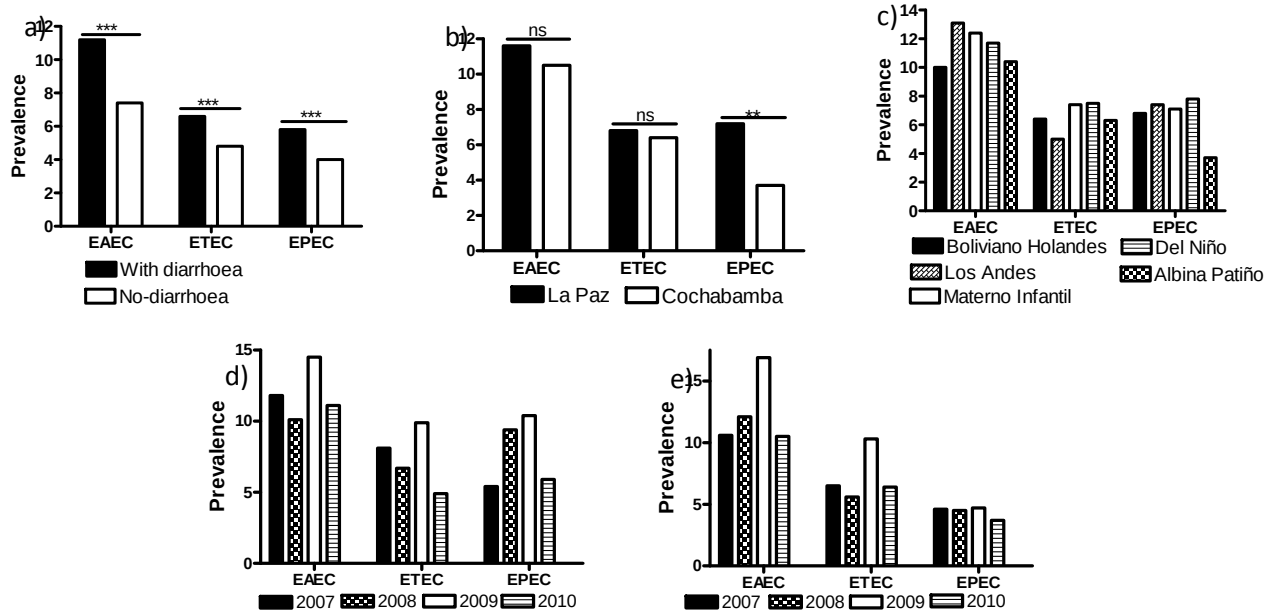

Figure 20. Prevalence of DEC categories in children <5 years of age during the study period (2007-2010) in Bolivia. a) Differences between children with diarrhoea and no-diarrhoea, b) differences between cities, c) differences between hospitals, d) year of distribution in La Paz, and e) year of distribution in Cochabamba.

This study was performed as a part of the rotavirus surveillance program in Bolivia but our study was focused on the prevalence of DEC pathogens. Rotavirus was identified in 36% of the children with diarrhoea during the whole study period (2007-2010), although this prevalence level is based on specimens collected between a pre-vaccine and post-vaccine period. Rotavirus vaccine was introduced in Bolivia in August 2008 as a national program in which all children were offered vaccination treatment for free. The prevalence of Rotavirus during 2007 was 48.6% but decreased during 2010 to 30%, reflecting a decrease in the prevalence due to the vaccine introduction. On the other hand, we observed a high prevalence of coinfections between rotavirus and DEC-categories; EAEC-rotavirus coinfection was the most prevalent accounting for 57.6%, followed by EPEC-rotavirus (26.1%) and ETEC-rotavirus (24.5%), whereas coinfections among different DEC categories were very low and found in only 1.9% of the children. Taken together, DEC and rotavirus

may account for 53% of all diarrhoea cases in Bolivia during 2007-2010. The remaining diarrhoea cases could be attributed to other pathogenic bacteria such as *Salmonella ssp.*, *Campylobacter spp.* and *Yersinia spp*., as well as different intestinal parasites and virus. Hence further studies are needed to determine a more complete aetiology of diarrhoea in Bolivia.

Prevalence of ETEC toxins

As a second aim of this thesis, we characterized all the ETEC isolates collected in the DEC study according to their toxin profile, to investigate if the distribution of the ETEC toxins has a special pattern in Bolivia compared to different parts of the world or in the region. Knowledge of the virulence factor distribution is important from a vaccine development point of view considering the virulence profile heterogeneity of ETEC strains. ETEC producing LT-only toxin were found in 40.5% of ETEC isolated from children with diarrhoea and in 56.4% in children without diarrhoea. STh-only was found in 39.5% of ETEC isolates from children with diarrhoea and in 27.3% of ETEC from children without diarrhoea; a combination of LT and STh was found in 20.1% in children with diarrhoea and 16.4% of children without diarrhoea. Our results suggest that STh-only strains are associated with diarrhoea, while LT-only strains are mainly found asymptomatic carriers; however LT-only strains are clearly able to cause disease. Variation in the prevalence of ETEC with different toxin profiles was found. Thus, LT-only was the most prevalent toxin during 2008 and 2009 and STh-only during 2007 and 2010. Similar strain fluctuations was observed worldwide, for instance STh-only strains were reported to be more prevalent in studies Bangladesh during 1996-1998 and 2002-2004 (Qadri *et al.*, 2000a, Qadri *et al.*, 2007), whereas LT-only strains were equally prevalent to STh-only strains during the flood on 2007 (Harris *et al.*, 2008). In Bolivia LT-only strains were more prevalent during 2002-2006 (Rodas *et al.*, 2011). In this study we did not determine the presence of ETEC STp prevalence due to an initial lack of primers and reference strains in Bolivia. Since STp-only strains are common and often occur in combination with the common colonization factors CS6 or CS14 we believe that the prevalence of ETEC reported in this study might have been underestimated.

Prevalence of Colonization Factors

So far, 25 different colonization factors have been described in ETEC, but in general CFA-I and CS1-CS6 have been shown to be the most prevalent ones (Qadri *et al.*, 2005). In reports from different countries, there is a wide variation in the reported prevalence of ETEC isolates that express CFs or express a described CFs (Gaastra & Svennerholm, 1996, Wolf, 1997). In our study 60% of the ETEC strains had an identified CF profile compared to 40% in the controls ($p<0.040$), suggesting that the presence of CFs could be related to development of disease. This percentage of identified CFs is comparable with other studies (Qadri *et al*., 2007, Rivera *et al.*, 2010, Giron *et al.*, 1995, Isidean *et al.*, 2011).

The most common colonization factors were CFA-I, CS1+CS3, CS14, CS5+CS6, CS17, CS21, CS6, CS7, CS13, CS12, CS2+CS3 and CS4+CS6 (Figure 21a), indicating that the CF profile of strains in Bolivia do not differ from other countries. The prevalence of CS21 (longus) alone was 4.7%, however CS21 was present in 27.4% of the isolates either alone or associated with another CF as distinguished by multiplex PCR. High prevalence of CS21 has also been reported in Brazil (33%)

(Nishimura *et al.*, 2002), Bangladesh (29%) (Qadri *et al.*, 2000b) and Argentina (20.7%) (Pichel *et al.*, 2002). Since the *lng*-operon encoding CS21 is highly variable, the phenotypic expression of CS21 was found to be lower than the genotypic identification (20%), this has also been commonly reported worldwide (Pichel *et al.*, 2002, Qadri *et al.*, 2000b, Rodas *et al.*, 2009). Since CS21 is usually associated with other CFs, it is unclear if it has a role in colonization. However, CS21 has been proposed to play a role in biofilm formation and autoaggregation, suggesting that is has a similar role as other type IV colonization factors found in pathogenic *E. coli* (Kolappan *et al.*, 2012, Mazariego-Espinosa *et al.*, 2010).

