Studies on the Expression and Regulation of Enterotoxins and Colonization Factors in Enterotoxigenic *Escherichia coli* (ETEC)

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2008

ISBN 978-91-628-7389-9
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Printed at Vasastadens Bokbinderi AB,
Göteborg, Sweden 2008

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ABSTRACT

Enterotoxigenic *Escherichia coli* (ETEC) is one of the most common causes of acute watery diarrhoea in developing countries, particularly among local children less than five years and is also the most common cause of diarrhoea in travellers to ETEC endemic areas. The infection is transmitted by ingestion of contaminated food and water and the disease is established in the small intestine. Colonization factors (CFs) on the bacterial surface mediate adhesion to the intestinal epithelium and diarrhoea is manifested by the actions of a heat-stable (ST) and / or a heat-labile (LT) enterotoxin. Two of the most common CFs in strains isolated world-wide are coli surface antigens 5 (CS5) and 6 (CS6). In this thesis the expression and regulation of these important virulence factors as well as the genetic variability among ETEC strains have been studied.

Using ETEC strains isolated directly from diarrhoeal stool specimens of Bangladeshi patients without sub-culturing the gene expression of the two enterotoxins as well as the two CFs were studied *in vivo*. By also quantifying the transcription levels of the respective genes after *in vitro* culture we found that there was no significant up- or down-regulation of transcription of the genes encoding ST (*estA*) or LT (*eltB*) *in vivo* as compared to *in vitro*; however, the CS5 operon was up-regulated 100-fold and CS6 operon 10-fold *in vivo*.

By culturing clinical strains under various conditions *in vitro*, ST, LT, CS5 and CS6 were shown to be differentially regulated by certain environmental factors, *i.e.* the presence of bile salts, lack of oxygen and different carbon sources (glycerol, glucose and amino acids). Thus, secretion of ST was down-regulated by glucose as carbon source under certain conditions but up-regulated by casamino acids, LT was only secreted in complex media in the absence of bile salts and presence of oxygen, phenotypic expression of CS5 on the bacterial surface was induced by bile salts and down-regulated by lack of oxygen, and expression of CS6 was up-regulated by lack of oxygen. An important finding was that the regulation of expression of these virulence factors does not seem to occur at the transcriptional level of the virulence operons.

A majority of wild-type LT-only ETEC strains that were genotypically positive for CS6, but that did not express CS6 on the bacterial surface, were shown to contain truncating mutations within the functional chaperone subunit. This mutation was predicted to severely affect the capacity of the chaperone to bind to the structural subunits, thus indicating a requirement for a functional chaperone for surface expression of CS6. In addition, a single-point mutation was identified in the non-coding region up-stream of the chaperone-encoding gene in these strains; this mutation was found in strains isolated in diverse geographical areas and belonging to different clonal groups.

By investigating the genetic relationship between ST-only CS6 positive strains isolated from children in a region highly endemic for ETEC, *i.e.* Guatemala, and adult travellers to the same region we found that these two groups may be infected by strains of the same genetic background and that ST-only CS6 positive strains belonging to several clonal complexes circulate in this area. We suggest that an ST-only CS6 positive ETEC strain belonging to the most common clonal complex, which was present during several years and found in strains isolated both from children and adults, may be considered as a candidate vaccine strain.

Keywords: ETEC, heat-stable enterotoxin, heat-labile enterotoxin, colonization factors, CS5, CS6, virulence gene expression, *in vivo* and *in vitro*, genetic variability.

ORIGINAL PAPERS

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

I Sjöling Å, Qadri F, **Nicklasson M**, Ara Begum Y, Wiklund G, Svennerholm AM
In vivo Expression of the Heat Stable (*estA*) and Heat Labile (*eltB*) Toxin Genes of Enterotoxigenic *Escherichia coli* (ETEC).
Microbes and Infection 8 (2006) *2797-2802*

II Sjöling Å, **Nicklasson M**, Stenberg J, Eriksson S
Gene expression, translation and secretion of the heat stable (ST) and heat labile (LT) toxins of enterotoxigenic *Escherichia coli* (ETEC) are regulated in response to different external stimuli present in the gastrointestinal tract.
Submitted for publication

III **Nicklasson M**, Sjöling Å, Qadri F, Svennerholm AM
Gene and protein expression of colonization factors CS5 and CS6 in Enterotoxigenic *Escherichia coli* (ETEC) after growth under different conditions *in vitro* and *in vivo*.
In manuscript

IV **Nicklasson M**, Sjöling Å, Lebens M, Tobias J, Janzon A, Brive L, Svennerholm AM
Mutations in the periplasmic chaperone leading to loss of surface expression of the colonization factor CS6 in enterotoxigenic *Escherichia coli* (ETEC) clinical isolates.
Accepted for publication in Microbial Pathogenesis

V **Nicklasson M**, Klena J, Rodas C, Bourgeois A, Torres O, Svennerholm AM, Sjöling Å
Genetic relationship of enterotoxigenic *Escherichia coli* ST/CS6 strains isolated from children living in Guatemala and adult visitors to Central America.
Submitted for publication

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CONTENTS

ABBREVIATIONS

cDNA	Complementary DNA
CF	Colonization factor
CFA	Colonization factor antigen
CS5	Coli surface antigen 5
CS6	Coli surface antigen 6
CsvR	Coli surface virulence factor regulator
CT	Cholera toxin
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
ETEC	Enterotoxigenic *Escherichia coli*
GM1	Monosialotetrahexosylganglioside; receptor for LT and CT
H-NS	Histone-like nucleoid structuring protein
ICDDR,B	International Centre for Diarrhoeal Disease Research, Dhaka
Ig	Immunoglobulin
LB	Luria Bertani culture medium
LT	Heat labile enteroxin
MAb	Monoclonal antibody
MLST	Multilocus sequence typing
M9	Defined minimal medium
mRNA	Messenger RNA
PCR	Polymerase chain reaction
QCRT-PCR	Quantitative competitive reverse transcriptase PCR
RAPD-PCR	Random amplification of polymorphic DNA PCR
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase PCR
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
ST	Heat stable enterotoxin
ST-398	(MLST) sequence type 398
TD	Travellers´ diarrhoea

INTRODUCTION

In 1885 the German bacteriologist and paediatrician Theodore Escherich discovered the rod-shaped Gram-negative bacterium *Bacterium coli commune*, later renamed to *Escherichia coli* (*E. coli*), which is the predominant facultative anaerobe of the normal flora of the human large intestine. The infant gastrointestinal tract is typically colonized by this organism within a few hours after birth, and for the rest of our lives we co-exist in harmony in a relationship where both parts benefit from each other [1]. However, six different groups of pathogenic *E. coli* strains exist that harbour various virulence factors which enable them to cause diarrhoeal disease; enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC) [2]. Among them, ETEC is the most common, particularly among children in developing countries, causing approximately 280-400 million diarrhoeal episodes in children under the age of five every year [3-5]. ETEC is also the most common cause of travellers´ diarrhoea in Asia, Africa and Latin America [6].

According to the World Health Organization (WHO), acute infectious diarrhoeal disease is the number two killer of children living in developing countries, accounting for approximately one fifth of all deaths in children under the age of five or 1.6-2.5 million childhood deaths in this age group every year [7, 8]. ETEC has been reported to be an important cause of mortality, causing an estimated 380.000 deaths in children under the age of five every year [4, 9].

ETEC DISEASE

Clinical features

Diarrhoea caused by ETEC is watery, without blood, and typically has an abrupt onset with an incubation period of 14-50 hours [4]. Adult patients may purge up to 10 litres per day and the diarrhoea is often accompanied by vomiting but not by fever. The loss of fluids and electrolytes results in dehydration which can be categorized from mild to severe [4]. The illness usually lasts for 3-4 days and is self-limited, but the more severe cases may require hospitalization. However, with adequate treatment of dehydration, *i.e.* intravenous rehydration therapy and / or oral rehydration solutions (ORS) the mortality is very low (< 1%) and the patients survive without any sequelae [4]. ETEC infections may also go completely unnoticed in short-term asymptomatic carriers; at any one time close to 50 million children below the age of five are colonized by ETEC but without showing any symptoms [4, 5]. However, ETEC is detected at least two or three times more frequently on average in symptomatic than asymptomatic children [4, 10]. ETEC disease is also a major problem within agriculture, particularly affecting cattle and post-weaning piglets, but animal ETEC strains do not cause disease in humans [11, 12].

General pathogenesis

ETEC disease is spread through ingestion of 10^6 to 10^{10} ETEC bacteria [4] and infection is established when the bacteria reach the small intestine. The disease caused by ETEC can be ascribed to the actions of two toxins produced by the bacteria; the heat-stable (ST) and / or the heat-labile (LT) enterotoxin, as well as adhesion molecules on the bacterial surface referred to as colonization factors (CFs). The events leading to ETEC diarrhoea are shown schematically below (Fig. 1). The nomenclature of the toxins is derived from the fact that LT looses its toxic activity after heat incubation while ST retains its activity after boiling. Both enterotoxins may induce diarrhoea independently each other and ETEC strains produce either ST only, LT only, or both toxins simultaneously [2, 4].

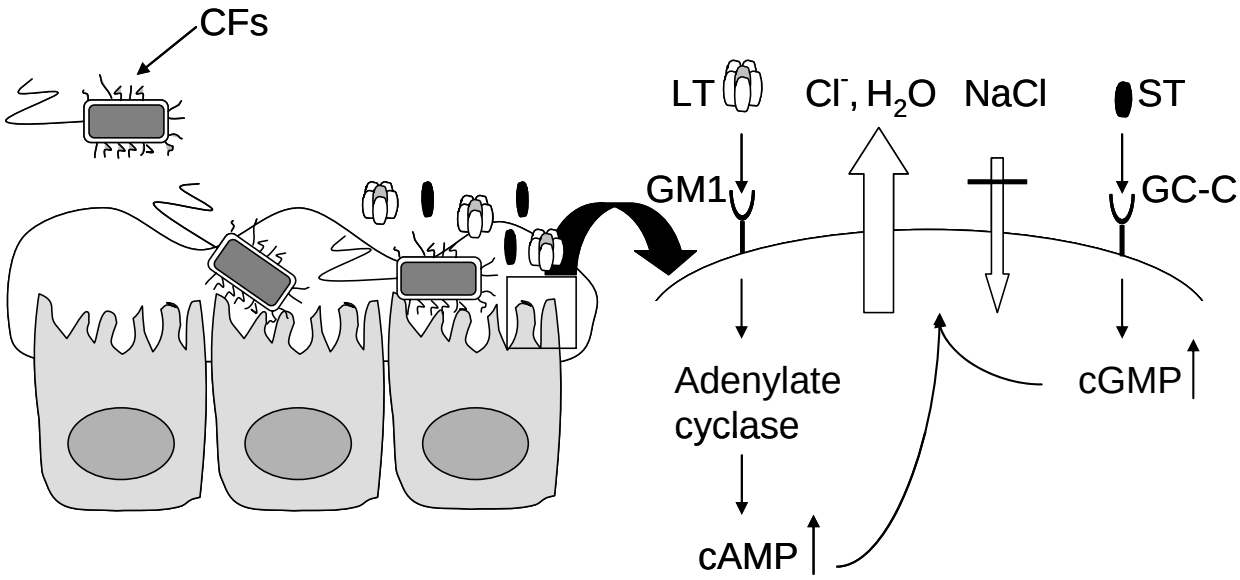

Figure 1. Pathogenesis of ETEC disease.

Colonization of the small intestine is mediated by the colonization factors (CFs) which constitute a diverse group of low molecular weight proteinaceous structures on the bacterial surface that bind to the enterocytes, thus mediating adhesion of the bacteria to the epithelium [11]. Studies in humans as well as animal models have demonstrated that CF-positive ETEC bacteria, but not their isogenic CF-negative mutants, are able to colonize the intestine and induce diarrhoea [4, 13]. LT and ST excert their toxic effects by binding to their respective receptors on the epithelial cell surface, leading to increased levels of cAMP and cGMP, respectively, ultimately resulting in the net secretion of water and electrolytes into the intestinal lumen.

ST

The heat-stable enterotoxins may be classified into two major phenotypes; the methanol soluble but protease resistant STI (STa), and the methanol insoluble but protease sensitive STII (STb) [14]. STI and STII differ both in structure and mechanism of action. ETEC strains infecting humans typically produce STI, although some human ETEC strains expressing STII have been reported [2, 14]. STI is divided

into two subtypes; STh and STp, named after their initial discovery in humans and pigs, respectively. Throughout this text, "ST" refers to STI.

ST is a non-immunogenic and low molecular weight peptide; STh and STp are approximately 2 kDa and consist of 18 aa (STh) or 19 aa (STp) [14]. Both STh and STp have been reported to cause diarrhoea in children as well as in adult travellers to different geographical areas [15]. Although relatively uncommon, STh and STp may be expressed in the same strain, and hence there are seven different possible combinations of toxins in strains infecting humans: LT, STh, STp, LT/STh, LT/STp, STh/STp, and LT/STh/STp.

STp and STh typically excert their toxicity by binding to the guanylate cyclase C (GC-C) receptor, a transmembrane enzyme located in the apical membrane of the intestinal epithelial cells. Binding to the extracellular domain of GC-C activates the receptor´s intracellular activity, resulting in increased intracellular cGMP levels. This in turn leads to activation and opening of the cystic fibrosis transmembrane conductance regulator chloride channel (CFTR) in the apical membrane by cGMP-dependent protein kinase II phosphorylation, resulting in elevated secretion of electrolytes and water, and to inhibition of NaCl and water absorption by blocking an apical Na/H exchanger [2, 14, 16-19]. The endogenous ligands for GC-C are guanylin and uroguanylin which are involved in normal gut homeostasis, and which are very similar to ST in function and structure, even though ST is more potent than guanylin in activating GC-C [20, 21].

LT

LT is an oligomeric protein of approximately 86 kDa and is similar to the cholera toxin (CT), both physiologically, structurally and antigenically and the proteins cross-react immunologically. The protein sequences share approximately 80% homology and they have superimposable tertiary structures. They both belong to a family of AB_5 toxins and consist of a pentameric ring of five identical binding (LTB) subunits of 11.5 kDa surrounding an active (LTA) subunit of 28 kDa, and have a similar mode of action [2, 22-24]. LT can be divided into two major serogroups; LTI, which is expressed by ETEC strains pathogenic for both humans and animals, and LTII, which is not associated with disease and is predominantly found in animal strains [2]. Throughout the remainder of this text, "LT" refers to LTI.

LT mainly mediates its toxic effect by irreversibly binding to the ganglioside GM1, as well as to glycoproteins, present on the apical surface of the enterocytes. This binding is mediated by the LTB subunits. Upon binding, the LT-GM1 complex is endocytosed and transported through the cell by a mechanism involving trans-Golgi vesicular transport. ADP ribosylation of the GTP-binding protein ($GS\alpha$) by the toxic LTA subunit activates adenylate cyclase leading to elevated levels of intracellular cyclic AMP (cAMP) and subsequent activation of cAMP-dependent protein kinase A,

which in turn phosphorylates and thereby stimulates chloride channels in the apical membrane, mainly CFTR. The net result is secretion of electrolytes and water and inhibition of NaCl absorption from villus tip cells [2].

Colonization factors (CFs)

The CFs constitute a diverse group of virulence factors; at least 25 CFs have been identified in ETEC strains infecting humans so far. They are designated as coli surface antigens (CS) with a number corresponding to their chronological order of identification, with the exception of colonization factor antigen I (CFA/I). Hence, the CFs are designated CFA/I, CS1, CS2, CS3, CS4, CS5, CS6, CS7, CS8, CS12, CS13, CS14, CS15, CS17, CS18, CS19, CS20, CS21 (also termed "longus") and CS22 [11, 25-27], while other CFs have not yet been given a designation.

Certain combinations of CFs seem to be preferred by ETEC strains, *e.g.* CS1, CS2 and CS3 are expressed in the combinations CS1 + CS3, CS2 + CS3, or CS3 alone. Similarly, CS4, CS5 and CS6 (sometimes referred to as the "CFA/IV group") are expressed in the combinations CS4 + CS6, CS5 + CS6 or CS6 alone. Some of the better characterized CFs can be subdivided into families based on their antigenic and genetic relationships, *i.e.* the CFA/I-like family (CFA/I, CS1, CS2, CS4, CS14, CS17 and CS19), in which the major subunits cross-react immunologically, the CS5-like family (CS5, CS7, CS13, CS18 and CS20), and a family of unique CFs (CS3, CS6, CS10, CS11 and CS12) without homology to any known CF [11].

The colonization factors are mainly fimbrial or fibrillar in structure, although some are non-fimbrial. The fimbrial CFs, *e.g.* CFA/I and CS1, are rigid, hairlike organelles consisting of hundreds of identical structural subunits. Fibrillae, *e.g.* CS5, have fewer subunits per helical turn and are therefore thinner and more flexible [11]. The receptors for most CFs have not been characterized in detail although some CFs are known to bind to glycoconjugates and glycoproteins present on eukaryotic cell membranes, *e.g.* CFA/I and CS1 - CS4. The diversity displayed by the oligosaccharides of these molecules are suggested to be responsible for the species, tissue and cell preferences of ETEC strains [11].

Novel virulence factors

In addition to the enterotoxins and CFs, novel putative virulence factors have been described for ETEC, but their roles in diarrhoeal disease have not yet been completely elucidated. The outer membrane proteins TibA and Tia, the serine protease EatA, the glycoprotein EtpA, and *leoA* (labile enterotoxin output) [28-31] were all initially identified in the classical ST/LT ETEC strain H10407 [32], which was originally isolated from a patient in Bangladesh with severe diarrhoea and which has since been regarded as a prototype for ETEC. The loci in H10407 encoding TibA and Tia have been shown to confer an ability to adhere to and invade human intestinal epithelial cells, even though ETEC are generally regarded as non-invasive mucosal

pathogens. TibA also promotes bacterial aggregation and biofilm formation [28-31]. EatA has been reported to somehow increase the virulence of H10407 and EtpA has been suggested to be involved in epithelial cell adhesion. In addition to H10407, EtpA has also been identified in other strains expressing CFA/I, CS1-3, CS14, or CS17, but not in strains expressing CS4, CS5 or CS6. The gene encoding EatA was identified in more than half of the clinical ETEC strains tested [33, 34].

EPIDEMIOLOGY

Spread of disease

ETEC is spread via contaminated food and water; in any situation with inadequate sanitation and drinking water facilities ETEC is often a major cause of diarrhoea [4]. In a recent study from our group ETEC was detected in the drinking water in two-thirds of the households in an urban community in Dhaka with generally poor living conditions and low socioeconomic status [35], and in a study conducted in villages in Egypt possession of a sanitary latrine in the family household significantly decreased the risk of ETEC among children up to the age of three [36]. According to WHO, around 1.1 billion people world-wide lack access to improved water sources and 2.4 billion have no basic sanitation. Other studies have shown that surface waters (rivers, lakes and ponds) in urban and rural Bangladesh are heavily contaminated with ETEC and that the toxin and CF profiles of environmental and clinical samples from the surrounding area were comparable, suggesting that surface water may contribute to the spread of ETEC [37].

ETEC is endemic in essentially all developing countries. In studies from Bangladesh, Egypt and Brazil the frequency of ETEC diarrhoea and asymptomatic infections has been reported to be elevated during warm periods of the year [4, 10, 38-42]. In Bangladesh, ETEC infections follow a distinct biannual seasonal pattern with one peak during the hot and dry months of April, May and June, and a second peak in September and October when the heavy monsoon rains have subsided, but they remain endemic throughout the year [4, 10]. In a recent birth cohort study of children in the urban community in Dhaka mentioned above, isolation of ETEC was higher during March to June than between July and October [10].

ETEC has also been suggested to be an important cause of acute watery diarrhoea in epidemics caused by floods [43]. In August 2007, Dhaka was struck by major floods and during this month the number of patients admitted in one day to the ICDDR,B hospital reached an all-time record of more than 1000 patients. During the height of patient admissions in the middle of the month, ETEC was identified in 15% of cases [44]. ETEC also has the potential to cause outbreaks in non-endemic countries; in fact, the first reported food-borne outbreak of ETEC in Europe occurred in Sweden in

1979 [45]. There have also been reports of ETEC transmission on board cruise ships [46].

ETEC among resident children in endemic countries

Children living in the developing world are predicted to experience 3.2 diarrhoeal episodes per year until their fifth birthday [4]. Out of these, an estimated 0.5 episodes are caused by ETEC [4, 5], which has been shown in most studies to be the most common bacterial enteric pathogen among children in developing countries, accounting for approximately 20% of cases [4, 39, 47]. After the first five years of life there is a drop in the incidence to approximately 0.1 ETEC diarrhoea episodes / year / child until 15 years of age [5]. While up to 400 million cases of ETEC diarrhoea occur every year in children less than five years old the corresponding figure in children 5-15 years old is 110 million cases [4, 5]. ETEC has also been reported to be the most common pathogen isolated from the first diarrhoeal episode experienced by infants in a cohort study of children less than three years in rural Egypt [39]. Finally, children in a birth cohort study in Dhaka who had experienced one or more episodes of ETEC diarrhoea were found to be significantly more growth stunted and malnourished at two years of age than those without ETEC disease [10], an association between ETEC disease and child development which may be of consequence for societies as a whole. However, undernutrition itself may also be an underlying cause of diarrhoeal mortality [8].

ETEC among resident adults in endemic countries

The first reports of ETEC were described in adults in 1971 [48]. After the initial decrease in ETEC infections among children between 5 and 15 years old, the incidence increases again in those over 15 years to an estimated 400 million cases per year [5] and approximately 25% of ETEC illness in Bangladesh is seen in adults [5, 49]. At the ICDDR,B hospital in Dhaka, ETEC has been reported as the second most commonly isolated bacterial pathogen after *V. cholerae* among patients > 65 years, and adults often present with more severe forms of ETEC diarrhoea than children and infants [4, 50].

Travellers´ diarrhoea

"Travel broadens the mind, but loosens the bowels". On top of being one of the most common bacterial causes of acute watery diarrhoea in children living in developing countries, ETEC is also responsible for most cases of Montezuma´s revenge (if in Mexico), Delhi belly (if in India), and Pharaoh's Curse (if in Egypt). Travellers´ diarrhoea (TD) is the most common infectious disease to affect travellers from industrialized countries to developing countries with a reported incidence of 20-66% during the first two weeks in the country of destination [7, 51]. TD is characterized by watery diarrhoea and may be accompanied by nausea, vomiting, abdominal pain and cramps, muscle aches, fever and weakness. Most cases of TD are self-limiting and mild and last for four days on average if untreated but 1% of cases last for more

than one month [51]. The incidence rate of TD is highest among infants and young adults and travellers who lack the gastric-acid barrier [4, 6]. A majority of TD cases (80-85%) at various destinations have been reported to be caused by bacterial pathogens [6] and ETEC is the single most common cause of TD in adult travellers in most studies worldwide [4, 6, 52, 53] and may be responsible for 20 to 40% of all TD cases [4]. TD caused by ETEC often results in a moderate clinical illness that interferes with daily activities although severe dehydration may occur in some cases.

Co-pathogens

Up to 40% of ETEC disease cases may be mixed infections [49], and this figure seems to increase with age in a study of patients admitted to the ICDDR,B hospital in Dhaka [49]**.** Rotavirus was the most common co-pathogen followed by *V. cholerae*, *Campylobacter jejuni*, *Shigella* spp. and *Salmonella* spp. Co-infection with rotavirus was the most common among young children, peaking at 6-12 months, whereas *V. cholerae* was common mostly in older children and adults [50]. In travellers, EAEC and *Campylobacter* spp. are common co-pathogens [4].

EPIDEMIOLOGY OF VIRULENCE FACTORS

Association between toxin and CF phenotypes

The proportions of ST-only, ST/LT, and LT-only strains vary between different studies and geographical areas. Roughly one third of all ETEC strains isolated globally have previously been reported to be ST-only strains, one third ST/LT and one third LT-only strains [4, 54]. In other studies the ST-only strains have been reported to constitute up to 50% of the strains [4, 10, 39, 49].

LT-only ETEC strains have been more frequently isolated (as compared to ST-only and ST/LT strains) from asymptomatic carriers than from patients, and have therefore been considered less pathogenic [4, 55]. However, this may possibly reflect the fact that in more than 90% of LT-only strains no known CFs have been detected, as compared to less than 40% of ST-only and ST/LT strains. In total, 25-50% of strains worldwide do not express any known CF [4, 49].

In diarrhoeal ETEC strains isolated worldwide, the most common CFs are CFA/I, CS1, CS2, CS3, CS4, CS5, and CS6, which have been detected at various frequencies in different parts of the world [4, 11, 54]. In many studies approximately 60-90% of ST/LT strains express CFA/I or CS1-CS6, whereas these CFs are expressed by approximately 40-70% of ST-only strains and are very rarely expressed in LT-only strains [4, 11].

CS6 is increasingly being identified in studies world-wide, both among adults and children [4, 49]. In studies on travellers´ diarrhoea in Jamaica, Kenya and India, as

well as in American travellers to Guatemala and Mexico, CS6 (alone or in combination with CS4 or CS5) was identified in 41-52% of all CF-positive strains making it the most common CF in these studies [56, 57]. CS6 (alone) was also the most commonly identified CF in children with ETEC diarrhoea (11.3%) in a paediatric diarrhoea study in Egypt as well as the most common among US military personnel deployed to Egypt [52, 58].

Phenotypic expression of CS6 is clearly associated with expression of ST (ST-only and ST/LT strains), and is rarely observed in LT-only strains [11, 54]. CS6 has been reported to predominantly be expressed alone (without CS5 or CS6); in a global study, CS6 occurred alone in 92% of strains expressing the CFA/IV group [54]. In a recent vaccine trial conducted in Mexico and Guatemala involving adult US travellers, ST-only strains expressing only CS6 were observed to predominate among those infected with CF-positive ETEC [57]. However, in studies where both genotypic and phenotypic methods are used LT-only strains which are negative for CS6 on the bacterial surface but positive when using genotypic detection methods have been identified in different geographical areas [59-61].

Diversity of ETEC strains

The various combinations of CFs and enterotoxins combined with the relative proportion and distribution of these virulence factors in different parts of the world indicate that ETEC comprises a highly diverse group of bacterial enteropathogens, which has proven to be a challenge to the development of an efficient vaccine. Another factor adding to the heterogenity of ETEC strains is the variability in the LPS (O serogroup) and flagellar antigens (H serotype) displayed on the bacterial surface; more than 100 different O serogroups and more than 30 H serotypes have been detected for ETEC strains isolated globally [54, 58]. Even though there are some O serogroups that are more prevalent, there are large geographical differences [9, 62].

IMMUNITY AND PROTECTION AGAINST ETEC INFECTIONS

Natural immunity and protection

The decrease in the incidence of ETEC-caused diarrhoea with age in endemic countries has suggested that natural protection may develop after repeated ETEC infections [3, 39]. ETEC infection results in intestinal secretory immunoglobulin (Ig)A (sIgA) as well as systemic IgA and IgG antibody responses against the CFs, LT (mainly against the LTB subunit) and the O antigen. Protective immunity may be mediated by the locally produced antibodies that prevent adhesion of bacteria and toxin action at the intestinal epithelium; the main immunologic protection against ETEC diarrhoea is presumed to be mediated by SIgA antibodies against the CFs [9, 62]. Studies in Mexico have shown a reduced risk of diarrhoea in infants after reinfection with ETEC strains carrying the same CFs as compared to different CFs [63], and in Bangladesh certain CFs, *e.g.* CS7 and CS17, have been found to be present at higher frequencies in children than in adults [4, 49]; these findings suggest that natural protective immunity against disease caused by an ETEC strain with a homologous CF profile may develop. In a birth-cohort study in an urban area of Dhaka, children with symptomatic or asymptomatic infections with ETEC strains expressing CFA/I, CS1 + CS3, CS2 + CS3 or CS5 + CS6 did not, or very rarely, experience a repeat episode of diarrhoea or infection by a strain with the same CF profile; however, infection with CS6-only strains did not seem to protect from subsequent CS6-only strains [10]. There have been different reports on the role of anti-LT immunity for protection against ETEC disease; while vaccination with the B-subunit of the cholera toxin (CTB) has been shown to be protective against ETEC strains that express LT [24], multiple episodes of LT-only diarrhoea are common [4] and symptomatic infections with LT-only strains did not seem to protect against reinfection of children with LT-only strains in Dhaka and Egypt [10, 39]; however, studies in Guinea-Bissau have suggested that infection with LT-positive ETEC strains provides protection against reinfection [64].

Vaccine strategies

The high mortality and morbidity rates of ETEC infections among local residents and visitors to endemic areas makes ETEC an important target for an efficient vaccine. According to prevailing dogma, such a vaccine should contain the most prevalent CFs [65], *i.e.* CFA/I and CS1, CS2, CS3, CS4, CS5 and CS6 in order to provide broad-spectrum protection against the majority of strains in most geographical areas. Such a vaccine also containing an LT toxoid may provide protection against approximately 80% of strains world-wide [9, 62].

Due to the high prevalence of diarrhoea causing ETEC strains expressing CS5 and CS6 worldwide, these CFs as well as ST and LT are the focus of this thesis. In the following text I will give a brief description of the expression of these virulence factors.

Expression and secretion of ST

STh and STp are encoded by the plasmid-borne *estA* and *st1* genes (GenBank accession numbers M34916 and M25607). The mature STh and STp proteins consist of 18 or 19 amino acids, respectively, and are nearly identical in the 13 amino acids that are required for enterotoxic activity. Six of these 13 amino acids are cysteines which form three intramolecular disulphide bonds [19], responsible for the heat-stable properties. The events leading to secretion of the mature toxin is shown schematically below (Fig. 2).

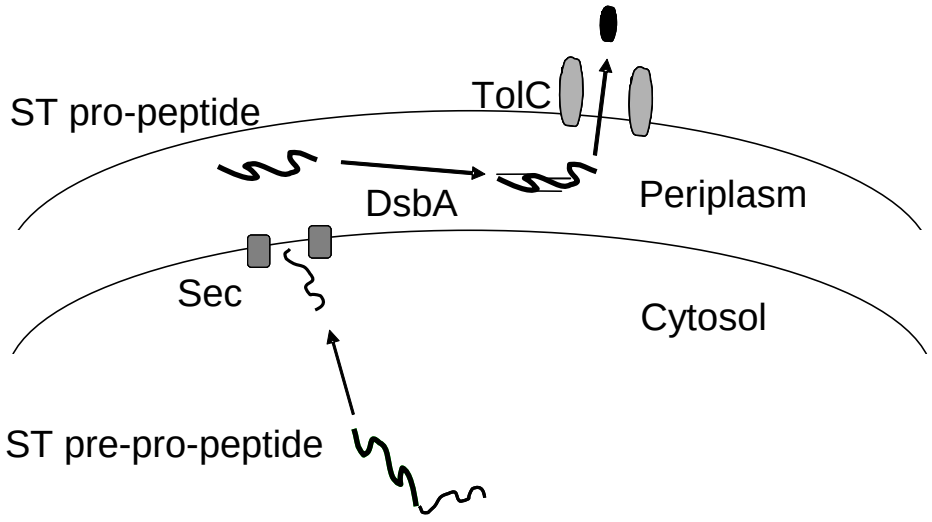

Figure 2. Secretion of ST.