We observed that the distribution of CFs fluctuated over time (Figure 21b); CS14 was the most common CF on ETEC strains in 2007, whereas CFA/I+CS21 were the most prevalent CFs on ETEC during all other years. The prevalence of CS1+CS3+CS21 strains increased significantly during 2010. CS14 ETEC have also been isolated in high frequencies from diarrhoea patients in previous studies in Central America and Egypt (Paniagua *et al.*, 1997, Peruski *et al.*, 1999, Shaheen *et al.*, 2009) and CS14 seems to be an emerging CF circulating worldwide. Comparison of the CF distribution on ETEC in Bolivia showed that even if CFA/I and CS1-CS6 are the most common CFs, there is a variation in their prevalence. Furthermore, other not as common CFs were observed in increased prevalence during specific time periods such as our finding that CS14 emerged in 2007, and CS17, that was common in Bolivia during 2002-2006 (Rodas *et al.*, 2011) or CS12 (2006-2007) in Peru (Rivera *et al.*, 2010)

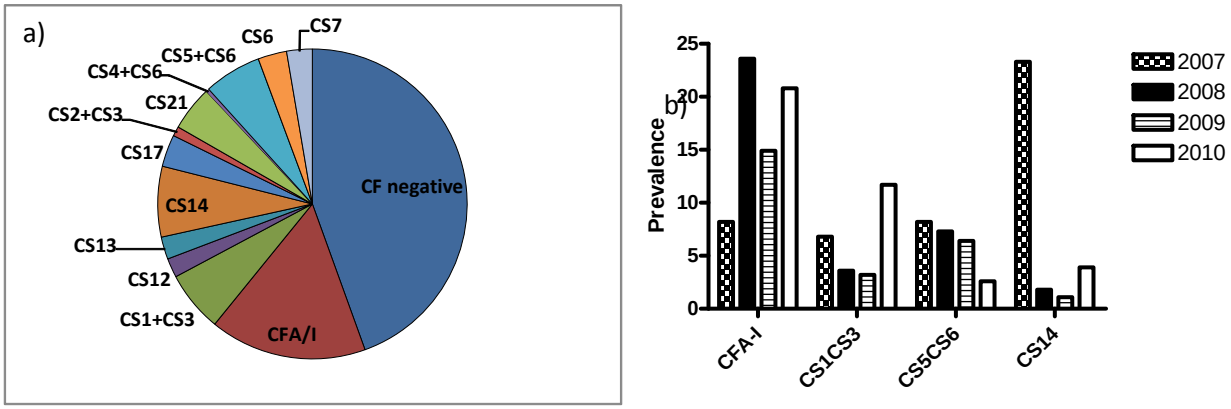

Figure 21. Distribution of the colonization factors in children with diarrhoea in Bolivia during 2007-2010. a) During the whole study period, b) distribution of colonization factors during the years of the study.

Association between enterotoxins and Colonization factors

Colonization factors are generally associated with a toxin profile. In our study we analyzed the correlation between identified colonization factors and toxin profile. We observed that the highest percentage of CF-negative isolates (68.6%) was found among LT-only ETEC. Similar results have been observed in different areas i.e. Bangladesh, Argentina, Nicaragua, Brazil, Egypt and Indonesia (Bueris *et al.*, 2007, Nunes *et al.*, 2011, Oyofo *et al.*, 2002, Qadri *et al.*, 2007, Viboud *et al.*, 1999, Vilchez *et al.*, 2009, Wierzba *et al.*, 2006). The most prevalent colonization factors CFA/I and CS14 were mainly present on STh-only ETEC strains, while CS1+CS3 and CS5+CS6 were present on LT/STh ETEC from children with diarrhoea. Hence the toxin-CF profiles in Bolivian ETEC strains are similar to other ETEC isolates worldwide.

Prevalence of putative virulence genes

We also tested the ETEC strains isolated in this study for six different putative virulence genes (*clyA, eatA, leoA, tia, tibC* and *East-1*). Among those genes, *clyA* was the most common and was present in 92.6% of the ETEC isolates. Other studies have shown that Cytolysin A (ClyA), a pore-forming hemolytic protein is commonly present in non-pathogenic *E. coli* and has also been found in other DEC categories (Ludwig *et al*., 2004), questioning its direct role for ETEC virulence. The rest of the other putative virulence genes were isolated in a lower prevalence in the ETEC isolates. We propose that these putative virulence genes do not have a main role in the virulence of ETEC, but might be involved in the ETEC virulence process either in adhesion and invasion of the bacteria such as *tia*, *tibA* and *eatA* or helping in the mechanism of the LT toxin with *leoA* or STh toxin with *East-1*.

We also analyzed if there was a correlation of the suggested virulence genes with a toxin or colonization factor profile. We found a significant association of *east-1* and *tia* with LT/STh ETEC strains and of *eatA* with STh-only ETEC strains suggesting that the genes that codified for these putative virulence factors could be present on the sample plasmids and/or chromosomal backgrounds. No correlation was observed with colonization factors.

Age and sex distribution

When comparing the sex distribution of children infected with the different DEC categories, there was no significant difference between girls and boys; however there were more DEC cases in boys, around 60%, compared to 40% in girls. A possible explanation for this tendency could be that due to cultural reasons boys are more often brought to hospital when they are sick.

Analysis of the age of all children with DEC diarrhoeas mainly occurred in children less than 2 years of age. DEC infections were most prevalent in children between 6 and 17 months, whit the highest peak of ETEC in children between 12 and 17 months. We observed that the peak of ETEC infections occurred in older children than EAEC and EPEC infections which peaked in children between 6 and 12 months (Figure 22a) as it has also been shown in previous studies (Ochoa *et al.*, 2009, Qadri *et al.*, 2000a, Ratchtrachenchai *et al.*, 2004, Spano *et al.*, 2008). We also observed that rotavirus infections were present in younger children (mean age 10.2 months), compared to DEC infections (mean age 14 months). Accumulative percentage analysis showed that 73% of diarrhoea cases caused by rotavirus occur in children up to 12 months and 97% in children up to 24 months while 57% of the DEC cases occur before 12 months and 88% before 24 months.

In general a low frequency of diarrhoeas was observed during the first 6 months of age, that could be explained by the protection of maternal antibodies against bacteria (Stoll *et al.*, 1986); lower frequencies of infection in children older than 2 years may be related to protective immunity induced by previous DEC infections (Pathela *et al.*, 2006).

After analyzing the age distribution of ETEC with respect to other diarrhoea pathogens, we analyzed the toxin distribution among the ETEC strains in the study population. No significant difference on the age distribution was observed but certain patterns seemed to be present. LT-only ETEC isolates were equally distributed in the younger (<2 years) and the older children (2-5 years) (40.8% and 40.7%, respectively), however, STh-only ETEC were more common in children <2 years than in

children 2 to 5 years (40.4% and 25.9%, respectively) and LT/STh ETEC strains were more frequent in the older than the younger children (33.3% and 18.9%, respectively) (Paper II). We also analyzed the main ETEC peaks to see if there was a variation in the toxin distribution of ETEC. We observed that in children from 12-17 months LT-only ETEC infections were more common, in comparison to ETEC strains isolated from children between 18-23 months old where STh-only ETEC strains were more prevalent (Figure 22b).

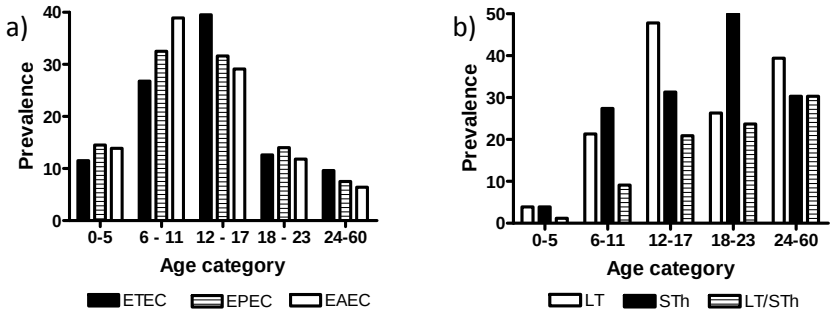

Figure 22. Age distribution of a) Different DEC categories, b) different toxin profile of ETEC isolated different age groups of children.