STh and STp are both synthesized as pre-pro-peptides of 72 amino acids which are processed into the mature protein during export from the cytosol. The pre-pro-peptides carry N-terminal signal peptides which are removed by signal peptidase after translocation across the inner membrane by the Sec machinery of the general secretory pathway (GSP). The resulting pro-peptide of 53 amino acids is released into the periplasm where the three disulphide bonds in the C-terminus are formed with the assistance of the GSP disulfide isomerase DsbA [66]. Secretion of the ST pro-peptide through the outer membrane is mediated by the TolC outer membrane protein transporter whereby the proregion is removed to release the mature toxin [67].

Expression of LT

LT is encoded by the plasmid-borne *eltAB* operon (GenBank accession number J01646) encoding the LTA and LTB subunits. The events leading up to the assembly of the AB_5 holotoxin are depicted in Fig. 3.

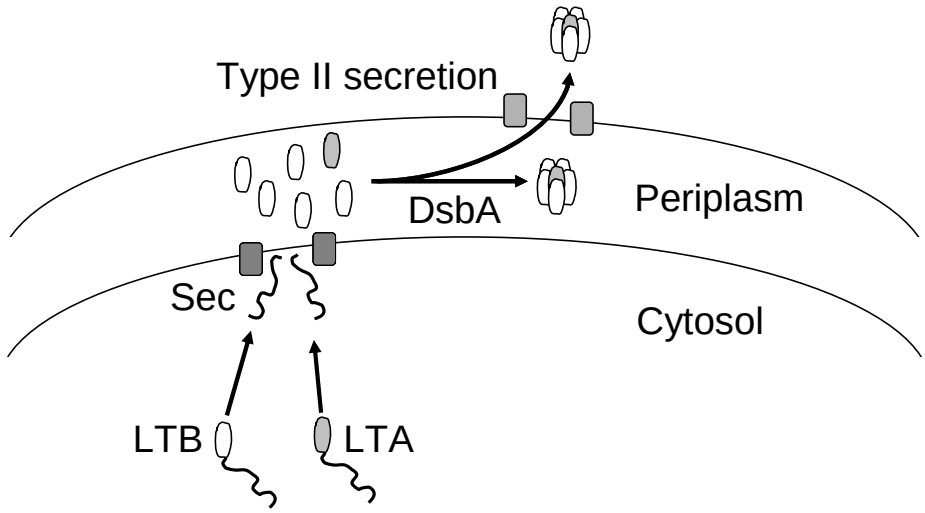

Figure 3. Assembly and secretion of LT.

The LT A and LT B subunits are synthesized as precursor proteins with typical N-terminal signal peptides, and are translocated separately across the inner membrane by the Sec machinery. After translocation across the inner membrane the mature subunits are released into the periplasm, where the subunits are assembled non-covalently into the LT holotoxin (consisting of one toxic A subunit and five B-subunits) with the assistance of DsbA [68]. Secretion of LT through the outer membrane has been proposed to be mediated by the Type II secretion system (sometimes referred to as the main terminal branch of the GSP), as shown in the classical ETEC prototype strain H10407 [69, 70].

The figure above shows LT being retained in the periplasm; originally, ETEC bacteria were believed to be deficient in the secretion of the toxin and ETEC was thought to retain the majority of produced LT in the periplasm [69]. A more recent study has indicated that different strains have different capacities to secrete the toxin under laboratory conditions, and that the ability of wild-type ETEC LT-only strains to secrete free LT was associated with their ability to cause water secretion in rabbit ileal loops [71]. LT has also been reported to be secreted in a polarized fashion from the bacterial cell [72].

Upon secretion, LT has been shown to have the ability to bind to the 3-Deoxy-D-manno-octulosonic acid (Kdo) core sugars of the *E. coli* lipopolysaccharides (LPS) via the LTB subunit and may thus remain associated to the outer cell membrane or to outer membrane vesicles [73, 74]; LT has been reported to be secreted in association with outer membrane vesicles shed from the bacterial surface and was detected both in the lumen of the vesicles and bound to the vesicle surface [75, 76].

The putative virulence gene *leoA* ("labile enterotoxin output") has been reported to be involved in specific LT secretion pathways in strain H10407; deletion of the gene caused a buildup of LT in the periplasm, a decrease in secreted LT and a decrease of toxic activity *in vivo* [77]. However, the role of this gene in the pathogenesis of most LT-expressing ETEC strains remains unclear; in our hands it was only identified in 2 clinical strains, one of which was an ST-only strain, out of more than 70 tested from Bangladesh, Guatemala and Egypt (Sjöling and Nicklasson, unpublished results). On the other hand, one of the genes in the *gsp* gene cluster (*gspD*) encoding the Type II secretion apparatus was identified in all of more than 30 strains tested. Similar data have also been reported for Brazilian strains [71].

Expression of CS5

According to the most recent report regarding the morphology of CS5, this colonization factor is a 2 nm flexible fibrillar structure, devoid of any tip-associated structures [11, 78], but it has also been reported to consist of two fine fibrils arranged in a helical structure. The CS5 operon (PubMed accession number AJ224079) consists of six genes encoding a major subunit (CsfA), a minor subunit (CsfD), an outer membrane usher (CfsC), two chaperones (CsfB and CsfF), and a protein involved in pilus length regulation (CsfE) [79-82]. It is not known whether CsfA or CsfD is responsible for adhesion but CsfD has been suggested to add flexibility to the CS5 structure. The molecular weight of the mature major subunit is 18.6 kDa [81]. A summary of a proposed model of surface expression of CS5, which is the first description of a dual-chaperone system for any human ETEC pilus, is shown schematically on the following page (Fig. 4) [81].

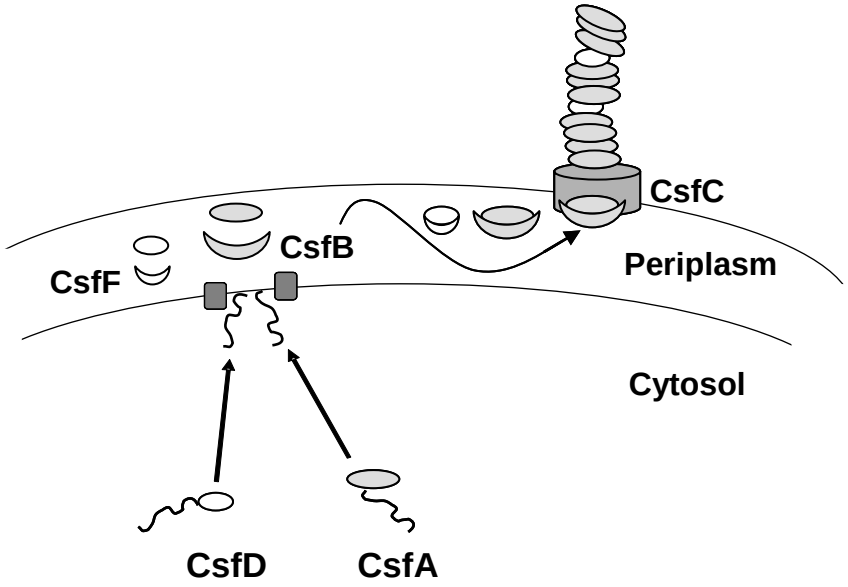

Figure 4. Model of expression of CS5 on the bacterial surface.
The first step in CS5 surface expression is translocation of the CS5 structural subunits across the inner membrane, which occurs by the Sec machinery. In the periplasm, the major subunit CsfA is bound to the chaperone CsfB and the minor subunits CsfD and CsfE are bound to the chaperone CsfF. Assembly of CS5 has been proposed to be initiated by binding of the minor subunit-chaperone complex (CsfD-CsfF) to the outer membrane assembly protein CsfC, resulting in translocation of CsfD across the outer membrane. Elongation of the CS5 structure occurs by several deliveries of the major subunit CsfA in complex with the chaperone CsfB (CsfA-CsfB) to CsfC and incorporation of CsfA into the growing CS5, as well as further interactions between CsfD-CsfF and CsfC; the rate of incorporation of CsfA and CsfD has been suggested to depend on the stoichometric ratio of the two subunits in the periplasm. The elongation is terminated when a CsfE-CsfF complex interacts with CsfC, believed to result in irreversible association of CsfE with CsfC and thereby preventing further incorporation of CsfA and CsfD (not shown). However, CsfF and not CsfE has been shown to be rate-limiting for the determination of pilus length. Figure adapted from [81].

Expression of CS6

Unlike most other CFs, CS6 is non-fimbrial. Its exact morphology has not been determined but it has been suggested to be a very fine fibril [11, 78, 83]. CS6 is also unusual in that it is composed of two major antigenically distinct structural subunits (CssA and CssB) [11, 83]; most other CFs, *e.g.* CS5, consist of a single major subunit and one or more minor subunits. The molecular weights of CssA and CssB are 14.5 and 16.0 kDa, respectively.

The operon for biosynthesis of CS6, *cssABCD*, contains four open reading frames encoding the two structural subunits (CssA and CssB), a periplasmic chaperone (CssC), and a molecular usher (CssD) and was first described in 1997 [83]. The entire operon has been sequenced in two LT-only strains expressing CS5 and CS6 (GenBank accession number UO4844, strain E10703) and CS4 and CS6 (GenBank accession number UO4846, strain E8755) [83]. The amino acid sequences from the two strains

differ at 11 positions in CssA and at 4 positions in CssB. The organization of the operon is shown below (Fig. 5).

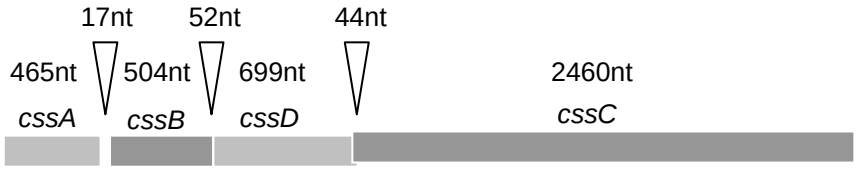

Figure 5. The CS6 operon (based on PubMed accession number UO4844).
The operon contains four open reading frames and two untranslated intragenic regions, one between *cssA* and *cssB* encoding the structural subunits and one between *cssB* and the chaperone gene (*cssC*), whereas there is a region of overlap between *cssC* and the gene encoding the usher (*cssD*). The untranslated region after *cssB* contains a sequence of dyad symmetry (six nucleotides downstream from *cssB*) [11, 83]. nt; nucleotides.

Similarly to many other virulence genes in *E. coli*, the CS6 operon and the operons encoding CFA/I, CS1, CS2 and CS3 have a much lower GC content (approximately 34% for CS6) than is normal for other *E. coli* genes, as well as a codon usage that is seen for *E. coli* genes that are expressed at low or very low levels. These CF operons, including the CS6 operon, are flanked by insertion sequences suggesting a non-*E. coli* origin [11, 83].

Phenotypic expression of CS6 on the bacterial surface starts by transportation of the structural subunits from the cytosol across the inner membrane to the periplasmic space and a presumed model of the surface expression is depicted on the facing page (Fig. 6).

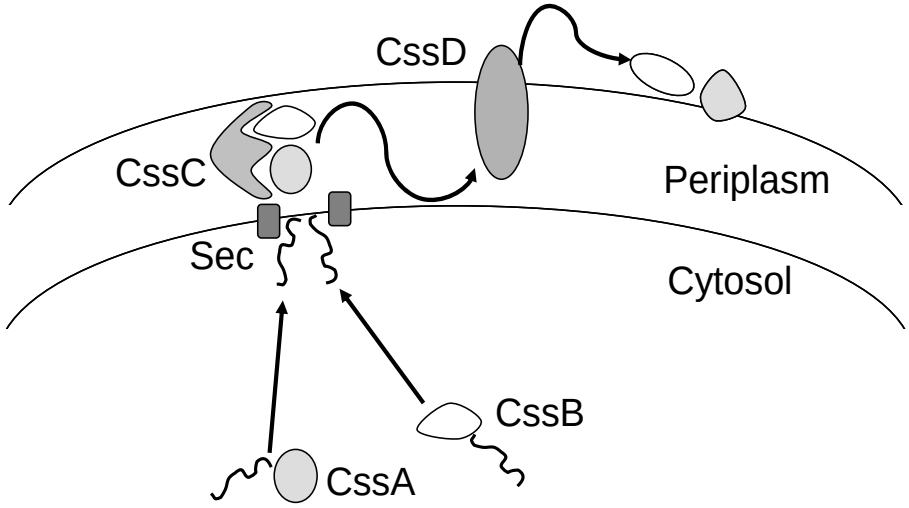

Figure 6. Model of expression of CS6 on the bacterial surface.
All four genes include a typical signal sequence for exported proteins and it can be assumed that translocation of the structural subunits across the inner membrane is mediated by the Sec machinery of the general secretory pathway (GSP) [84]. Once the subunits are released into the periplasm the chaperone subunit (CssC) is believed to protect the structural subunits from proteolytic degradation and to transport them across the periplasm to the outer membrane, where the usher (CssD) is believed to translocate CssA and CssB to the bacterial surface. However, phenotypic expression of CS6 does not seem to require the entire CssD since CS6 was detected on the bacterial surface in recombinant strains where only the N-terminal one-third of *cssD* was present [83].

AIMS OF THE THESIS

The overall aims of this thesis were to study the genotypic and phenotypic expression of the ETEC virulence factors ST, LT, CS5 and CS6 *in vivo* and *in vitro*, and to determine the genetic variability of ETEC strains.

The specific aims were:

- To investigate the relative transcription levels of the genes encoding ST (*estA*) and LT (*eltB*) of ETEC strains *in vivo* and *in vitro*.

- To investigate the relative transcription levels of the genes encoding CS5 (*csfD*) and CS6 (*cssB*) *in vivo* and *in vitro*.

- To identify environmental factors in the human intestine that may up- or down-regulate the transcription, production, and secretion of ST and LT or phenotypic expression on the bacterial surface of CS5 and CS6 *in vitro*.

- To try to explain lack of phenotypic expression of CS6 on the bacterial surface of genotypically CS6-positive LT-only ETEC strains.

- To determine the genetic relationship between ST-only CS6 positive ETEC strains infecting children and travellers to the same ETEC endemic area.

MATERIALS AND METHODS

This section describes the bacterial strains and methods used in this work. More detailed descriptions of the methods used can be found in the papers and manuscripts included at the end of this thesis.

BACTERIAL STRAINS AND CULTURE CONDITIONS

The clinical bacterial strains used in this thesis were either derived from diarrhoeal stool specimens collected at the hospital of the International Centre for Diarrhoeal Disease Research (ICDDR,B) in Dhaka, Bangladesh (I, II and III) or collected in India and Guinea-Bissau (IV). The details of these strains as well as two recombinant strains used in Paper IV are listed below (Table 1).

Table 1. Wild-type and recombinant ETEC strains included in the thesis.

Paper	Strain	Origin	Toxin profile	Colonization factors	References
Clinical strains isolated from stool specimens from adult patients at the ICDDR,B hospital.					
I	D16	Bangladesh	STh + LT	CS5 + CS6	*This study*
I	D17	Bangladesh	STh + LT	CFA/I	*This study*
I	D19	Bangladesh	STh + LT	CS5 + CS6	*This study*
I	D21	Bangladesh	STh + LT	CS1 + CS3	*This study*
I	D22	Bangladesh	STh + LT	CFA/I	*This study*
I	D24	Bangladesh	STh + LT	CFA/I	*This study*
I	D36	Bangladesh	STh + LT	CS2 + CS3	*This study*
I	D39	Bangladesh	STh + LT	CS5 + CS6	*This study*
I	D40	Bangladesh	STh	CS6	*This study*
I	D42	Bangladesh	STh + LT	CS5 + CS6	*This study*
I	D44	Bangladesh	STh + LT	CS5 + CS6	*This study*
II, III	2527507	Bangladesh	STh + LT	CS5 + CS6	*This study*
II, III	2533435	Bangladesh	STh + LT	CS5 + CS6	*This study*
II, III	2545618	Bangladesh	STh + LT	CS5 + CS6	*This study*
II, III	2619767	Bangladesh	STh + LT	CS5 + CS6	*This study*
ETEC strains from India and Guinea-Bissau.					
Paper	**Strain**	**Origin**	**Toxin profile**	**CS6 phenotype / genotype**	**References**
IV	GB60	Guinea-Bissau	LT	CS6- / *cssABCD*	[85]
IV	GB76	Guinea-Bissau	LT	CS6- / *cssABCD*	[85]
IV	Ind290	India	LT	CS6- / *cssABCD*	[59, 61]
IV	Ind325	India	LT	CS6- / *cssABCD*	[59, 61]
IV	Ind329	India	LT	CS6- / *cssABCD*	[59, 61]
IV	Ind380	India	LT	CS6- / *cssABCD*	[59, 61]
IV	Ind424	India	LT	CS6- / *cssABCD*	[59, 61]
IV	Ind598	India	LT	CS6- / *cssABCD*	[59, 61]
IV	GB35	Guinea-Bissau	LT	CS6+ / *cssABCD*	[85]
IV	GB112	Guinea-Bissau	LT	CS6+ / *cssABCD*	[85]
IV	GB124	Guinea-Bissau	STp	CS6+ / *cssABCD*	[85]
IV	WSO14862	Egypt	LT	CS6- / *cssABCD*	*This study*
IV	WSO14889	Egypt	LT	CS6- / *cssABCD*	*This study*
Recombinant strains.					
IV	GB60::pJTCS635		LT	CS6+ / *cssABCD*	*This study*
IV	GB60::pJTCS6329		LT	CS6+ / *cssABCD*	*This study*

In addition to the above strains, 24 CS6 positive ST-only strains isolated in Mexico and Guatemala were included but the details of these strains are given in Paper V.

Collection of stool specimens and preparation of *in vivo* and *in vitro* samples (I, II and III)

The procedure for collection of acute watery diarrhoeal stool specimens from randomly selected adult patients at the ICDDR,B hospital is outlined below (Fig. 7).

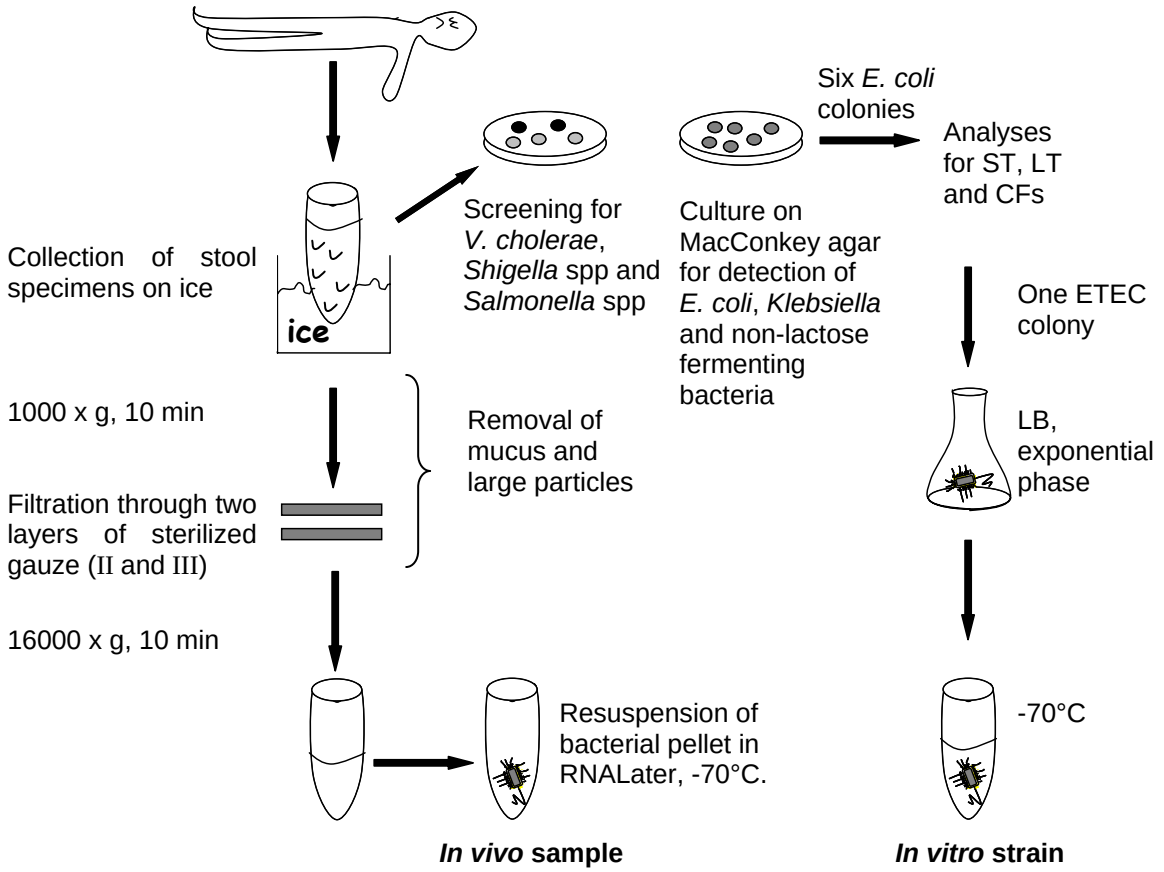

Figure 7. Collection and characterization of *in vivo* and *in vitro* ETEC samples from clinical stool specimens. The stool specimens (10-50 ml) were immediately placed on ice and brought from the hospital wards to the laboratory where they were screened for *V. cholerae*, *Shigella* spp and *Salmonella* spp by standard methods [49, 86]. Presence of lactose-fermenting, *e.g. E. coli* and *Klebsiella*, and non lactose-fermenting bacteria was determined by culture of the stool on MacConkey agar. For detection and characterization of ETEC strains, six lactose-fermenting colonies resembling *E. coli* were individually tested for ST and LT by ELISA assays and against an array of MAbs specific for the CFs CFA/I, CS1-8, CS12, CS14, CS17 and CS21 by dot-blot assays [87-89]. The toxin genotype was determined by PCR analyses of DNA from a pool of the six colonies for *estA* (STh), *st1* (STp) and *eltB* (LT). For ETEC-positive stool specimens, one ETEC colony was grown to exponential phase in LB medium and stored at -70°C in 20% glycerol; these samples are referred to as the "*in vitro* strains". The corresponding "*in vivo* samples" were prepared from the stool specimens as illustrated, stored in RNALater® (Qiagen) and shipped on dry ice to Göteborg for total RNA extraction.

Culture media and conditions (I, II, III and IV)

Bacterial strains were grown to exponential phase in LB (I) or CFA (IV) for transcriptional analyses, or overnight under several different aerobic and anaerobic conditions (II, III and IV) for transcriptional and / or phenotypic analyses. Aerobic media, *i.e.* CFA (1% casamino acids, 0.15% yeast extract, 0.41 mM $MgSO_4$, 0.04 mM $MnCl_2$) [90], LB (1% tryptone peptone, 0.5% yeast extract, 0.17 M NaCl) 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. Anaerobic CFA and minimal basal media [91, 92] were prepared in collaboration with the Deep Biosphere Laboratory at the Institute of Cell and Molecular Biology-Microbiology, Göteborg University. All culturing was performed at 37°C.

Construction of recombinant strains (IV)

Two plasmids containing the CS6 operon (*cssABCD*) were constructed by cloning of *cssABCD* from the two wild-type ETEC strains GB35 and Ind329 into a low-copy number expression vector (pJTtac; J Tobias, unpublished); resultant plasmids (pJTCS635 and pJTCS6329) are illustrated schematically in Fig. 8. The recipient strain was the wild-type strain GB60; wild-type and recombinant strains are listed in Table 1.

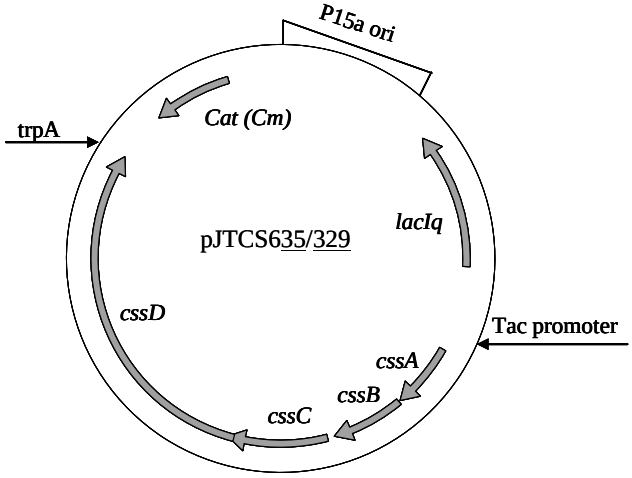

Figure 8. Schematic illustration of recombinant pJTtac vector with *cssABCD*.

trpA; tryptophan transcription terminator, *Cat* (Cm); chloramphenicol resistance gene, P15a ori; origin of replication, *lacIq*; Tac promoter regulatory gene.

PHENOTYPIC METHODS

Quantification of ST and LT (II)

ST was quantified by a GM1-based inhibition ELISA and LT was quantified by a GM1-based ELISA assay, using an anti-ST MAb and an anti-LTB/CTB MAb, respectively, and GM1 as coating antigen (II). An ST-CTB conjugate was used in the inhibition step in the ST assay, which is based on competition for the MAb between the conjugate and toxin in the sample.

Detection and quantification of CS5 and CS6 (III and IV)

Detection of CS6 on the bacterial surface was performed by dot-blot analyses essentially as described [61] using a concentration of 2×10^{10} bacteria per ml and the monoclonal antibody (MAb) CS6-2A:14 [61], which recognizes the two structural subunits CssA and CssB (IV).

Presence of CS6 in whole-cell lysates and periplasmic preparations was detected by SDS-PAGE and immunoblot assays (IV) using two MAbs that recognize CssA and CssB, respectively. A MAb directed against the heat-shock protein DnaK (Assay Designs, Inc., Ann arbour, US) was used as a control of protein load. Whole-cell bacterial lysates were prepared from bacteria cultured overnight on CFA agar by a method utilizing thermal cell lysis, SDS as detergent, and DL-Dithiothreitol as reducing agent (IV).

Periplasmic preparations were made using a previously described protocol with slight modifications [93] by treating the bacterial cells with a sucrose solution containing EDTA followed by cold-shock treatment with a hypotonic solution of $MgCl_2$. Presence of periplasmic proteins was verified by a chromogenic reaction between alkaline phosphatase and 4-nitrophenyl phosphate disodium (Sigma-Aldrich). Protein concentrations were determined spectrophotometrically and the samples were stored at -20°C.

Quantification of phenotypic expression of CS5 and CS6 on the bacterial surface after *in vitro* culture in different growth media was performed by inhibition ELISA assays (III). This method has been described previously for CS6 [59] and is based on competition between bound antigen and antigen in the bacterial samples for a MAb against CS5 (CS5-5-1:1) or CS6 (CS6-2A:14), respectively.

Determination of serum antibody responses (III)

Antibody titers of IgA and IgG isotypes against CS5 and CS6 were determined by ELISA assays as previously described [94] using purified CS5 or recombinant CS6 as coating antigens.

Protein modelling (IV)
Protein modelling was performed in collaboration with Dr Lars Brive at the Department of Cellular and Molecular Biology, Göteborg University, and is described in Paper IV.

GENOTYPIC METHODS

Isolation of total DNA (II, III, IV and V)
Total DNA was isolated from bacterial cells by the DNeasy® Tissue Kit (Qiagen) (II, III and IV) or by the "rapid boil" method (V). DNA concentrations were determined spectrophotometrically and DNA samples were stored at -20°C. The isolated DNA was used for sequencing (IV and V), construction of recombinant strains (IV), and establishment of standard curves for real-time RT-PCR (II and III).

Toxin multiplex PCR (V)
Toxin multiplex PCR for STh (*estA*), STp (*st1*) and LT (*eltB*) was performed using a protocol developed in our group [87].

Isolation of total RNA (I, II, III and IV)
Total RNA was isolated from 1 x 10^9 bacterial cells in exponential phase (I and IV) or stationary phase (II and III) after culture *in vitro* and from *in vivo* samples by using the RNeasy® Mini Kit (Qiagen). The non-cultured *in vivo* bacteria preserved in RNALater® were thawed on ice and spun down at 4°C before RNA extraction using an appropriate volume of lysozyme and RLT buffer depending on the size of the pellet. Integrity of the RNA and absence of DNA after DNase treatment was determined by gel electrophoresis. Concentrations were determined spectrophotometrically and samples were stored at -70°C.

RT-PCR (IV)
For detection of transcription of the CS6 operon, RT-PCR was performed using gene-specific first-strand cDNA (*cssA*, *cssB*, *cssC* and *cssD*) which had been synthesized from DNase-treated total RNA with the Sensiscript® RT Kit (Qiagen) immediately prior to the RT-PCR assay (IV). Negative controls without reverse transcriptase (-RT) were prepared in parallel with the cDNA from the same amount of RNA for all samples and were included in subsequent analyses.

Quantification of virulence gene expression; QCRT-PCR (I)
In Paper I, quantification of gene transcription (*estA* and *eltB*) in *in vivo* and corresponding *in vitro* samples was performed by QCRT-PCR as previously described for quantification of gene transcription in *Helicobacter pylori* [95] but with modifications, *i.e.* using fluorescent labelled forward primers and separation by

capillary gel electrophoresis on an ABI310 DNA sequencer (Applied Biosystems). Total DNase-treated sample RNA and a known amount of an RNA competitor specifically constructed for each gene were reverse transcribed into cDNA in the same gene-specific RT-PCR reaction. Thereafter the target cDNA and the competitor cDNA were co-amplified in the same PCR reaction using fluorescent forward primers labelled with 6-FAM, HEX or TET that allowed detection of the respective genes on the ABI310. The competitors were shorter than the expected length of the correct cDNA fragments but were amplified by the same PCR primers. The *E. coli* 16S gene *rrsH* was used as an external reference to normalize the expression values of *estA* and *eltB* and to account for possible degradation of RNA in the *in vivo* samples prior to RNA extraction.