Severity of the disease

Considering that all DEC categories may cause severe diarrhoea, we were interested to study if there was a difference in the severity of disease caused by different DEC categories and between ETEC with different virulence profiles. For this purpose, we used a modified Vesikari score with a maximum possible score of 20 to assess the severity of disease in children with single infections, in the absence of other diarrhoeal pathogens (EAEC, EPEC, EIEC, EHEC, ETEC and rotavirus) and from those who had complete clinical information. In the calculation of the score we included duration of diarrhoea, maximum frequency of stools per day, maximum frequency of vomits per day, dehydration, treatment by oral or intravenous rehydration, presence of metabolic acidosis and electrolytic disequilibrium. Our scores were defined as mild (0 to 8 points), moderate (9 to 14 points), and severe (15 to 20 points) (Paper I and II).

The Vesikari scores for the DEC categories were fairly similar: 11.65 for ETEC, 10.80 for EAEC and 10.72 for EPEC-infected children. We observed a difference in severe cases between children infected with ETEC (15.7%) and children infected with EAEC (11.8%; p=0.005), suggesting that diarrhoeas caused by ETEC may be more severe. We also compared the severity of ETEC diarrhoea cases according to their toxin profile, but the scores were also similar: 11.02 for LT-only isolates, 12.3 for STh-only isolates, and 11.8 for LT/STh ETEC isolates. Finally, we compared all DEC cases with non-DEC (children who had diarrhoea due to an agent other than DEC). Diarrhoeas caused by non-DEC (12) were significantly more severe than those caused by DEC (11; p=0.017). (Paper I). Additional analysis of the Vesikari score between diarrhoeas caused by rotavirus reported a score of 14 for rotavirus. Comparison of the Vesikari score for rotavirus and DEC cases showed that 60% of the diarrhoea cases caused by DEC were moderate compared to 49.8% for rotavirus. Severe diarrhoeas caused by rotavirus accounted to 49.4% compared to 38% of diarrhoeas caused by DEC (data not shown), suggesting that diarrhoea caused by rotavirus may be more severe than cases caused by DEC.

Analysis of seasonality

In Bolivia there are two seasons, the cold-dry season from April to September and the warm-rainy season from October to March. Studies have shown that the seasonal peak of viral infections occurs during the cold-dry months (Parashar *et al.*, 1998), whereas the probability of bacteria causing diarrhoea is higher during the warm-rainy season (Elliott & Dalby-Payne, 2004, Grassly & Fraser, 2006, Pascual & Dobson, 2005). The peak of hospitalization due to diarrhoea in Bolivia was present in May and June. During this period, higher numbers of DEC and rotavirus infections were observed as well as higher prevalence of coinfections with DEC-rotavirus. In contrast, infection rates of ETEC and EPEC were significantly higher during the warm-rainy season compared to the cold-dry season, while EAEC did not differ between seasons (Figure 23a). Previous studies in our laboratory in Bolivia have reported that EAEC together with rotavirus has a main peak during the dry-cold season in May, and a second bigger peak during the warm-rainy season (unpublished data).

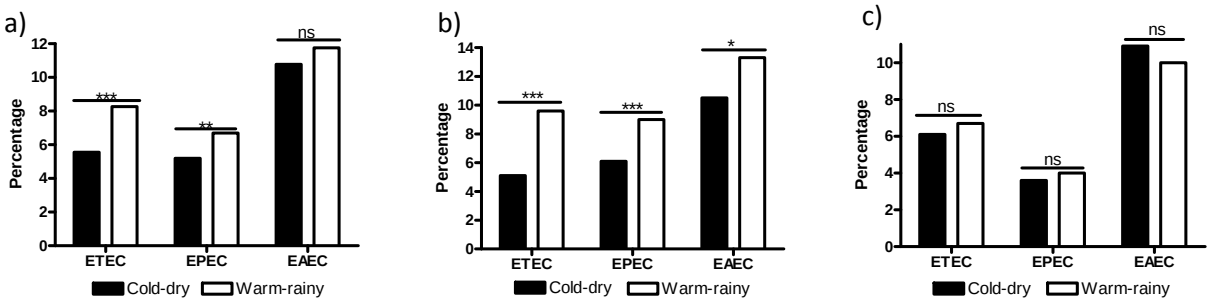

Figure 23. Differences in the prevalence of DEC categories between cold-dry (April-September) and warm-rainy (October to March) in a) the whole population, b) in La Paz and c) in Cochabamba.

We did not find any differences in the seasonal distribution of DEC categories between cities, besides the geographic differences between La Paz and Cochabamba, but we observed a significant association of ETEC, EPEC and EAEC with the warm-rainy season in La Paz (Figure 23b), which was not present in Cochabamba (Figure 23c). It is thus possible that other factors than temperature and precipitation may affect the distribution of the DEC seasonality in Cochabamba.

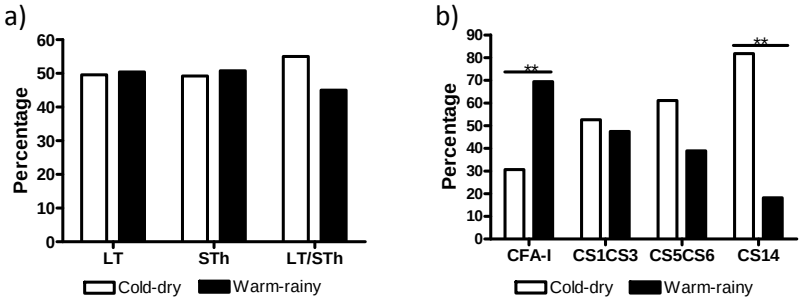

Figure 24. Prevalence of ETEC toxin profile and colonization factors during the cold-dry (April-September) and warm-rainy season (October to March). a) toxins, b) colonization factors.

Considering that ETEC is more prevalent during the warm-rainy season, we investigated if there was a seasonal distribution of both toxins and colonization factors. The distribution of the toxin profiles of ETEC strains was rather similar between seasons (Figure 24a), with the exception that LT/ST ETEC strains that were most common in April-May. Among colonization factors, CS14 ETEC isolates were associated with the cold-dry season (81.8%; $p<0.05$) compared to the warm-rainy

season (18.2%), and CFA/I ETEC isolates were highly associated with the warm-rainy season (69.4%; $p<0.05$) than with the cold-dry season (30.6%) (Figure 24b).

Antibiotic resistance

We also analyzed the susceptibility of antibiotic in all DEC isolates from children with and without diarrhoea for 9 different antibiotics: chloramphenicol, amoxicillin sulbactam, nalidixic acid, ampicillin, trimethoprim-sulfamethoxazole, tetracycline, cefoxitin, cefotaxime and ciprofloxacin. The study revealed high rates of antibiotic resistance among the different DEC categories, in particular resistance to AMP, TET, and STX. We observed that trimethoprim-sulfamethoxazole and tetracycline resistance were found significantly more often in strains from children with diarrhoea than from children without diarrhoea ($p<0.005$); 93% were resistant to at least one antibiotic. The DEC strains were commonly resistant to AMP (93.8%), STX (74.8%), TET (55.5%), and SAM (41.4%). Low levels of resistance were observed against CHL (16.5%), NAL (15.3%), CTX (2.7%), FOX (1.6%) and CIP (0.8%). Multidrug resistance, defined as resistance to three or more antibiotics, was present in 62.2% of the strains, probably due to inappropriate consumption of these drugs.

DISCUSSION EPIDEMIOLOGICAL STUDIES

The epidemiological studies in this thesis constitute the first comprehensive analysis of the prevalence, seasonality, antibiotic resistance, and severity of diseases caused by diarrhoeagenic *Escherichia coli* (DEC) in Bolivia, as well as the molecular characterization of ETEC strains isolated during a long study period. In Bolivia, diarrhoeas are estimated to cause approximately 13.000 to 15.000 of deaths every year, being the second cause of mortality in children under 5 years of age (WHO, 2008). Therefore, a better understanding of which enteric pathogens are responsible for the burden of diarrhoea morbidity and mortality are required. This information will be important for the planning of treatment and use of vaccines.