Quantification of virulence gene expression; real-time RT-PCR (II and III)

In Papers II and III quantification of gene transcription (*estA*, *eltB*, *csfD*, *cssB*, and *gapA*) was performed by real-time RT-PCR assays by absolute quantification using a PCR-product based standard curve covering the concentration range of 10-10^7 gene copies / 2 μl. The standard curve was generated by ten-fold serial dilutions of a known amount of gene-specific PCR product (calculated as described in Paper I), which had been amplified by conventional PCR using the real-time PCR primers. cDNA was synthesized from a maximum of 200 ng of DNase-treated total RNA by using the QuantiTect Reverse Transcription Kit (Qiagen) to allow for quantification of transcription of different genes in a single sample, and negative controls without reverse transcriptase were prepared in parallel from the same amount of total RNA for each RNA sample. The assays were performed on an ABI 7500 using SYBR® Green I (Applied Biosystems) as detector and newly designed primers for *csfD* and *gapA* (III), primers cssBF1 and cssBR1 (IV, Table 2), and real-time PCR primers for *estA* and *eltB* previously developed in our group [35]. The specificities of the primers were determined by a BLAST search of the NCBI genome database. The real time RT-PCR assays were run in 20 μl reactions using a maximum of 20 ng cDNA or 2 μl standard curve DNA, 8 pmol of each primer and Power SYBR® Green PCR Master Mix (Applied Biosystems). After an initial 10 min denaturation step at 95°C, the reactions were subjected to 45 cycles comprising 15 sec of denaturation at 95°C and 60 sec of annealing and elongation at 60°C, followed by a dissociation step for melting temperature (T_M) analysis of each amplification product to confirm amplification specificity [35]. Quantification was performed by using the instrumental software with default settings for threshold and baseline values (Applied Bioystems). All individual experiments were run in duplicates. Samples, standard curve and controls without reverse transcriptase were analyzed in the same assay and results were compensated for contaminating DNA as described in Paper III.

Genetic typing (IV and V)

The genetic relationship between wild-type strains was determined by random amplification of polymorphic DNA (RAPD)-PCR (IV) or multilocus sequence typing (MLST) (V). RAPD-PCR utilizes a single short primer with an arbitrary chosen sequence, combination with a low annealing temperature. In this case a primer previously used for ETEC [40, 96] was used. A difference of two or more bands in the banding patterns between two strains was interpreted as an indication that the two strains were different [97]. The banding patterns were visually inspected on three gels and by two individuals; on each inspection the strains were grouped into the same RAPD profiles.

The MLST typing method is based on sequencing of internal regions of a number of housekeeping genes [98]. The MLST scheme used in Paper V was developed by Mark Achtman for analyses of *E. coli* strains and includes seven housekeeping genes; *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA* [99]. The conditions and primers for PCR amplification are described on a website maintained by the Max-Planck Institut für Infektionsbiologie (http://web.mpiib-berlin.mpg.de/mlst/dbs/Ecoli). Purified PCR products were shipped to Germany for sequencing in both directions at the MWG sequencing facility (MWG Biotech, Martinsried, Germany), using the same primers as for PCR amplification. Analyses of the MLST results was performed in collaboration with Dr John Klena at the US Naval Medical Research Unit in Cairo and is described in Paper V.

Sequencing of *cssABC* (IV and V)

Sequencing of *cssA*, *cssB* and *cssC* and non-coding intergenic regions was performed in both directions using the primers listed in Table 2 and purfied PCR products amplified using primers cssAF1 and cssDR1 (IV) or cssBF2 and cssCR3 (V). The relative positions of the primers are shown schematically in Fig. 9. In Paper IV sequencing was initially performed using the 1.1 Big Dye Ready Reaction Mix and an ABI310 Genetic Analyzer (Applied Biosystems) in our department. The results were confirmed by sequencing at the Swegene Göteborg Genomics resource facility on an ABI3730 (Applied Biosystems) using the 3.1 Big Dye Ready Reaction Mix; both methods are based on incorporation of fluorescence-labelled nucleotides into a growing PCR product which induces termination of elongation, followed by capillary polyacrylamide gel electrophoresis of the products. Analysis of the sequence data is described in Papers IV and V.

Table 2. Primers used for sequencing of *cssABC* (IV, V).

Primer	Sequence	Direction	Position [a]	Paper
cssAF1	5′-GGCAGCCATGCCAGAACAGA-3′	Forward	43-62	IV
cssBF1	5′-CAGGAACTTCCGGAGTGGTA-3′	Forward	520-539	IV
cssBF2	5′-GATTCTGGCGCTGGTAAGTT-3′	Forward	819-838	IV, V
cssBR1	5′-CTGTGAATCCAGTTTCGGGT-3′	Reverse	652-671	IV
cssCF1	5′-TCAATGCCACCAACAGAAAA-3′	Forward	1354-1373	IV
cssCR1	5′-CATCCCCGAATGCTGATATT-3′	Reverse	1417-1436	IV
cssCR3	5′-CTTTCCATTTTTATGCCATTTT-3′	Reverse	1503-1524	V
cssDR1	5′-CCTGATGGTAGGAATCGTGAGTCAAAA-3′	Reverse	1837-1863	IV

[a] Refers to position relative to the *cssA* start site in the previously published CS6 sequence (GenBank accession number UO4844).

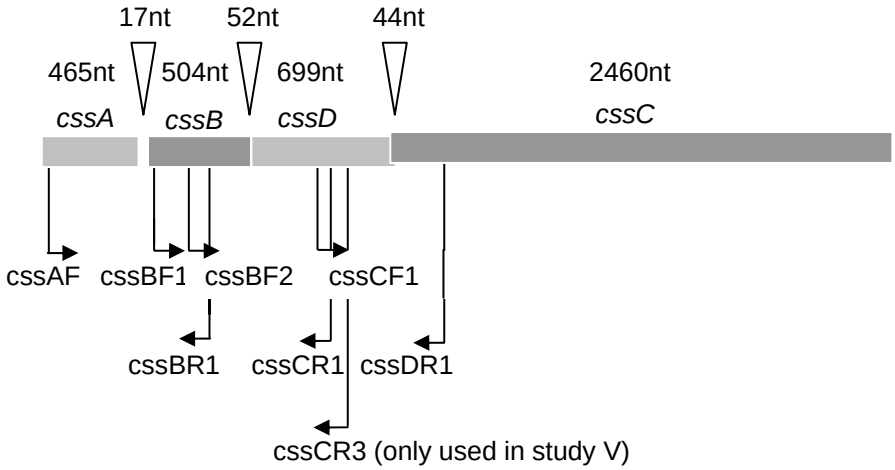

Figure 9. Relative positions of the primers used for sequencing of *cssABC* (IV and V). The lengths of non-coding intergenic and overlapping regions are shown as inserts (GenBank accession number UO4844).

Statistics (I, II and III)

In Paper I the paired non-parametrical Wilcoxon test was used for statistical analyses. In Papers II and III we used the non-parametrical Mann-Whitney test. The statistical analyses were performed using the Prism software system (GraphPad Software Inc, San Diego, California). $P < 0.05$ was considered significant.

RESULTS AND COMMENTS

Collection of clinical ETEC samples (I, II and III)

The first aim of my studies was to investigate the expression of the enterotoxins ST and LT and colonization factors CS5 and CS6 during infection of the human intestine. This was performed by quantifying the transcription levels of *estA* (ST), *eltB* (LT), *csfD* (CS5) and *cssB* (CS6), respectively, in *in vivo* bacterial samples, *i.e.* bacteria isolated directly from diarrhoeal stool specimens without *in vitro* sub-culturing. For comparison the transcription levels were also quantified in the corresponding *in vitro* strains after further culture *in vitro*.

Optimization of collection of *in vivo* samples (I and III)

The *in vivo* samples were derived from diarrhoeal stool specimens that were collected at the hospital of ICDDR,B in Dhaka from adult patients with acute watery diarrhoea. In Paper I the *in vivo* samples were collected by staff at the Immunology Laboratory and hospital ward staff at ICDDR,B during two ETEC peak seasons in 2001 and 2002. In the spring of 2005 I was given the opportunity to visit ICDDR,B for collection of the *in vivo* samples to be included in Paper III and corresponding *in vitro* samples to be included in Papers II and III. During this collection period we succeeded in optimizing the protocol for collection of *in vivo* samples so as to reduce the amount of mucus present in the samples and to decrease the degradation of the RNA, which had previously been a problem that forced us to exclude several samples collected for analysis of *estA* and *eltB* gene transcription *in vivo* (I). The improvement in sample preparation was achieved by adding an initial step of filtration through two layers of sterilized gauze (Fig. 7 in Materials and methods), and by decreasing the time-span from collection of stool to resuspension of the *in vivo* samples in RNALater® from several hours to just above one hour.

Epidemiology of ETEC strains (II and III)

In connection to my visit to Dhaka in 2005, a total of 127 stool specimens were collected during the peak ETEC season between April and June. Out of these, 34 were positive for ETEC (26.8%), which is similar to data from a larger study including 1800 patients performed at the ICDDR,B hospital from April 2003 to October 2005, where the corresponding number was 18% [94]. One of the 34 stool specimens contained an ETEC strain positive for CS6 (2.9%), one contained a CS4 + CS6 positive strain (2.9%), and five specimens contained a CS5 + CS6 positive strain (14.7%). In other words, a total of 20.6% of the ETEC strains expressed CS4, CS5 and / or CS6, in consistency with previous findings worldwide [54]. All CS5 + CS6 positive strains expressed both ST and LT.

The operons encoding ST, LT, CS5 and CS6 are transcribed in disseminated (*in vivo*) bacteria (I and III)

Upon collection, the *in vivo* samples were stored in the RNA preserving buffer RNALater® and shipped to Sweden on dry ice. After arrival in Göteborg total RNA was extracted and converted into cDNA to determine the transcription levels of the four virulence factors. In the first study (I) we investigated the transcription of the genes encoding STh (*estA*) and the LT B-subunit (*eltB*).

Transcription of *estA* (ST) and *eltB* (LT) *in vivo* (I)
In this study eleven of the *in vivo* samples collected in 2001 and 2002 (Table 1, Materials and methods) were included; total RNA was extracted from 23 samples but 12 were excluded due to observed degradation of the RNA or since they contained detectable levels of bacterial DNA, as determined by PCR analysis for the 16S gene *rrsH* and / or detectable mRNA from the human *β-actin* gene.

The *in vivo* samples were analyzed by QCRT-PCR and it was found that both *estA* and *eltB* were transcribed in the disseminated bacteria isolated directly from diarrhoeal stool. Also, one or both toxins were detected in a majority of the stool specimens, indicating production of the toxins during infection.

Next, we wanted to determine whether the toxin genes were up- or down-regulated under the *in vivo* conditions in disseminated stool as compared to under laboratory conditions *in vitro*, *i.e.* LB medium. In order to account for the fact that the *in vivo* samples may also contain RNA from other bacterial species than *E. coli*, the mRNA levels in the *in vivo* and *in vitro* samples were normalized against the RNA levels of the *E. coli* 16S rRNA gene *rrsH*. Using this approach, we found that in three of the ST/LT samples both genes were transcribed at higher levels in the *in vivo* samples than after culture in LB, in six samples both genes were transcribed at lower levels, and in one sample *estA* was transcribed at a lower level whereas *eltB* was transcribed at a higher level *in vivo*. In the ST-only sample included (D40, Table 1) *estA* was also transcribed at a lower level *in vivo*. Thus, we could not determine any significant up- or down-regulation in the transcription level *in vivo* of either *estA* or *eltB* as compared to *in vitro* (Fig. 10). However, since stool specimens are heterogenous and may contain other pathogenic and commensal *E. coli*-like species also expressing the *rrsH* gene, it is possible that the actual transcription levels per bacterium *in vivo* were in fact underestimated in some samples.

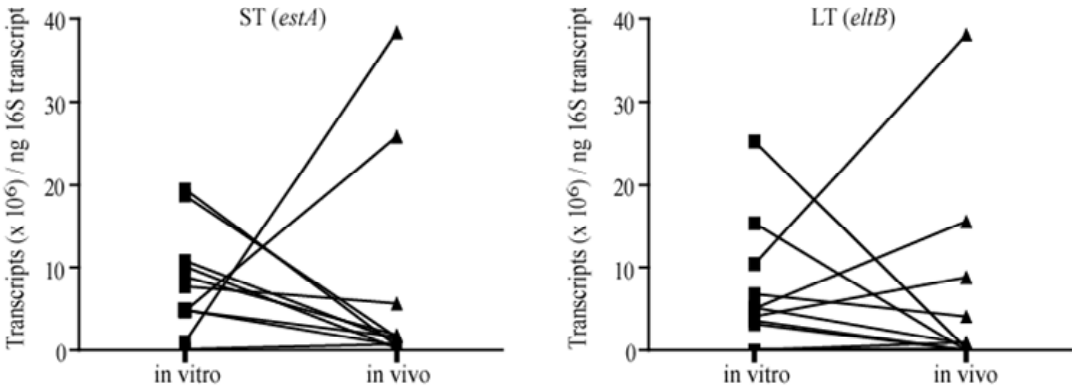

Figure 10. Transcription levels of *estA* and *eltB in vivo* and *in vitro* (I).

Transcription of *csfD* (CS5) and *cssB* (CS6) *in vivo* (III)

In Paper III, instead of using the QCRT-PCR method, we developed real-time RT-PCR assays for absolute quantification of the genes encoding the CS5 minor subunit (*csfD*), the CS6 structural subunit CssB (*cssB*), and the *E. coli* housekeeping gene *gapA* which was included as a control for intact *E. coli* mRNA in the *in vivo* samples. In the case of CS5 the minor rather than the major subunit was chosen as target in order to avoid cross-amplification of the major subunit of CS7, which is genetically and immunologically related to CS5 [11].

The transcription levels of *csfD* and *cssB* were quantified in three of the CS5 + CS6 positive ST/LT *in vivo* samples (2527507, 2533435 and 2545618) collected at ICDDR,B in 2005 and in a CS5 + CS6 positive ST/LT *in vivo* sample (2619767) collected in 2006. Transcription of *csfD* and *cssB* was detected in all samples except for 2533435, and this was not due to degradation of the mRNA in this sample since it was positive for *gapA* mRNA.

In order to determine whether transcription of the genes encoding CS5 and CS6 in the disseminated bacteria reflected phenotypic expression of CS5 and CS6 during infection, the IgA and IgG responses to both CS5 and CS6 were measured in serum samples collected from the three patients enrolled in 2005, *i.e.* 2527507, 2533435 and 2545618. At early convalescence, *i.e.* on day seven from hospitalization, the patients infected with strains 2527507 and 2545618 had developed strong IgA and IgG responses to both CS5 and CS6. On the other hand, the patient infected with strain 2533435 only reacted modestly, which was in agreement with the finding that the corresponding *in vivo* sample was negative for transcription of the CS5 and CS6 operons. Interestingly, however, the patient infected by strain 2533435 had a co-infection with *V. cholerae*, and further studies are needed to determine if presence of

this pathogen affects expression of the ETEC colonization factors, or if transcription and a strong immune response could simply not be detected due to presence of too few ETEC bacteria.

Similarly to the samples collected in 2001 and 2002 (I), the number of ETEC bacteria in the stool specimens collected in 2005 was not known, and due to the presence of commensal and / or other pathogenic *E. coli*-like species in the clinical stool specimens the use of an *E. coli* reference gene for normalization is not the optimal solution to determine how much of the total RNA in these *in vivo* samples that was actually derived from the respective ETEC strains. Therefore, in Paper III we instead calculated the ratio between the absolute transcription levels of *csfD* and *cssB* in the three *in vivo* samples that were positive for transcription, *i.e.* 2527507, 2545618 and 2619767. Thus, the CS5 operon (*csfD*) was found to be transcribed at a higher level than the CS6 operon (*cssB*) in all three samples; the average ratio between the transcription levels of *csfD* and *cssB* was found to be 108:1 (182:1 for 2527507, 65:1 for 2545618 and 76:1 for 2619767).

For comparison, the corresponding *in vitro* strains were cultured under a variety of growth conditions *in vitro* described in the next section (Fig. 11). Similarly to the disseminated bacteria, *csfD* was found to be transcribed at higher levels than *cssB* under all *in vitro* conditions tested, but the average ratio between the transcription levels of *csfD* and *cssB* was found to be 10-fold lower *in vitro* (11:1) as compared to *in vivo* (108:1). Taken together, these results indicate a 10-fold shift towards transcription of the CS5 operon in comparison with the CS6 operon in disseminating ETEC bacteria, which may indicate that transcription of the CS5- and CS6-encoding operons is not co-regulated.

When sample 2619767 was collected in 2006 an additional step was added to the collection procedure in order to allow for comparison of absolute transcription levels between the two genes, which was achieved by estimating the transcription levels in a single ETEC bacterium. Thus, DNA was extracted from 1 ml of the stool after removal of mucus (Fig. 7, Materials and methods) and the number of *estA* gene copies in this volume was determined by real-time PCR to be 1.046×10^7. ETEC strains have previously been shown to carry between one and two *estA* gene copies per bacterium [35], and we therefore assumed that 1 ml of stool from the patient infected with strain 2619767 contained approximately 10^7 bacteria. Since the RNA from this sample was also extracted from 1 ml of the stool after removal of mucus the number of total *csfD* and *cssB* transcripts in 1 ml, *i.e.* in 10^7 bacteria, could be calculated based on our real-time RT-PCR results. The number of transcripts in a single bacterium was also calculated after *in vitro* culture. For *csfD* the number of transcripts per bacterium was found to be 1.26×10^{-2} *in vivo* and 1.81×10^{-4} on average *in vitro*, and for *cssB* the number of transcripts was found to be 1.66×10^{-4} *in vivo* and 1.4×10^{-5} *in vitro*. These results suggest an approximately 100-fold up-regulation of

csfD and a 10-fold up-regulation of *cssB* under *in vivo* conditions in disseminated stool as compared to under laboratory conditions. This experiment was only performed in one strain, but the results are supported by the approximately 10-fold shift towards *csfD in vivo* in all the three strains tested as reported above.

In conclusion, these results indicate that although both *csfD* and *cssB* seem to be up-regulated *in vivo* compared to *in vitro* there is a preferential up-regulation of the CS5 encoding operon in disseminated bacteria, which may suggest that the environment in the human intestine favours transcription of *csfD* rather than *cssB*.

ST, LT, CS5 and CS6 are differentially regulated by environmental factors *in vitro* (II and III)

In vitro culture conditions and experimental set-up

In order to study the effect of the host environment on the expression of ST, LT, CS5 and CS6, the four clinical strains collected in Dhaka in 2005 and 2006 were cultured under various *in vitro* conditions designed to pin-point some of the environmental factors present in the gastro-intestinal tract, *i.e.* lack of oxygen, presence of bile salts and the carbon source. In addition, the classical ETEC strain H10407 was included in Paper II. The experimental setup and the growth conditions tested are outlined in Fig. 11.

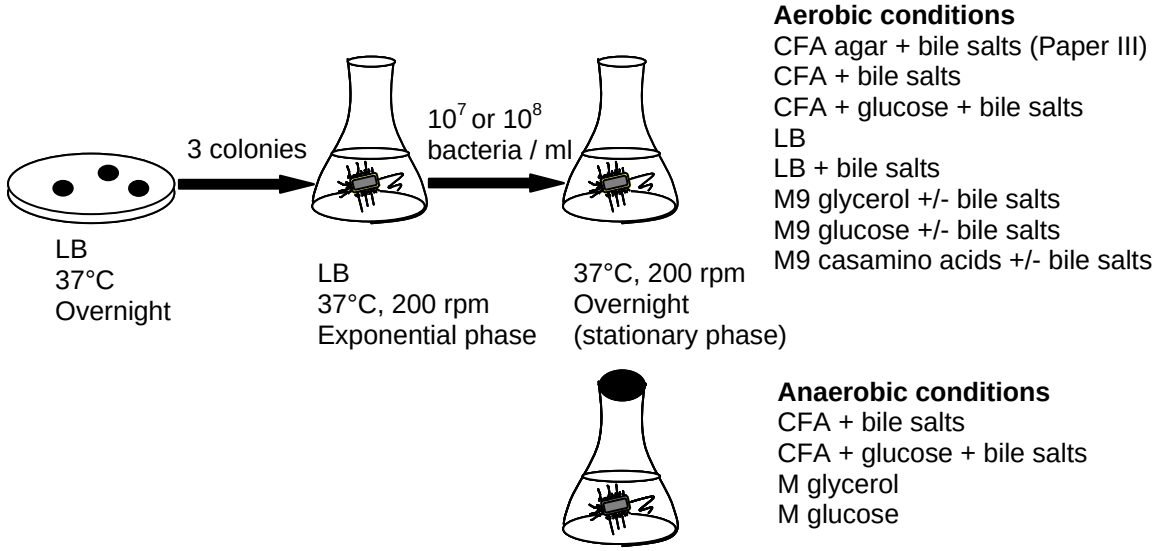

Figure 11. Experimental setup of *in vitro* growth condition studies (II and III).
The strains were first cultured on LB plates overnight (37°C). On the following day three colonies were picked and cultured in 10 ml LB medium with aerated shaking (37°C, 200 rpm) for 3-5 hours to reach the exponential phase. The bacteria were transferred to 20 ml of the culture media to be tested at an initial concentration of 10^7 or 10^8 bacteria / ml and were cultured overnight with shaking (37°C, 150 rpm). Cultures were initially set to pH 7. Ox-bile extract, mainly consisting of sodium glycocholate and sodium taurocholate, was added at a concentration of 0.15% to LB and CFA or 0.015%, a concentration used by other groups (personal communication), to M9 media. CFA; [90], LB; Luria Bertani broth, M9; defined minimal medium, M; basal minimal medium [91].

ETEC, like all *E. coli*, are facultative anaerobes, meaning that they are able to adjust their metabolic pathways to the available oxygen levels in order to generate energy by either aerobic or anaerobic respiration or by fermentation, depending on the available electron acceptors in the environment. A striking aspect of conventional culture conditions in the laboratory is the vigorous shaking with optimal aeration as opposed to the anaerobic conditions present in the small intestine, which is the site of ETEC infection. Instead of using the commonly used method of simply sealing a culture flask filled completely with culture media in order to obtain an anaerobic environment *in vitro* [100], we collaborated with Dr Sara Eriksson at the Deep Biosphere Laboratory at Göteborg University who prepared the anaerobic media used in studies II and III, using the same protocol as for culture media for obligate anaerobes.

Expression of ST after *in vitro* culture (II)
Using real-time RT-PCR the *estA* gene was found to be transcribed in all media. Significantly higher levels were found under anaerobic growth conditions than under aerobic conditions. However, the highest levels were reached in aerobic and anaerobic minimal media with glycerol.

Secretion of ST was determined in the supernatant from 1 ml of bacterial culture and adjusted to ng / 10^9 bacteria. However, the total production of ST could not be quantified since the MAb used in the ELISA assay only detects the mature toxin, *i.e.* secreted ST. ST was found to be secreted from the clinical strains in all LB and CFA media tested and the optimal media for ST secretion were found to be aerobic complex media, *i.e.* LB and CFA, which both contain amino acids.

Even though aerobic M9 minimal media were less favourable for secretion of ST than LB and CFA (Fig. 12A), we were able to establish that the preferred carbon source for ST secretion was casamino acids, since secretion of ST was significantly higher in aerobic minimal media (M9) supplemented with casamino acids than in M9 with glycerol or M9 with glucose, respectively (Fig. 12B). The casamino acids were added to the minimal media at the same concentration as to the CFA media, *i.e.* 1% (w/v), indicating that the presence of amino acids in the LB and CFA media contribute to the optimal ST secretory conditions.

In minimal media ST secretion could not be detected at all under anaerobic conditions, *i.e.* in basal minimal media supplemented with glucose or glycerol. However, the bacteria did not grow well in these media, and the absence of detectable levels of ST secretion may simply be due to presence of too few bacteria in the bacterial culture. Also, anaerobic basal minimal medium supplemented with casamino acids, which may be expected to favour ST secretion, was not tested. However, in anaerobic CFA media, which contains amino acids, secretion of ST was detected, although the levels were lower than in aerobic CFA media (Fig. 12A).

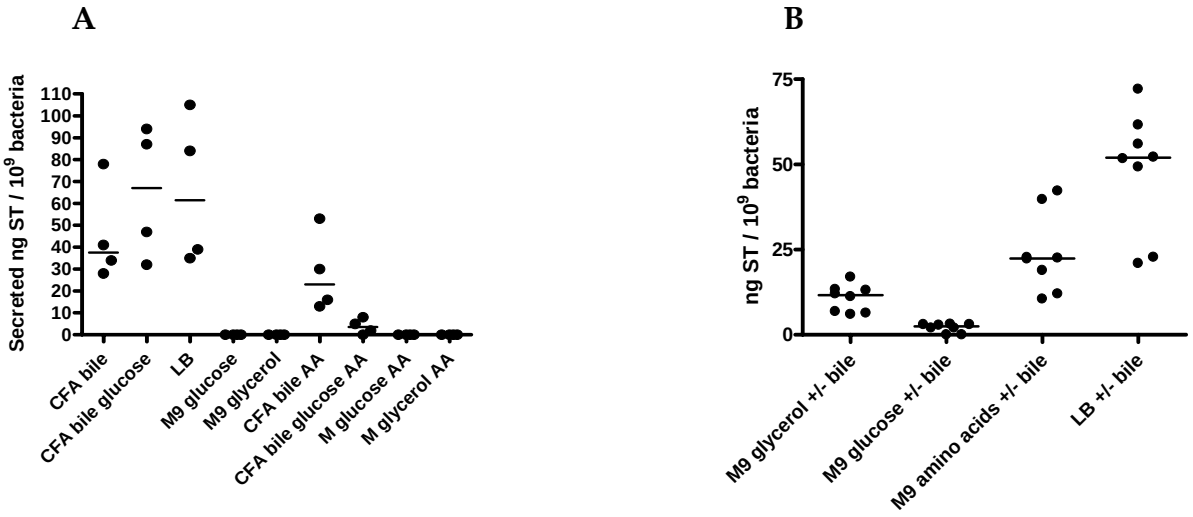

Figure 12. Secretion of ST in clinical ETEC strains after culture *in vitro* (II).
AA; anaerobic conditions.

Secretion of ST has previously been reported to be regulated by catabolite repression, *i.e.* to be down-regulated by addition of glucose to the growth medium or using glucose as the sole carbon source [101, 102]. The same applies to secretion of STII which has been reported to be decreased by addition of 1% glucose as compared to 0.1% glucose to the growth medium, whereas addition of glycerol did not affect the level of ST secretion [103]. In Paper II we found that ST was secreted at significantly lower levels when glucose was utilized as primary carbon source in aerobic minimal M9 media than when either glycerol or casamino acids were used. Also, addition of glucose to CFA media significantly down-regulated ST secretion under anaerobic conditions, but this was not observed in the presence of oxygen. In order to try to determine whether this regulation of ST secretion by glucose occurs at the transcriptional level, the transcription levels of *estA* were compared and found to be lower when the bacteria were grown in M9 glucose than in M9 glycerol or M9 casamino acids. However, this difference was not significant; hence, we were not able to establish that the STh-encoding gene itself is subject to catabolite repression.

Expression of LT after *in vitro* culture (II)
Similarly to ST, secretion of LT was measured in the supernatant from 1 ml of bacterial culture. By also measuring the amount of LT in the corresponding sonicated bacterial pellet, the total level of produced LT could be determined. In contrast to ST, LT was only found to be secreted into the growth medium in aerobic LB and aerobic CFA media without added bile salts. No secretion was detected from the clinical strains after culture in any of the anaerobic conditions. In order to pin-point the role of the lack of oxygen we grew the strain H10407, generally considered a "high-secretor" when it comes to LT [71], in aerobic and anaerobic LB. Whereas LT was

secreted in aerobic LB, secretion was not detected in the absence of oxygen, indicating a specific requirement for oxygen for LT secretion. Despite the presence of oxygen, however, LT was also not secreted by the clinical strains in any of the aerobic minimal media included, *i.e.* M9 supplemented with glycerol, glucose or casamino acids with and without added bile salts, and the same result was obtained when we grew strain H10407 in aerobic minimal media, *i.e.* M9 glucose and M9 glycerol without added bile salts. These results suggested a preference of complex media for secretion of LT and that presence of a specific carbon source such as amino acids, which are present in LB and CFA, is not enough to support LT secretion.

LT secretion was inhibited by presence of bile salts in LB and CFA media in the strains tested. Bile salts are part of the antimicrobial host defence system in the gut, acting through lipid emulsification and solubilization and membrane disruption [104]. However, the inhibition of LT secretion by bile salts observed in this study was not due to decreased viability of the bacteria since culture in LB with and without bile salts did not result in lower viable counts. Thus, we conclude that in the present study, the ETEC bacteria required presence of oxygen, absence of bile salts and a complex medium in order to secrete LT.

As mentioned in the Introduction, ETEC were originally considered to be inefficient secretors of LT [69], but in a more recent study the ability of ETEC bacteria to secrete LT has been reported to vary from strain to strain [71]. This is supported by the present study, since we found that approximately 50% of the total LT was secreted from the clinical strains and in strain H10407, under optimal *in vitro* conditions, *i.e.* aerobic LB. The lowest level of secretion was found in strain H10407 (42% secretion), historically considered to be a high LT-secretor, whereas the highest level was found in strain 2533435 (71%), indicating that LT may indeed be secreted from the bacteria under optimal conditions *in vitro*.

Production of LT and transcription of the *eltAB* operon has been reported to be optimal at 37°C and transcription to be negatively regulated at low temperatures by the histone-like nucleoid structural protein (H-NS), a global gene regulator commonly involved in regulation of environmentally controlled genes in *E. coli* and other gram-negative enteric bacteria [105]; H-NS has been reported to bind to silencer regions both up-stream and within the LT operon [106, 107]. In the present study, *eltB* was transcribed in the clinical strains under all conditions tested, although the levels of transcription were lower in aerobic CFA and LB media than in minimal and anaerobic media; this may be due to H-NS mediated negative regulation which has previously been reported for LB [107]. LT was produced under all condtions tested except for in anaerobic CFA where production was completely shut down.

Expression of CS5 after *in vitro* culture (III)

In general, relatively little is known about the regulation of CS5 expression. The CS5 operon apparently does not encode any transcription regulators, but has been suggested to be positively regulated by the plasmid-encoded Coli surface virulence factor regulator (CsvR) [108]. CsvR is homologous to the positive regulators of CFA/I (CfaD) (87%), CS1 and CS2 (Rns) (86%), and CS4 (CsfR) and is able to functionally substitute for CfaD to some extent in recombinant strains, even though CS5 is not a part of the CFA/I-like family like the others. CfaD, Rns and CsvR are all part of the large AraC/XylS family of transcriptional regulators [109], and CfaD and Rns have been shown to activate transcription of the respective CF operons by relieving suppression of transcription by H-NS [110-112]. However, the exact role of CsvR in wild-type strains has not been determined [108].

In similarity with several other CFs, *i.e.* CS7, CS8, CS12, CS14 and CS17 [87], phenotypic expression of CS5 on the bacterial surface under standard laboratory conditions, *i.e.* CFA agar, requires the addition of bile salts to the growth medium [87]. Similarly, in the present study CS5 was only detected on the bacterial surface after culture in the presence of bile salts and it was shown that bile salts are required for surface expression of CS5 in both LB and minimal media under aerobic conditions, although minimal media only supported low surface expression. In anaerobic CFA media very low levels were detected in the presence of bile salts (Fig. 13). This is the first study to show that the requirement of bile salts for phenotypic expression of CS5 on the bacterial surface is not due to regulation on the transcriptional level of the CS5-encoding operon, since *csfD* was transcribed under all conditions tested and was not significantly affected by the presence or absence of bile salts.