Enteric infections are restricted to geographic and seasonal patterns related to the degree of sanitation and clean drinking water (Boschi-Pinto *et al.*, 2008). In order to be able to estimate the burden of diarrhoeal disease in Bolivia in the most susceptible population, our study was conducted in a hospital-based rotavirus surveillance program which included 5 different hospitals in 2 geographically different cities during a 4 year period (2007-2010). Children seeking care at these hospitals were covered under the Universal Mother and Child Insurance System (SUMI), which provides free primary maternal and child care in Bolivia. Most of these families come from low socio-economic levels from suburbs with high growth rates with deficient infrastructure in water source service and insufficient sewage disposal systems.

Our findings of a high prevalence of DEC in stool specimens from children without diarrhoea may be attributed to the characteristics of the pathogens as well as host and environmental factors. Bacteria may persist in the host for longer periods after causing disease and strains that are circulating in human populations may be heterogeneous, with some strains being more prone to cause disease. Development of disease may also depend on host factor susceptibility for different strains and on the amount of inoculums needed to cause the disease (DeBoer *et al.*, 2012). In addition, the development of immunity to previous infections probably contributed to lower infection prevalence of symptomatic DEC in older control children. It would have been interesting to determine the bacterial load in children with and without diarrhoea to determine if a certain threshold is found in sick children suffering from DEC diarrhoea. However, in our study detection of the pathogens was done by ordinary PCR, so it was not possible to measure differences in bacterial load in fecal specimens between children with diarrhoea and without diarrhoea.

Considering that rotavirus is the most common diarrhoea pathogen in Bolivia, we would have expected a change on the prevalence with respect to DEC after the introduction of rotavirus vaccination in Bolivia on 2008. Despite the decrease in the rotavirus prevalence from 46 to 30% during the study period, rotavirus was still more prevalent than DEC and caused more severe diarrhoea cases. However, we show that there is a seasonal distribution difference between rotavirus infections and DEC, where DEC is significantly associated with the warm-rainy season whereas viral infection was more frequent during the cold-dry season. Interestingly, we did not find significant difference in the prevalence of DEC infections between the two cities of La Paz and Cochabamba with very different climates, suggesting that DEC pathogens are equally distributed in different geographic regions in Bolivia and other parameters than climate are associated with the prevalence.

We reported a relatively low ETEC prevalence (7.6%), compared to previous studies in the region (Torres *et al.*, 2001, Viboud *et al*., 1999, Vilchez *et al*., 2009). It has been previously shown that there is a difference in the prevalence of ETEC and rotavirus between hospital-base and community level study designs; ETEC prevalence was found to be higher in a community level study compared to a hospital-based study, while rotavirus showed the opposite behavior (Boschi-Pinto et al., 2008). However, a similar prevalence of ETEC infections (9%) as in our study has previously been reported in Bolivia (Rodas *et al*., 2011) and Peru (5.3%) (Rivera *et al*., 2010) in hospital-based studies. It is also important to consider that an underestimation of ETEC prevalence might have occurred in the study since we did not analyzed ETEC strains producing STp. Further studies of the prevalence of diarrhoea pathogens at the community level in Bolivia, and the inclusion of analysis for STp are needed to have a more exact estimation of the prevalence of ETEC in Bolivia. However, our studies provide important information about ETEC molecular characterization and epidemiology, showing that the virulence profiles found in the Bolivian population are similar to the ones found in the region and in other parts in the world.

An important part of our study was the analysis of the severity of the disease between DEC categories and among ETEC virulence profiles. Although the most important parameter to measure severity of diarrhoea is dehydration; we used a modified Vesikary score which employs additional parameters to estimate severity of disease. It has been reported that mixed infections, which might represent a high percentage of diarrhoeal episodes are not necessarily more severe compared with infections caused by a sole pathogen (Petri *et al.*, 2008). However, in our study we only included diarrhoea cases caused by a sole pathogen searched for. We were not able to show significant differences in the severity of disease between DEC pathogens but we could showed that severe cases (Vesikari score >17) caused by ETEC were more common than severe cases caused by EPEC and EAEC.

In previous studies LT-only ETEC strains have been shown to cause less severe diarrhoeal illness compared to ST-only and LT/ST ETEC strains. This has partly been due to the high percentage of lack of defined colonization factors in the LT-only strains, suggesting that CFs may play an important role in the pathogenesis of ETEC diarrhoea (Evans *et al.*, 1978, Lumish *et al.*, 1980, Qadri *et al*., 2005). In our study LT-only ETEC strains were more common in children without diarrhoea and were more often CF negative than STh and LT/STh strains; however, we were not able to show any significant differences in the severity of the disease that could be directly attributed to a certain virulence profile among ETEC isolates. Hence our results suggest that in Bolivia LT-only strains are as virulent as LT/STh and STh-only strains.

Diarrhoea is a self-limited disease that often does not require antibiotic for treatment. The recommended treatment for diarrhoeas is the use of oral rehydration solutions (O'Ryan *et al.*, 2005). However, we observed a high rate of antibiotic resistance in our DEC isolates especially to trimethoprim-sulfamethoxazole, which is a first choice of treatment of persistent diarrhoeas in Bolivia. We found a significantly higher resistance in strains isolated from children with diarrhoea compared to children without diarrhoea. Increased resistance of enteric bacterial pathogens can be attributed to an inappropriate and excessive use of antibiotics due to the easy access. Additionally, it has been shown that recent use of antibiotics during the previous 4 weeks is a documented factor for

development of infection or colonization with resistant bacteria (DeBoer *et al*., 2012). It has been acknowledged worldwide that there is a need to restrict the use of antibiotics in order to constrain the emerging antibiotic resistance that develops in bacterial pathogens. However easy access and cultural habits probably would even increase use of antibiotics in the coming years unless limited access and stringent prescription is reinforced in countries with unlimited access.

In conclusion, our findings may be relevant for future approaches to reduce the burden of diarrhoeal disease in Bolivia, and contribute information for future vaccine development considering that ETEC and rotavirus are among the pathogens that WHO has given priority for vaccine development, because of the contribution of these infections to most pediatric mortality and severe morbidity in developing countries.

MOLECULAR STUDIES

In the second part of the thesis we studied the role of different host environmental factors i.e. glucose, bile, osmolarity and pH in the production and secretion of the ETEC toxins, as well as the role of the global transcription factor CRP on the regulation of the toxins under certain growth conditions.

Effect of the host environmental factors on the regulation of ETEC toxins.

The first aim on this second part of the thesis was to study the impact of host environment on the expression of ETEC toxins LT and STh. Our experiments were set up to study both the total toxin production and the secretion. We defined the production as the total amount of toxin present in both the periplasm and in the external milieu. Secretion was defined as the percent of toxin present in the external milieu compared to total production. In the case of the STh toxin, we were not able to determine STh total production because the monoclonal antibodies we used for the detection did not recognize the prepeptide forms of STh. Therefore, we were only able to detect the active peptide that is secreted from the bacteria and it is detected in the supernatant. The general experimental setup and the growth conditions tested during the thesis are outlined in Figure 25.