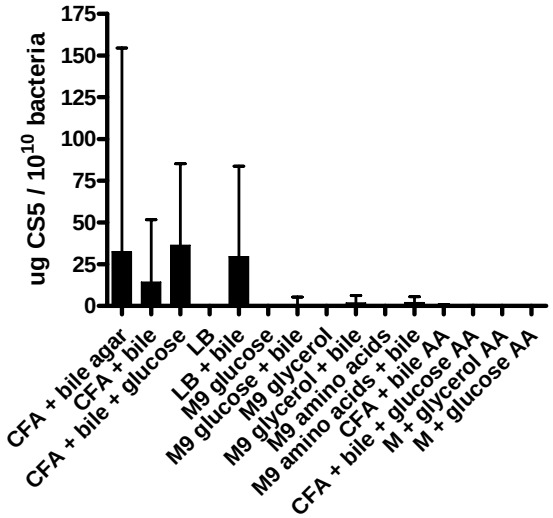

Figure 13. Expression of CS5 on the bacterial surface *in vitro* (III).
AA=Anaerobic conditions.

The effects of bile salts was found not to be due to disruption of the bacterial membrane since, as previously mentioned, the viable counts were not affected by the presence of bile salts. Surface expression of CS5 was significantly down-regulated by lack of oxygen under the conditions tested, *i.e.* CFA, whereas the transcription levels of *csfD* were not significantly affected by the oxygen status in CFA or minimal media. The carbon source used did not significantly influence either phenotypic or transcriptional levels of CS5.

Taken together, the CS5-encoding operon was found to be transcribed under all conditions tested and not significantly altered by any of the environmental factors included in this study. Hence, our results do not support that the CS5 operon is directly subject to transcriptional regulation, but rather that it is constitutively expressed while exposure of the CS5 fibrillae on the bacterial surface is induced by bile salts and inhibited by lack of oxygen.

Expression of CS6 after *in vitro* culture (III)

In contrast to CS5, no positive regulator has been suggested for CS6, which has been reported to be expressed equally well on the bacterial surface in recombinant *V. cholerae* transformed with the CS6 operon as in the wild-type ETEC strain, and presence or absence of the gene encoding Rns did not affect the expression of CS6 [113]. On the other hand, an increase in the number of CS6 operons has been reported to result in an increase in CS6 surface expression in *V. cholerae* [113] and in *E. coli* [J Tobias et al., to be published]. CS6 protein expression has also been reported to be temperature-dependent, *i.e.* CS6 was not detected after growth at 17°C and is optimally expressed at 37°C [83].

CS6 was expressed on the bacterial surface and *cssB* was transcribed under all *in vitro* culture conditions tested. CS6 surface expression or transcription of *cssB* was not significantly affected by presence or absence of bile salts or the carbon source, except for M9 supplemented with amino acids, a condition that clearly did not favour CS6 surface expression. However, this is the first study to show that both phenotypic expression of CS6 on the bacterial surface and transcription of the CS6 operon is up-regulated by anaerobic conditions.

Summary (II and III)

The results from the *in vitro* culture studies are summarized in the Table below.

	Bile salts	Oxygen	Carbon source
Transcription of *estA*	Not affected	Up-regulated by lack of oxygen	Not affected
Secretion of ST	Not affected	Down-regulated by lack of oxygen in CFA in the presence of glucose; not affected in CFA without glucose [a]	Down-regulated by glucose and up-regulated by casamino acids as primary carbon source [b]
Transcription of *eltB*	Not affected	Not affected	Not affected
Secretion of LT [c]	Requires absence of bile salts	Requires oxygen	Not affected
Transciption of *csfD*	Not affected	Not affected	Not affected
Surface expression of CS5	Induced [d]	Down-regulated by lack of oxygen	Not affected
Transcription of *cssB*	Not affected	Up-regulated by lack of oxygen	Not affected
Surface expression of CS6	Not affected	Up-regulated by lack of oxygen	Not affected

Only significant effects are included; "Not affected" = no significant difference, "up-" and "down-regulated" refer to significant changes.

[a] The effect of an anaerobic environment in minimal media on the secretion of ST could not be determined.

[b] ST was also down-regulated by addition of glucose to CFA media under anaerobic conditions, but not under aerobic conditions.

[c] LT was only secreted in complex media.

[d] Presence of bile salts was required for surface expression of CS5 under aerobic conditions.

Lack of phenotypic expression of CS6 on the bacterial surface in LT-only ETEC strains (IV)

While CS6 was transcribed and phenotypically expressed in the ST/LT strains in Paper III under all *in vitro* growth conditions tested, the aim in Paper IV was to investigate the mechanism behind the lack of expression of CS6 on the bacterial surface in certain LT-only ETEC strains that were positive for the CS6 operon (*cssABCD*). The strains were identified in India at the beginning of the nineties [59, 61], and more recently in Guinea-Bissau [64] (Table 1). To rule out the possibility that this phenomenon was only present in a single circulating clone the eight strains were analyzed by RAPD typing and found to belong to five different RAPD types.

The lack of surface expression was found to be a consistent trait after culture in a variety of culture conditions as determined by a dot-blot assay using a MAb directed against the two structural subunits CssA and CssB. We also investigated whether the

CS6 operon was being transcribed and established that the CS6 operon had not been silenced since transcription was detected for all four genes, *i.e. cssA*, *cssB*, *cssC* and *cssD*, in all eight strains tested (six from India and two from Guinea-Bissau) (Table 1).

To investigate the possibility that even though the genes were transcribed they may contain mutations that could affect the functions of the protein products, a part of the CS6 operon was sequenced in both directions in the eight LT-only strains that were genotypically positive but phenotypically negative for CS6, as well as in one ST-only and two LT-only ETEC strains from Guinea-Bissau that were positive for phenotypic expression of CS6 on the bacterial surface (Table 1). The gene encoding the usher subunit, *cssD*, was not sequenced since surface expression of CS6 has been reported not to require the entire CssD protein [83], which is supported by recent results from our group when testing ETEC mutants defective in CssD [J Tobias et al., submitted for publication].

When analyzing the sequences we found that a majority (6/8) of the CS6 non-expressing LT-only strains contained single-point mutations within the chaperone-encoding gene *cssC*, leading to premature stop codons. One of the two remaining CS6 non-expressing LT-only strains (Ind290) contained a premature stop codon within the gene encoding the structural subunit CssA, whereas in one of the strains, Ind598, no premature stop codon was identified. The sequences were submitted to the GenBank database with accession numbers DQ538380-DQ538390.

By construction of recombinant strains we confirmed that a CS6 operon encoding a truncated chaperone subunit resulted in reduced surface expression of CS6. One of the LT-only strains that were genotypicaly positive but phenotypically negative for CS6, GB60, was used as recipient strain. Under the control of the Tac promoter, CS6 was expressed on the bacterial surface at higher levels when GB60 was transformed with an intact CS6 operon (*cssABCD*) from GB35 (GB60::pJTCS635, Table 1) than when GB60 was transformed with the CS6 operon from Ind329 (GB60::pJTCS6329) containing a premature stop codon in the chaperone gene.

Protein database searches revealed that the CS6 chaperone subunit CssC is highly similar to the non-pilus chaperones SafB from *Salmonella enterica* [114] and Caf1 from *Yersinia pestis* [115]. By using protein structure modelling based on the known structures of these proteins, CssC was predicted to consist of an L-shaped structure of two immunoglobulin-like domains connected by a short loop (Fig. 14).

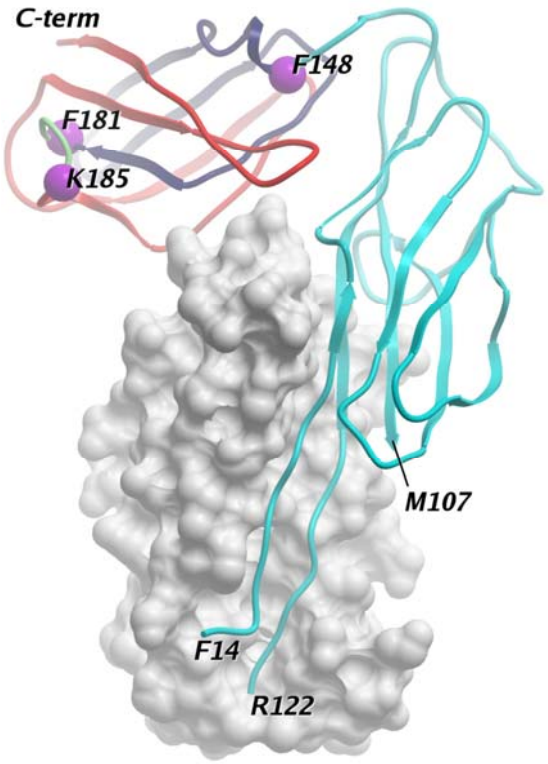

Figure 14. Model of the CS6 chaperone subunit (CssC) and its predicted interaction with the structural subunits (IV). CssC is shown as a ribbon; the positions of the truncations in CssC are indicated as magenta spheres.

By using this model we determined that the premature stop codons in *cssC* resulted in either entire loss of the C-terminal Ig-like domain (GB60 and GB76; truncated at amino acid F148, Fig. 14), which is likely to result in less affinity to CssA and / or CssB, or loss of the four β-strands (Ind380 and Ind424; truncated at F181, and Ind325 and Ind329; truncated at K185), which is likely to result in a more unstable chaperone [116].

Finally, protein analyses using MAbs against the two respective structural subunits showed that neither CssA or CssB were present in the periplasm of the six strains with premature stop codons in *cssC*, suggesting that even if the structural subunits are translated in the cytosol they are probably degraded in the periplasm due to the absence of a functional chaperone (Fig. 2, Paper IV). This has previously been described for uropathogenic *E. coli* (UPEC), where absence of the periplasmic pilus chaperone PapD leads to aggregation and digestion of the fimbrial subunits by periplasmic proteases and subsequent lack of surface expression [117].

Interestingly, both CssA and CssB were also absent in the periplasmic preparations of the two strains that did not contain truncating mutations in *cssC*. However, all eight LT-only strains from India and Guinea-Bissau that were genotypically positive but phenotypically negative for CS6, as well as two such strains from Egypt, contained a nucleotide shift from A to T in the untranslated region up-stream of *cssC* close to the putative ribosome binding site of *cssC*. A tentative explanation to the loss of phenotypic CS6 expression on the bacterial surface in the absence of a truncated chaperone is therefore that this nucleotide polymorphism results in lower affinity of the ribosome and hence reduced levels of the chaperone peptide, but this remains to be proven.

Genetic variability of ETEC strains (IV and V)

The fifth study also focused on strains carrying the CS6 operon, but in this study we determined the genetic variability in a set of phenotypically CS6 positive ST-only ETEC strains. This combination of virulence factors has recently been found to predominate among travellers to Mexico and Guatemala [57]. The study was undertaken in order to investigate whether this was due to the travellers being infected by a single circulating ST-only CS6 positive clone and if similar ST/CS6 clones were found in resident children living in the same region.

A region of the CS6 operon found to contain several silent single nucleotide variations as well as the A/T polymorphism in the untranslated region up-stream of *cssC* was sequenced. Similarly to the phenotypically CS6 positive LT-only and ST-only strains in Paper IV, it was found that all 24 ST/CS6 strains analyzed in Paper V contained an A nucleotide at this position; this supported our previous finding that an A at this position is associated with CS6 phenotypically positive strains while a T is associated with CS6 phenotypically negative strains.

The analyzed CS6 sequence was found to be identical in 23 of the ST/CS6 strains included in Paper V and in the only ST/CS6 strain included in Paper IV (GB124). Interestingly, analysis by toxin multiplex PCR revealed that these strains were all STp positive, while the remaining strain included in Paper V found to contain another CS6 sequence was positive for STh.

Multi Locus Sequence Typing (MLST) analysis [99] was used to determine the sequence type of the ST/CS6 strains and revealed that several sequence types were present among the ST/CS6 strains. The most commonly identified MLST sequence type, *i.e.* ST-398, was found in all seven strains isolated from children and also in strains isolated from three of the travellers. Hence, we could show that ST/CS6 ETEC strains of the same sequence type can infect both children and travellers and spread

between the two patient groups since the sequence type (or clonal coplex) ST-398 was found to circulate in Guatemala during at least 6 years.

By comparison with previously entered MLST sequence types on the *E. coli* MLST website, it was deduced that other pathogenic *E. coli* such as EAEC, EIEC and an *E. coli* isolated from a urinary tract infection may be of the same MLST sequence type as ETEC. Similar results were obtained in Paper IV; ETEC strains of varying virulence profiles, *i.e.* ST-only and LT-only CS6 positive strains as well as LT-only strains that were phenotypically positive or negative for CS6, were found to belong to the same RAPD types, respectively. These results suggest that the genetic *E. coli* background is not a good predictor of the *E. coli* virulence profile.

GENERAL DISCUSSION

Enterotoxigenic *Escherichia coli* (ETEC) is one of the most common causes of acute diarrhoea in developing countries, both among local residents and international travellers to these areas. In order to understand the pathogenesis of ETEC it is important to answer how the virulence factors are regulated at the site of infection where the disease is manifested. In comparison to other more thoroughly studied enteropathogens such as *V. cholerae* [118, 119], relatively little is known about the regulation of most ETEC virulence factors, especially during infection of the human intestine. One of the aims of this thesis was to investigate how the expression of the ETEC enterotoxins ST and LT as well as two common colonization factors, CS5 and CS6, is regulated by the environment within the human intestine during the infection process. This was done by quantifying the expression levels of these virulence factors in ETEC bacteria disseminated from patients with acute diarrhoea without subculturing (*in vivo* samples) and in corresponding organisms after culture under different conditions *in vitro* (I, II and III).

When ETEC bacteria enter their human host they encounter a range of environmental changes, beginning with a temperature shift to 37°C and the acidic conditions of the stomach. In the small intestine, which is the site of ETEC colonization, the acidic pH is neutralized by secretion of a solution rich in sodium bicarbonate from the pancreas and the intestinal mucosa [120]. As the ETEC bacteria enter the duodenum of the small intestine they are exposed to bile secreted from the gall bladder; the concentration of the bile salts present in the bile is decreased along the small intestine as they are absorbed and redelivered to the liver [104]. Also, as the bacteria move further away from the stomach the oxygen levels decrease. During digestion in the stomach and the small intestine, carbohydrates, fat and proteins are broken down into glucose, glycerol, fatty acids and amino acids and gradual up-take of these nutrients occurs along the small intestine. The ability of enteropathogens to phenotypically express virulence factors in response to such environmental signals in the intestine may be essential for the bacterial pathogenesis, and regulation of virulence genes in response to environmental stimuli has been reported for many species [121-123].

To understand how ETEC are regulated during infection we first analyzed ETEC bacteria disseminated directly in diarrhoeal stool, and found that the genes encoding the virulence factors ST, LT, CS5 and CS6 are transcribed as the bacteria are disseminated from the host. While there was no significant up- or down-regulation of transcription of the genes encoding the toxins in the disseminated *in vivo* bacteria as compared to after culture *in vitro*, transcription of both the CS5 and CS6 operons was up-regulated in the *in vivo* bacteria. The CS5 operon was consistently transcribed at higher levels than the CS6 operon, both *in vivo* and under laboratory conditions, but interestingly the CS5 operon seemed to be 10-fold more up-regulated than the

CS6 operon in the disseminated *in vivo* bacteria, indicating that the conditions in diarrhoeal stool may be more beneficial for transcription of the CS5 operon than the CS6 operon. Speculatively, this up-regulation could be relevant for the pathogenesis or spread of ETEC infection. However, further studies are needed to elucidate the adaptation of ETEC bacteria to the environment outside the host. For instance, disseminated *V. cholerae* bacteria have been shown to display greatly enhanced infectivity as compared to *in vitro* grown bacteria, but this hypervirulent state was not due to transcriptional up-regulation of the virulence genes encoding the cholera toxin (CT) or the toxin-coregulated factor (TCP) [124].