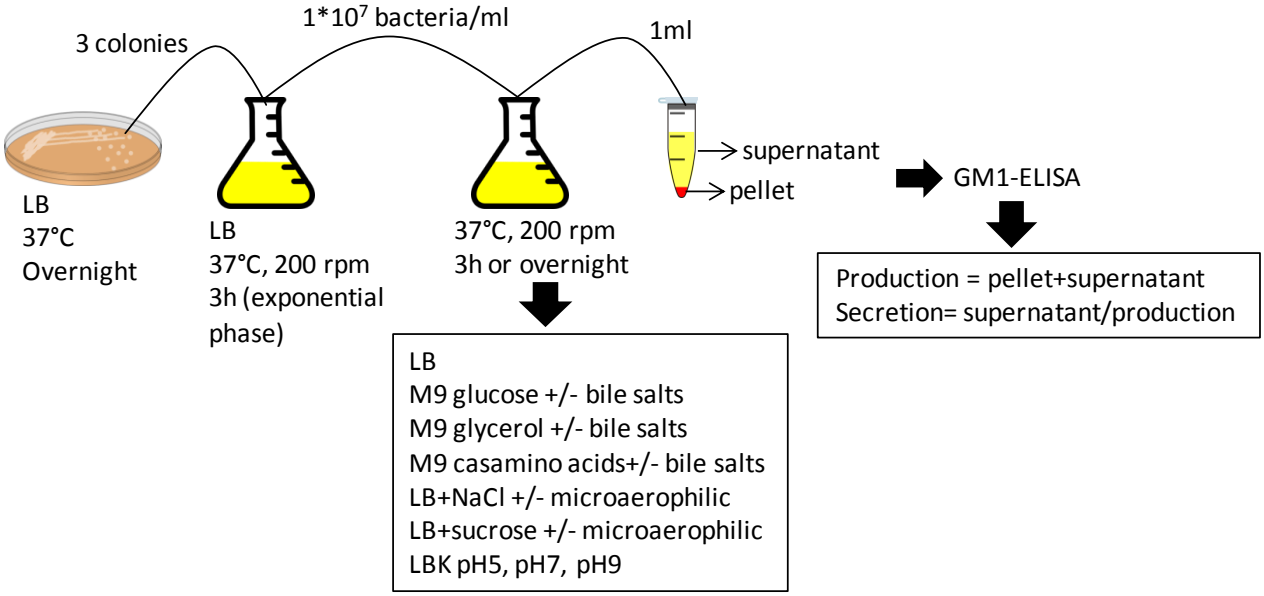

Figure 25. Experimental setup of *in vitro* growth conditions for the analysis of host environmental factors. The strains were first cultivated in LB plates overnight. Three colonies were picked and cultured in 20 ml of LB media for 3 hours until they reached exponential phase. The bacteria were transferred to 50 ml of the culture media to be tested at an initial concentration of 10^7 bacteria/ml and cultured for 3 hours or overnight depending on the growth conditions. 1 ml of the culture was collected and centrifuged at 13.000 rpm for 5 min, the supernatant was collected in a new tube and the pellet was resuspended in PBS and sonicated. Both pellet and supernatant were analyzed by GM1-Elisa for LT and GM1-inhibition Elisa for ST toxin quantification. Total production and secretion were adjusted to ng/10^9 bacteria. Microaerophilic conditions were assessed by exposing the liquid culture to a gas mixture consisting of 10% CO2, 5% O2, and 85% N2 and the flask was then immediately sealed to avoid evaporation of the gas mixture.

Expression of LT after *in vitro* culture

We studied the effect of various carbon sources using glucose, glycerol and casamino acids on the production and secretion of LT toxin. We tested four different LT/STh strains isolated from diarrhoea cases in Bangladesh. In the experiment, we used minimal media (M9) supplemented with glucose, glycerol or casamino acids. Secretion of the LT toxin was completely inhibited under these conditions. However, LT production was favored in the presence of glucose compared to casamino acids but did not vary with respect to glycerol. We also analyzed all these conditions in the presence of bile salts. Bile salts are part of the antimicrobial host defense system in the gut, acting through lipid emulsification and solubilization and membrane disruption but they can also be a signal for pathogens that have reached the small intestine. We observed that the presence of bile in combination with glycerol increased LT production. However we were not able to establish a direct role of the bile salts on the regulation of LT toxin (Paper III).

In order to try to determine whether inhibition of LT secretion was due to the carbon-source conditions or by the M9 media, we tested the secretion of LT toxin using LB-only and M9 media supplemented with glucose and glycerol in the laboratory strain H10407. We observed that LT secretion was favored in LB-only media but completely abolished in M9 media demonstrating that inhibition of LT secretion was due to the M9 media conditions. Thus, we conclude that ETEC bacteria are favored by the presence of glucose as a main carbon source for the production of LT and needs a complex medium in order to secrete LT (Paper III).

In the intestine ETEC is believe to encounter osmotic stress as well a decreasing oxygen levels which might have a function in the regulation of the toxins. A previous study has reported that the presence of NaCl and sucrose combined with microaerophilic conditions increased the expression of LT toxin (Trachman & Yasmin, 2004). Opposite to this previous findings, in our study the production and secretion of the LT toxin was not affected neither by the addition of the osmolytes NaCl nor sucrose and no difference between aerobic and microaerophilic conditions was observed. The only significant variation we observed during this experiment was decreased levels of LT toxin when bacteria were grown in LB-only media with microaerophilic conditions compared to aerobic conditions. Hence, our study suggests that high osmotic stress might have a minor effect on LT regulation and that the osmolarity present in the LB media is optimal for the secretion of LT toxin (Paper III).

We also considered the effect of pH of the toxin expression taken into account that ETEC is exposed to a wide range of pH when it passes through the stomach to the duodenum to reach the site of infection. The E2863 LT-only ETEC strain was grown in LBK media buffered to pH 5, 7 and 9. Total LT production and secretion were pH dependent and increased with the pH and reached its highest values at pH 9. However, secretion was inhibited at pH 5 (Paper IV). Extrapolating these results to the situation in the human gastrointestinal tract, ETEC will start to express LT when the temperature reaches 37 C but the bacteria will adapt to acid stress when they pass the stomach and goes through acid adaptation resulting in absence of secretion of LT toxin although the toxin is expressed and present in the periplasm. When bacteria reaches further down in the gut and is exposed to alkaline environments, the production and secretion of the LT toxin will increase reaching their highest levels. In general ETEC are considered to be inefficient secretors of LT and to vary from

strain to strain (Hirst *et al.*, 1984, Lasaro *et al.*, 2006). In our study we were able to show that alkaline pH induces secretion of LT toxin in much higher levels than the ones previously reported in strains grown in normal culture conditions.

Expression of STh after *in vitro* culture

Considering that both ETEC toxins are exposed to the same host factor conditions when they pass through the gastrointestinal tract, we also analyzed the effect of carbon source on the secretion of the STh toxin. Contrary to our findings on LT toxin, we were able to establish that the preferred carbon source for STh secretion was casamino acids. The positive effect of casamino acids on the secretion of STh was additionally increased in the presence of bile salts. Casamino acids have been previously reported to increase the production of porcine heat stable toxin STb (Erume *et al.*, 2010), in this study we showed similar effect of casamino acids in the human STh toxin (Paper III).

Secretion of STh toxin has previously been reported to be down-regulated by catabolite repression when glucose is added to the media or used as a sole carbon source (Alderete & Robertson, 1977, Bodero & Munson, 2009, Martinez-Cadena *et al.*, 1981). We found that STh secretion was significantly inhibited when glucose was used as a carbon source compared to glycerol and casamino acids. Our results corroborated previous findings where addition of glucose to the media decreased the expression of STb toxin in comparison to glycerol which did not affect STb secretion (Busque *et al.*, 1995) suggesting that both STh and STb toxins are regulated by CRP and supporting that similar host factor conditions are needed for the secretion of STh as well as STb toxins although they cause disease in different hosts.

Osmolarity condition experiments to quantify STh secretion revealed a new important finding where the presence of sucrose significantly increased the secretion of STh toxin. This condition was even higher in the presence of microaerophilic conditions. In contrast, increasing concentrations of NaCl did not have any effect on STh toxin secretion. High osmolarity conditions have shown to induce the production of HrdC protein, a TolC homologous protein involved in ST secretion (Tokunaga *et al.*, 2004). We propose that higher osmolarity conditions caused by sucrose might have an effect on the TolC function favoring the secretion of STh. In the table below we summarize our findings on the effect of host factor conditions on the production and secretion of the ETEC toxins.

Table 2. Summary of the effect of the different growth conditions tested in our in vitro studies on the production and secretion of LT and STh toxin.

Condition	LT-production	LT-secretion	ST secretion
M9 Glucose	+++	0	Inhibition
M9 Glycerol	+	0	+
M9 Aminoacids	+	0	+++
M9 Bile	Bile-glycerol +++	0	Bile-glucose +++ Bile-aminoacids +++
LB NaCl	No effect	No effect	No effect
LB Sucrose	No effect	No effect	+++
LB Microaerophilic	No effect	Decreased	+++
LBK pH 5	+	0	NT
LBK pH 7	+++	+	NT
LBK pH 9	++	+++	NT

NT: not tested

Role of the global regulator CRP in the regulation of LT toxin.

Under our previous observation that the presence of glucose in the media increases the production of LT toxin, we were interested in the role of the cyclic AMP receptor protein (CRP). CRP is a global transcription factor involved in catabolite regulation. In the presence of glucose the levels of cAMP decrease and CRP cannot bind DNA in the absence of its cAMP cofactor, thus favoring the expression of *eltAB* (Bodero and Munson 2009; Paper IV). However, the role of CRP on the production and secretion of LT toxin is not well understood. To answer this question, we constructed CRP mutant strains in the LT/STh/STp H10407 and LT-only E2863 strains. Then, we measured LT toxin by GM1-Elisa in both the pellet and supernatant. Similar results were found in experiments using both strains. LT total production was significantly higher in the mutant strains (E2863ΔCRP and H10407ΔCRP) than in the wild-type strains (E2863 and H10407). To confirm that the regulation was also affected at the level of transcription, we quantified the number of *eltAB* transcripts per bacterium by using qPCR. We found that the numbers of *eltAB* transcripts per bacterium were significantly lower in the wild-type strain compared to the mutant strain. Taken together these results confirmed that CRP is a negative regulator of LT production through transcriptional regulation of *eltAB* gene. Moreover, LT secretion was positively regulated by CRP. Contrary to our result of LT production, secreted LT levels were higher in the wild-type strain compared to the mutant strains in the experiments performed in both strains E2863 and H10407. Therefore, we report for the first time that secretion of LT is positively regulated by the global regulator CRP (Paper IV).

Further, we were interested in verifying our findings *in vivo*. We used an infant mice model of 3 days old pups to determine small intestine colonization and LT secretion *in vivo*. First, we performed competition assays where we were able to show that the mutant strain is deficient in colonization compared to our wild-type strain. Secondly, groups of mice were infected separately either with the wild-type or the mutant strain and sacrificed after 3 or 18 hours of the infection. The intestine was collected in two individual samples, the upper and lower part and secretion of LT was measured in the supernatant of each centrifuged intestine. Interestingly, LT secretion was significantly higher in the wild-type strain compared to the mutant strain at 3 hours and very low secretion levels were observed at 18 hours. As a result, we could confirm that CRP is a positive regulator of LT secretion *in vivo,* similarly to what we showed in our previous studies *in vitro* (Paper IV).

Regulation of the toxins by CRP under host factors.

After detecting that CRP has a role on the regulation of both the production and secretion of LT toxin, we were interested in studying if the role on the regulation of LT toxin by CRP is affected by different host factor environmental factors. We set up experiments where both the wild-type and CRP mutant strains were analyzed with different host factor components. For carbon source analysis we used glucose, for osmolarity LB media was supplemented with NaCl and sucrose and for pH LBK was buffered to pH 5, 7 and 9. A summary of the results obtained during the experiments are shown in the table below.

Table 3. Regulation of the LT toxin by CRP under different host factor conditions

Condition	Production		Secretion	
	Wild-type	ΔCRP	Wild-type	ΔCRP
M9 Glucose	Lower	Higher	0	0
LB NaCl	ns	ns	ns	ns
LB Sucrose	ns	ns	Higher	Lower
LBK pH 5	Lower	Higher	0	0
LBK pH 7	Lower	Higher	Higher	Lower
LBK pH 9	ns	ns	Higher	Lower

Ns: no significant difference

The presence of glucose and CRP are closely related and are reported to have an antagonistic function. In the absence of glucose in the media, the levels of cAMP rise allowing the formation of the cAMP-CRP complex, which activates or represses transcription. We demonstrated that CRP is a negative regulator of *eltAB,* the operon encoding the LT toxin, and hence we believed that the expression of LT is turned on in the presence of glucose when the levels of cAMP decreased. However, there is a discrepancy among studies whether LT toxin is either positively or negatively regulated by the addition of glucose in the media (Bodero & Munson, 2009, Gibert *et al.*, 1990, Gilligan & Robertson, 1979). In this study, we were not able to disprove this complicated regulatory model since our results did not show a direct correlation of glucose and CRP (Figure 26). We showed that in the absence of glucose when CRP levels are higher the mutant strain expressed higher levels of LT as expected, but addition of 0.4%.glucose in the media also significantly increased the production of LT in the mutant strain compared to the wild-type strain although levels of CRP should be low in this condition (Paper III). These results could be explained by that low levels of CRP was present even in the glucose media but interestingly we found that addition of glucose did not increase LT production in the wild-type, confirming previous findings (Kansal *et al.*, 2013) and more importantly glucose significantly increased the production on LT per bacterium in the mutant strain. Consequently, we propose that other regulators besides CRP must be involved in the regulation of LT toxin in the presence of glucose and that LT regulation is more complicated than previously anticipated (Paper III).

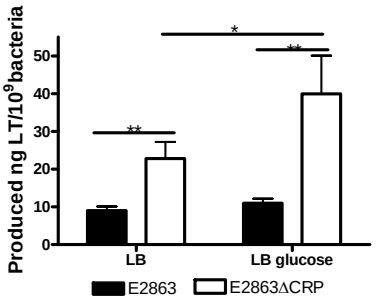

Figure 26. Effect of glucose and the cyclic receptor protein (CRP) in the production of LT toxin.

Next, we analyzed the effect of osmolarity on the regulation of both LT and STh toxins. Both wild-type and mutant strains were grown in LB media supplemented with high concentrations of NaCl (171 nM and 300mM) and sucrose (600mM). No difference in the production and secretion between the wild-type and mutant strains was observed in any of the conditions in either LT or STh toxins, indicating that the regulation of the toxins under high osmolarity conditions is CRP independent (Paper III).

Finally, we assessed the effect of pH in relation to CRP regulation on LT toxin. Similar to our previous finding in unbuffered LBK media, the total production in the mutant strain was significantly higher than in the wild-type strain at pH5 and pH7, however, no difference was found at pH 9. These results were equally found with GM1 Elisa and q-PCR, suggesting that LT production is CRP-dependent at pH 5 and pH 7 and CRP-independent at pH 9 (Paper IV). Possible explanations to the absence of regulation by CRP at pH 9 are that the CRP molecule might not bind at the promoter and inhibit LT expression when the cytoplasmic pH increases to slightly more alkaline. A second hypothesis could be that other transcription factors may bind to the promoter and directly inhibiting or promoting CRP binding depending on pH regulated signals. A third hypothesis is that changes in DNA topology by DNA binding proteins occurs at the promoter site as a response to external pH thereby influencing the regulation of the *eltAB* operon.

A pH dependency on LT secretion has been reported in previous studies (Gilligan & Robertson, 1979, Hegde *et al.*, 2009, Kunkel & Robertson, 1979, Minami *et al.*, 1984, Mundell *et al.*, 1976). It has also been observed that in LB media pH becomes alkaline at stationary phase and that addition of glucose to LB media acidifies the media (Gilligan & Robertson, 1979, Kunkel & Robertson, 1979). Under this consideration, we were interested in study the effect of CRP in the secretion of LT toxin at different pH ranges. We showed that LT secretion was significantly higher in the wild-type strain compared to the mutant strain at pH 7 and pH 9, whereas secretion of LT was completely inhibited at pH 5. We conclude that LT secretion is CRP dependent but requires neutral to alkaline pH for secretion. In addition the higher the pH the more secretion hence there must be a pH dependent regulation on secretion as well.

Effect of growth and viability under pH conditions

Under the observation of the direct effect of pH on the production and secretion of LT toxin, we were interested in analyzing growth rate in the bacteria at the different pH conditions. We registered the OD_{600} of bacteria at 3, 5 hours and from overnight culture in LBK-only media and LBK buffered at pH 5, 7 and 9. We observed that initial bacteria growth at pH 5 was very low possibly due to acid stress adaptation; however, it reached the highest OD value among all pH ranges in overnight cultures. In contrast, pH 9 growth curve was very low at all points compared to pH 5 and pH 7. Taken together our results suggest that bacteria growth curve is not proportional to LT toxin production and secretion.