ST and LT were found to be present in diarrhoeal stool (I), thus indicating that they are produced during infection. In the case of CS5 and CS6, transcription in the disseminated bacteria could be linked to phenotypic expression of the CFs during infection as indicated by immune responses to both CS5 and CS6 at late convalescence (III). Hence we could show that the virulence factors ST, LT, CS5 and CS6 were transcribed and produced during infection of the human host. However, we also wanted to investigate the regulation of expression of the virulence factors during the infection process, and in this thesis the aim was to pin-point the effects of bile salts, lack of oxygen and the carbon source on the levels of transcription and production and / or secretion of ST and LT or surface expression of CS5 and CS6. This was achieved by culturing clinical ETEC strains under various conditions *in vitro*. Based on the results from these studies we propose the following (and yet very incomplete) model for phenotypic expression of ST, LT, CS5 and CS6 during the infection process (Fig. 15).

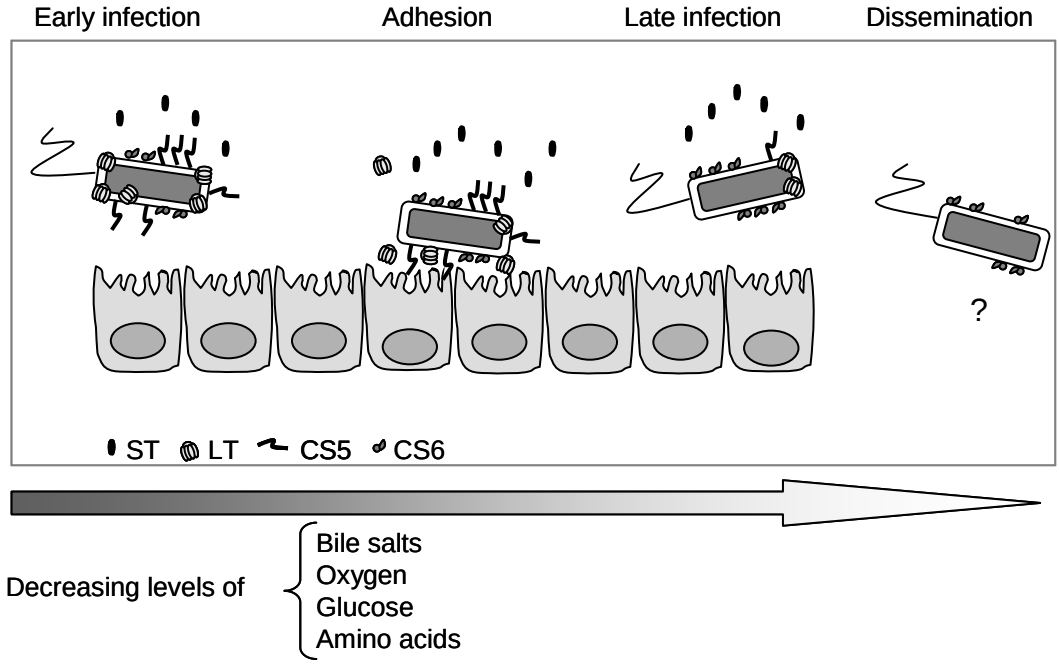

Figure 15. Proposed model of regulation of expression of ST, LT, CS5 and CS6 during the infection process (I, II and III).

During early infection, the presence of bile salts in the duodenum would be expected to induce surface expression of CS5 but inhibit secretion of LT, whereas secretion of ST and surface expression of CS6 is not affected by bile salts and may be assumed to be phenotypically expressed. During adhesion to the intestinal epithelium, CS5 and CS6 are expressed on the bacterial surface. Even though secretion of LT was - somewhat surprisingly - only found to occur under aerobic conditions in our studies, infection with LT-positive ETEC strains gives rise to local and serum immune responses [125, 126] and the toxin must therefore be exposed to the immune system during infection. This could occur by secretion in free form [71], in a polarized fashion [72] or bound to vesicles [75, 76, 127], or by association to LPS on the bacterial surface [73]. However, in these studies we did not investigate the membrane fractions for presence of LT. During the later stages of infection, surface expression of CS5 would be expected to decrease due to decreased levels of bile salts as well as increased anaerobic conditions as the bacteria move further away from the stomach along the small intestine. On the other hand, contrary to CS5, the increasingly anaerobic conditions would be expected to support increased surface expression of CS6, which may therefore be up-regulated during late infection. However, based on our results, CS6 may be expressed on the bacterial surface during the entire infection process since none of the *in vitro* conditions tested resulted in abolished surface expression. Similarly to CS6, ST may be secreted during all stages of infection since it was secreted under totally anaerobic conditions in complex media and was never completely inhibited in the presence of glucose, even though it was found to be catabolite repressed, as previously suggested [101], in anaerobic complex media and aerobic minimal media. The concentration of glucose may be assumed to decrease at the later stages of infection due to the absorption of nutrients, resulting in increased secretion of ST. Secretion of ST was also found to be induced by amino acids, indicating that ST is secreted in response to digestion of proteins. Hence there is probably an optimal level of amino acids where glucose levels are low that are optimal for ST secretion inside the human intestine.

The above model of ETEC pathogenesis involving ST, LT, CS5 and CS6 is based on a greatly simplified view of the environment in the human intestine. For instance, in this work a standardized ox-bile extract was used, but the exact composition of bile has been reported to vary in different populations and to depend on the nutritional status of the host [104]. Similarly, the relative concentrations of glucose and amino acids may vary greatly depending on the nutritional status and diet of the individual. It is also possible that the concentrations of nutrients and bile salts are different in the intestinal lumen and in the mucosa. In addition, other environmental signals that were not included in this work such as pH, iron and osmolarity are also known to affect expression of virulence factors in other enteropathogens [122, 123]. Our model for ETEC infection should therefore only be considered as a hypothetical presentation of the effects of the particular environmental factors studied in this thesis.

A major finding of this work was that the regulation of ST, LT, CS5 and CS6 by the environmental signals studied did not seem to occur at the transcriptional level of the respective operons, since the transcriptional levels were not correlated with the phenotypic expression levels, with the exception that CS6 surface expression and transcription of *cssB* were both up-regulated under anaerobic conditions. Since LT is similar to CT both functionally and structurally, it is interesting to note that the CT-encoding operon (*ctxAB*) seems to be regulated on the transcriptional level by anaerobic conditions [100], while the LT-encoding operon did not seem to be regulated on the transcriptional level in our studies (II). Further studies are needed to elucidate the mechanisms of regulation of ST, LT, CS5 and CS6 by the environmental factors described in this work, but we hypothesize that the regulation occurs at the secretion / transportation level. For instance, LT was found to be produced under almost all culture conditions but only secreted under certain conditions, and while surface expression of CS5 was induced by bile salts the level of transcription was not affected by presence or absence of bile salts. The *gsp*-cluster encoding the Type II secretion system believed to be responsible for secretion of LT through the outer membrane [68] has been reported to be suppressed by H-NS in strain H10407 [128]. However, recent results from our group indicate that there is no correlation between the secretory ability and the transcription level of the operon encoding the *gsp*-cluster secretion system responsible for secretion of LT across the outer membrane. For ST, which was found to be secreted at varying levels in the studies reported in this thesis, it is interesting to note that TolC, involved in secretion of ST, has been reported to be affected by the oxygen levels and the pH, suggesting that the secretion mechanisms are affected by environmental factors [129]. Hence, while several studies on expression of virulence factors by enteropathogens during infection have been restricted to analyses of transcription, our studies emphasize the importance of studying the phenotypic expression and the transportation systems as well.

Another major finding in this work was that while CS6 was constitutively expressed on the bacterial surface (III), its phenotypic expression is dependent on a functional chaperone subunit (IV), which acts by protecting the structural subunits from degradation in the periplasm during transportation to the bacterial surface [83]. Thus, a majority of clinical LT-only strains that carried the CS6 operon but did not express CS6 on the surface under various *in vitro* conditions were shown to contain truncating mutations within the chaperone gene and not to be able to assemble the CS6 molecule in the periplasm. All these LT-only CS6 phenotypically negative but genotypically positive ETEC strains also contained a single-point mutation within the untranslated region upstream of the chaperone-encoding gene, but this mutation was not present in LT-only and ST-only strains expressing CS6 on the bacterial surface. The function of this mutation remains to be determined, but one speculation is that it may be involved in blocking ribosome binding and subsequent translation of the chaperone subunit. Interestingly, this mutation was identified in LT-only

strains isolated in various parts of the world, *i.e.* India, Guinea-Bissau and Egypt, and over a period of at least ten years, indicating a functional relevance.
The LT-only CS6 phenotypically negative but genotypically positive ETEC strains were found to belong to different RAPD types (clones) (IV), and using MLST analyses (V) it was found that STp/CS6 positive strains may belong to several different clonal complexes. Taken together, these studies suggest that on top of the previously reported diversity in O and H antigens in ETEC strains [54], ETEC strains harbouring the same combination of virulence genes may constitute a diverse group of *E. coli* regarding the genetic background. Our results (IV and V) support the previously suggested theory that the CF-toxin profile alone cannot be considered a reliable indicator of clonality [40] and hence, to a large extent, the virulence plasmids have been acquired and not only inherited from common ETEC ancestors [130].

Due to the high burden of ETEC disease in developing countries, an efficient vaccine is greatly needed both for the local population, in particular children below 5 years, but also for visitors from non-endemic areas to ETEC endemic areas. The ultimate goal of the research performed by our group is to develop an effective ETEC vaccine that can be used by these different patient groups. According to current opinion, an efficient vaccine should optimally include the most prevalent CFs [65] that are expressed during infection. Therefore the initial studies of this thesis dealt with regulation of the common colonization factors CS5 and CS6, with a special emphasis on CS6 which is increasingly being identified across the world [4], as well as the two enterotoxins, since they they are the hallmarks of ETEC disease.
Since ST-only CS6 positive strains are increasingly being identified in epidemiological studies both in children and in travellers [57, 58], the aim of the fifth study of this thesis was to investigate the genetic variability of ST-only CS6 positive strains isolated from local children in a highly ETEC endemic area, *i.e.* Guatemala, and from American visitors to the same region, *i.e.* Guatemala and Mexico. The results of this study suggest that an ETEC strain belonging to the most commonly identified MLST sequence type in this study, *i.e.* ST-398, may be considered a candidate vaccine strain since strains belonging to this sequence type were isolated from different target groups for a vaccine.

In this work we have found that ETEC virulence with respect to ST, LT, CS5 and CS6 are influenced by bile salts, glucose and amino acids which indicate that malnutrition and diet may influence the outcome of ETEC disease. We have also established that both local children and international travellers to ETEC endemic areas may be infected by ETEC strains belonging to the same clonal complex. In conclusion, this work illustrates the importance of studying the regulation of phenotypic surface expression and secretion of ETEC virulence factors as well as the variability of ETEC strains and their virulence for increased understanding of the pathogenic mechanisms of this heterogenous enteropathogen as a background for different intervention strategies.

ACKNOWLEDGEMENTS

"Arriving at one point is the starting point to another" (John Dewey). I would like to take this opportunity to sincerely thank everyone who has supported me in different ways to reach this point. Without you this thesis would never have happened! I would especially like to thank:

Ann-Mari Svennerholm, min ena huvudhandledare, för att du antog mig som doktorand och för att du så frikostigt har delat med dig av din stora forskningserfarenhet. Din aldrig sviktande entusiasm för det här projektet har betytt mycket. Tack också för att du skickade mig till Dhaka!

Åsa Sjöling, min andra huvudhandledare, för många givande diskussioner, för all tid och kunskap som du generöst har delat med dig av, och för att du har visat mig att inga problem är för stora för att lösas, och att man dessutom oftast kan lösa dem med ett skratt.

Ett jättestort tack till er båda för allt ert engagemang och all uppmuntran under de här åren! Jag är jätteglad över att ha fått ha er båda som handledare.

Firdausi Qadri, for welcoming me to the ICDDR,B and giving me the opportunity to collect my samples there. A big thank you! Thank you also to **Yasmin Ara Begum** and **Ashraf Chowdhury** (and others at the Immunology lab) for taking such good care of me while I was in Dhaka. The sari party was something special!

Wim Gaastra, for introducing me to the world of CS6 in such a friendly manner, way back when I was still at the very beginning of my ETEC life.

Michael Lebens. Mike, thanks for your patience with all my questions and for many discussions, especially about the never-ending story of CS6. I´ve enjoyed working with you!

Gudrun Wiklund, för ditt stora tålamod och kunnande på labb som du generöst har delat med dig av! Det har varit väldigt trevligt att jobba ihop.

Johanna Nyström, för tips, råd och framförallt pepp-mail inför ramskrivandet. Tack också för mycket trevligt rums-delande under flera år, det var roligt!

All ETEC people in our department: **Anna L, Ann-Mari, Biljana, Claudia, Gudrun, Ingrid, Josh, Kerstin, Mike, Susanne K, Åsa Lo, Åsa S,** and (well, almost ETEC!) **Anders** and **Bert,** and all other members (past and present) of the Hp/ETEC group, for creating such a friendly and happy atmosphere. I would also like to thank all PhD

students, and everyone else at Microbiology and Immunology, for making our department such a nice place to be!

Sven Britton, för en inspirerande kurs i Ghana.

Maria Santamaria and **Per Olcen**, for an inspiring internship in Geneva, and my friend **Anna R** for sharing this adventure with me.

Mina gamla kursare: **Anna M, Anna R, Albina, Anders, Mia** och **Tania** (och alla som hör till!), för gemenskap och vänskap som håller i sig. Jag är jätteglad att få tillhöra vårt gäng!

Aessi, for long and lasting friendship. **Alex, Elvira and David** for everything that we have shared, and will share! **Anja,** för att du både är en vän och som en storasyster för mig. **Eva** och **Karin,** för att ni är ni (och för att ni drog med mig till Norge!)

Alla i **Husgruppen,** för oaserna på torsdagskvällarna (även om det inte har blivit så många för min del den senaste tiden…), kräftskivor, och allt annat trevligt.

Birgitta och Ulf, Britt och Leif, Britt-Marie, Elsy och **Elvi.** Ni har betytt mycket för mig under de här åren.

The **Hellberg family**, for always making me feel so welcome in your warm home.

Joel, världens bästa bror, för allt som vi har delat genom åren, för att du skämmer bort mig och för att du alltid tar dig tid att hjälpa mig med mina dator-bekymmer! ☺

Mamma och pappa, för att ni är så bra!! Ni betyder oerhört mycket för mig. Tack för att ni alltid finns där och stöttar mig vad det än gäller, och för att ni ställer upp så mycket för mig. Tack också för att ni alltid uppmuntrar mig att förverkliga mina mål och drömmar, och för att ni alltid tror att jag ska klara det.

Martin ♥ Tänk att du finns i mitt liv!! Tack för att du är världens gulligaste, och för att du finns vid min sida!

The studies included in this thesis were supported by the Swedish Agency for Research and Economic Cooperation (Sida-SAREC), the Marianne and Markus Wallenberg Foundation, the Swedish Research Council (grant16X-09084), the Adlerbertska Scientific Foundation, and the Magnus Bergwall Foundation.

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