Then, we tested bacteria membrane integrity under the presumption that high secretion found at pH 9 could be due to membrane leakage. Hence, we measured membrane integrity using a live/dead viability kit by flow cytometry which stains live and dead bacteria in different colors. We did not found significant differences between pH ranges, however, bacteria at pH 9 started to take up some of the dead membrane dye earlier than the others suggesting a change of membrane permeability at this pH. It is possible that some conformational changes in the membrane composition occur under alkaline stress conditions.

Expression of bacteria under the growth curve

During our experiments, samples were obtained at different points in the bacteria growth curve, at 3 hours for mid exponential phase, 5 hours for late exponential phase and from overnight cultures for stationary phase. When bacteria were grown in minimal media, samples were collected from overnight cultures due to low bacteria growth rate at these conditions. In contrast, when we used LB or LBK media only or with different supplements i.e. glucose, NaCl, sucrose and buffered at pH 5, pH 7 and pH 9; samples were collected at the different time points. Our results showed that higher gene expression occurred between 2 and 3 hours when qPCR analysis was performed, whereas the analysis using GM1 Elisa suggests that higher values of both production and secretion were registered at 3 and 5 hours. Interesting, our *in vivo* studies also showed higher levels of LT secretion in mice sacrificed after 3 hours compared to mice sacrificed at 18 hours, confirming that LT is expressed at early time points in exponential phase both *in vitro* and *in vitro*. Taken together, we were able show that analysis of ETEC toxins in the bacteria growth culture at early times of growth could detect higher gene expression, production and secretion compared to overnight cultures when experiments are normally performed.

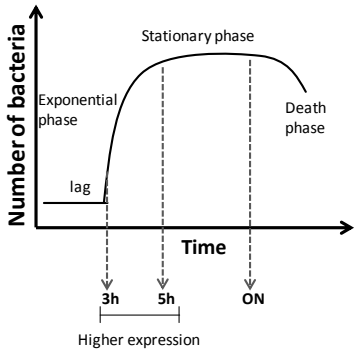

Figure 27. Schema of the bacteria growth curve in relation with the different time point of sampling.

Proteome analysis on the wild-type and CRP mutant strains at different pH conditions.

Fluctuations in pH has a major effect on the outer membrane and periplasm of gram negative bacteria which is exposed to the extracellular pH (Wilks & Slonczewski, 2007) while the cytoplasmic membrane functions as a barrier to maintain a cytoplasmic pH of approximately 7.6 (Zilberstein *et al*., 1984). To evaluate the protein expression profile of the surface proteome at different pH conditions we employed an LPI^{TM} Hexalane FlowCell (Nanoxis Consulting AB, Sweden). By immobilizing intact cells in the flow cell and subsequently digesting the exposed proteins enzymatically, the surface proteome is targeted. The generated peptides were labeled using a tandem mass tag kit (TMT, Thermo Scientific), thus enabling evaluation of up-and-down regulations of the expressed proteins during the various pH conditions. The labeled peptides were analyzed by ESI-LC-MS/MS (Proteomics Core Facility, www.cf.gu.se/Proteomics) and the appropriate ratios between different pH conditions were calculated. We used a strict cut-off of 2-fold increase or decrease in ratios between conditions and analyzed E2863wt and E2863ΔCRP at pH 5, 7 and 9. To identify target proteins that could potentiate secretion of LT we looked specifically for proteins that were differentially expressed in conditions that were favorable for secretion compared to its corresponding unfavorable condition.

Comparing wt pH 9/wt pH 7, wt pH 7/ wt pH 5, wt pH 9/Δ pH 9, wt pH 7/Δ pH 7 and finally Δ pH 9/ /Δ pH 7 where the denominator was significantly deficient in LT secretion in all comparisons indicated that there was a tendency of downregulation of the osmoprotectant trehalose in conditions favoring LT secretion especially alpha-trehalose-phosphate synthase (OtsA) was decreased while trehalose-6-phosphate hydrolase (TreC) was increased. Furthermore proteins involved in the electron transportation chain in the inner membrane showed a tendency to be changed in conditions that favored secretion. For instance WrbA that regulates proton transport over the inner membrane was downregulated in 4 of 5 conditions that favored secretion.

The proteome analysis also indicated that the NADH-ubiquinone oxidoreductase Nuo that exports H+ over the inner membrane was decreased in both the wild type and CRP mutant in response to pH 9 while only the wild-type seemed to respond to pH 9 with increased expression of ATP synthase (AtpA AtpF), Since growth at pH 9 poses a specific type of stress where the activity of ATPase will pump H+ into the cytosol to maintain intracellular pH homeostasis, an upregulation of ATPase probably reflect adaptation to alkaline stress. The results indicate that components of the electron chain (Flavoprotein WrbA and Nuo) and the proton motive force that generates ATP, by transport of protons from the periplasm to the cytosol through ATPase syntase, are deregulated in CRP mutants which also are defective in LT secretion. The results might indicate that controlled regulation of alkaline periplasmic pH is important for LT secretion.

The disulfide bridge protein dsbA has been implicated in assembly of the LT holotoxin by forming disulfide bonds between the B subunits (Wulfing & Rappuoli, 1997, Yu *et al*., 1992, Hirst *et al*., 1994) while degP removed misfolded proteins (Wulfing & Rappuoli, 1997). Both dsbA and degP have been reported to be induced by alkaline pH (Malhotra *et al*., 2000, Thede *et al*., 2011). Spontaneous assembly of the B subunits has also been shown, and recently it was shown that spontaneous assembly is pH dependent and increase with increasing pH, no assembly was detected below pH 7 (Zrimi *et al*., 2010). Since the periplasmic space has similar pH as the surrounding environment (Wilks & Slonczewski, 2007) this could partly provide an explanation to the pH dependence shown in this study. This is corroborated by the proteomic analysis that indicated a deregulated proton motive force in the CRP mutant. Other studies have shown that among the genes repressed at the alkaline pH are genes encoding two respiratory chain complexes that pump protons outward during electron transport, Cyo and Nuo, while induced genes include those encoding the ATP synthase that imports protons during ATP synthesis, and CydA which generates a proton motive force without outward proton pumping (Maurer *et al*., 2005). These results are the same as we found at pH 9 and interestingly the E2863ΔCRP had a defective expression of these genes which indicate that lack of CRP might generate an abnormal response to the stress that alkaline pH poses. In the wild-type the alkaline stress response would transport protons from the periplasm into the cytosol in an attempt to compensate the inverted proton gradient and an increased level of ATPase would generate energy. If this pathway is deregulated in the CRP mutant the periplasmic pH might be different than in the wild-type. Indeed the optimal pH for release of LT has been proposed to be pH 8.6 (Hegde *et al.*, 2009, Kunkel & Robertson, 1979) and since we have shown that the E2863ΔCRP is deficient in LT secretion the periplasmic pH might be lower in a strain lacking CRP. This however needs to be further analyzed. However the lack of upregulation of ATPase in the E2863ΔCRP strain would result in less energy and we found that the mutant strain had a significantly lower growth rate than E2863wt at pH 9.

DISCUSSION MOLECULAR STUDIES

To understand the pathogenesis of ETEC bacteria generates important information that can be exploited towards developing methods of controlling infection, including vaccines and antibiotics, in the continuing fight against disease that is crucial for populations where diarrhoeas caused by ETEC are present. In this second part of the thesis our studies were focused on understanding how ETEC enterotoxins are regulated at the site of infection where the disease is manifested. To achieve this, we investigated how the production and secretion of LT and STh toxins are regulated by environment host factors within the human intestine during the infection process. This was done by culturing bacteria under different conditions *in vitro* i.e. glucose, glycerol, aminoacids, bile, NaCl, sucrose and different pH range; and by growing bacteria in either aerobic or microaerophilic conditions (Paper III and IV).

In a general context, ETEC spreads through fecal-oral route. After ingestion, bacteria pass through the stomach and duodenum before colonizing the lower part of the small intestine, exposing bacteria to a wide range of pH and environmental conditions. As bacteria move further down from the stomach the levels of oxygen decrease. In the upper part of the small intestine, where most of the digestion and nutrient absorption occur, high levels of sodium bicarbonate are released from the pancreas in order to neutralize the potentially harmful acid coming from the stomach. At the same time, protein, lipids and carbohydrates undergo digestions, being degraded in small peptides, aminoacids, fatty acids, glycerol, and into simple sugars such as glucose. Bile salts secreted by the liver and the gall bladder have a role in the digestion of lipids, they emulsify the triglycerides in the watery surroundings until the lipase can break them into the smaller components that are able to enter the villi for absorption (Thomson *et al.*, 2003b, Thomson *et al.*, 2003a). In addition, bacteria are exposed to a stress environment with high osmolarity and alkaline pH. Moreover, all these factors are known to induce adaptation response in bacteria (Blankenhorn *et al.*, 1999, Erol *et al.*, 2006, Weber *et al.*, 2006) and has been implied in virulence regulation of many pathogens (Altier, 2005, Hung & Mekalanos, 2005, Sue *et al.*, 2004).

LT and ST ETEC enterotoxins were found to be inversely regulated by some of the host environmental factors. LT toxin production was increased in the presence of glucose and LT secretion levels were detected in LB rich media compared to minimal media where the secretion was completely inhibited. In contrast, secretion of ST was induced by aminoacids and preferred by the combination of aminoacids and bile salts; however, it was inhibited in the presence of glucose. Finally secretion of ST toxin was favored by the osmoprotectant sucrose. Taken together, our results could suggest that LT and ST toxins can be temporally and spatially separated during the course of an infection. We hypothesize that toxin expression could be independent events that occur under specific host and environment conditions when infection to the host occur by LT/ST strains. However the new finding of this thesis that LT and STh secretion both occur optimally under conditions that induce CRP signaling adds additional complexity to virulence regulation in ETEC. Considering the phylogenetic diversity of ETEC, it is possible that ETEC strains have evolved separately in response to different environmental and host conditions. A discrepancy on the expression of ETEC toxins in response to external conditions has been recently reported. Different toxin gene expression was observed in the two most well characterized ETEC strains E24377A and

H10407; expression of *eltA* encoding LT toxin was oppositely regulated in response to glucose between strains. In addition, *estA* encoding ST toxin was upregulated in the presence of bile in E24377A whereas *eltA* was upregulted by bile salts in H10407 (Sahl & Rasko, 2012). All these observations highlight the difficulty of working with diverse isolates of ETEC and suggest that the study of one strain is not enough to extrapolate the results to all ETEC strains.

One of the strengths pointed out in our studies is the clear difference between the regulation of the production and secretion of LT toxin. Both events have shown not to respond to the same environmental signals and been differentially regulated by the global regulator CRP. Previous studies have suggested that delivery of toxins involves post-transcriptional events that coordinate the release of preformed enterotoxin from the periplasmic space of ETEC (Lee & Busby, 2012). However, our observations propose that regulation of the LT toxin secretion is an event that involves transcriptional regulation and response to environmental signals that might be independent to the regulation of LT production. This is supported by our observation where negative regulation of LT production present in normal LBK media, at pH 5 and at pH 7 by CRP was abolished in alkaline pH. Further studies are needed to explain the mechanism by which regulation of the production and secretion of the toxin occurs in the human intestine.

Different mechanisms by which LT is secreted have been described although most of them have been performed under normal growth conditions. It is possible that bacteria posses multiple mechanisms for effective delivery of the toxin to the exterior that are activated when bacteria encounter optimal conditions that favor a particular one. LT toxins has been reported to be secreted either as a free toxin (Lasaro *et al*., 2006) or associated with outer membrane vesicles (Horstman & Kuehn, 2000, Horstman & Kuehn, 2002). It has been observed that when vesicles were treated with high pH, most vesicles proteins were not released to a greater extent that with buffer alone (Horstman & Kuehn, 2000). Contrary to this observation, our studies show that higher secretion of LT, as a free toxin, was observed at alkaline pH. A similar observation was reported in cholera toxin (CT) where secretion of CT was reduced in growth media under pH 7 due to the accumulation of the toxin in the periplasm, but it was rapidly released at pH 8.5 (Saier, 1998). Additionally, preliminary studies in our group have shown that only low concentrations of LT was detected in vesicle preparations as compared with that detected in the corresponding growth culture supernatants.

Secretion of LT has also been related with the intimate bacteria-host cell contact, which it has been reported to be crucial for the activation of cAMP production in target epithelial cells (Lee & Busby, 2012, Roy *et al*., 2011). It has been shown that bacteria focus toxin secretion to one pole of the bacterium as it encounters the host epithelial which permits ETEC to deliver the toxin (Lee & Busby, 2012). It is possible that in a situation when both LT and ST toxins are present, they could polarize and secrete the toxins in different sides of the bacterium in response to specific stimuli that favors each toxin secretion; this would be an explanation on the antagonism response of LT and ST toxin expression to different environmental stimuli. Another hypothesis, considering that the secretion of the toxins respond to the same signals, at least with respect to CRP, might suggest that LT is expressed early in the gut whereas ST is instantly expressed at the site of infection. However, if this happens and how it happens needs to be elucidated.

This bacteria-host interaction has also been reported to cause morphological changes and variation on gene expression as the interaction matures and culminates in effective toxin delivery (Kansal *et al.*, 2013). We observed that expression of the *eltAB* gene occurred at early stages of the exponential growth phase and was barely detected at stationary phase using q-PCR analysis supporting a gene expression variation through time. Interestingly, Kansal *et al*. also report that bacteria-host interaction is modulated by CRP and c-di-GMP suggesting that modulation of genes by CRP allows bacteria to sense cAMP generated by host epithelial cells in response to stimulation by LT toxin and therefore it is possible that ETEC might provoke increase of cAMP which will be used to modulate the expression of other virulence genes (Kansal *et al.*, 2013) or increased secretion of LT (paper IV). Under this consideration, we might think that the antagonist regulation of the production and secretion of LT toxin could be used by the bacteria to sense the environment and deliver LT more effectively. The higher pH close to the epithelium might induce low levels of secretion of preformed periplasmic LT which might generate an increase of cAMP levels in the host resulting in high cAMP levels surrounding the bacterium, once ETEC senses the cAMP massive secretion is induced while the *eltAB* operon is repressed in a feedback loop allowing the bacterium to save resources.

Finally, it is important to consider that regulation of LT toxin might be a multifactorial process which is regulated by many genes that might express at different conditions. We have shown that LT toxin production is negatively regulated by CRP and that this regulation in inhibited at alkaline pH. We also reported no relation between regulation by CRP and presence of glucose in LT production, which we thought could have been functionally related. Finally, contradictory to previous studies (Trachman & Yasmin, 2004), we did not observe an effect of osmolarity on LT production. LT regulation might not only depend on CRP, other transcriptional regulators must have a role at different conditions. For instance, H-NS has been reported to be a negative regulator of LT production, which is independent of osmolarity and microaerophilic conditions (Trachman & Maas, 1998, Trachman & Yasmin, 2004). However it is possible that other additional proteins in ETEC facilitate the secretion of the toxin to the media. LeoA has been suggested to modulate LT secretion (Fleckenstein *et al.*, 2000) although it has been found in a low percentage in our study (Paper II). EatA has also been related to bacterial adhesion favoring delivery of LT to the epithelial cells EatA modulates adhering of ETEC by degradation of EtpA (Roy *et al*., 2011). EatA was present in 34.9% of the ETEC strains (Paper II); however, we did not estimate the impact of EtpA in our studies. EtpA has been reported to mediate adhesion between flagella and host cells (Roy et al., 2009). It is reasonable to assume that strategies to deliver LT and ST toxin payload have evolved as a multifactorial process where different regulators, proteins and mechanisms will be activated in response to specific conditions in a process that will allow ETEC to avoid host defense and deliver the toxin in the most advantageous way. There is still more to discover in ETEC!

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