

HEAT-STABLE ENTEROTOXIN B (STB) OF *ESCHERICHIA COLI*: STRUCTURE,
MECHANISM, AND ROLE IN INTESTINAL SECRETION, BARRIER DYSFUNCTION,
AND INFLAMMATION

by

Chisom Cynthia Ohalekwu

A critical review essay submitted in partial fulfillment of the requirements for degree of
Master of Science (Msc) in Chemical Sciences

The Faculty of Graduate Studies
Laurentian University
Sudbury, Ontario, Canada

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**CRITICAL REVIEW ESSAY DEFENCE COMMITTEE/COMITÉ DE SOUTENANCE
DE LA REVUE CRITIQUE**

Laurentian University/Université Laurentienne
Faculty of Graduate Studies/Faculté des études supérieures

Title of Essay Titre de l'essai	HEAT-STABLE ENTEROTOXIN B (STB) OF ESCHERICHIA COLI: STRUCTURE, MECHANISM, AND ROLE IN INTESTINAL SECRETION, BARRIER DYSFUNCTION, AND INFLAMMATION	
Name of Candidate Nom du candidat	Ohalekwu, Chisom Cynthia	
Degree Diplôme	Master of	
Program Programme	Chemical Sciences	Date of Defence Date de la soutenance April 21, 2026

APPROVED/APPROUVÉ

Critical Review Essay Examiners/Examineurs de la revue critique:

Dr. Mazen Saleh
(Supervisor/Directeur(trice) de l'essai)

Dr. Guangdong Yang
(Committee member/Membre du comité)

Approved for the Faculty of Graduate Studies
Approuvé pour la Faculté des études supérieures
Dr. Alain Simard

Dean of Graduate Studies and International
Doyen des Études supérieures et de la stratégie internationale
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Abstract

Enterotoxigenic *Escherichia coli* (ETEC) is a primary causative agent of diarrheal disease in humans and domesticated animals, leading to considerable worldwide disease burden, death, and financial consequences. Among ETEC's virulence determinants, the heat-stable enterotoxin b (STb) is a less researched component, distinguished by a special mode of action, unlike the other ETEC enterotoxins. STb is a 48-amino acid peptide enterotoxin encoded by the *estB* plasmid gene. The pathogenesis of STb involves the binding of the enterotoxin to the sulfatide receptor, leading to the oligomerization and formation of pores, which induce a rise in calcium, disruption of tight junctions, and finally, the induction of diarrhea. There is still a need to understand the structure of the pores, the complete range of receptors, and the exact role in the pathogenesis of the disease, especially in humans. This review aims to summarize the current understanding of the heat-stable STb, including the structure, genetics, and pathophysiology, and the gaps in the current knowledge.

Keywords: ETEC, *Escherichia coli*, STb, heat-stable enterotoxin, sulfatide, calcium signaling, intestinal secretion, diarrhea, tight junction, pore-forming toxin, vaccine.

Acknowledgement

I begin by offering my deepest and most sincere gratitude to the Almighty God, who in His infinite mercy and benevolence has given me life, good health, and a sound mind, and has enabled and blessed me to successfully complete this review essay. To Him, therefore, all the glory and honor are due.

I would like to express my sincere and deepest appreciation to my supervisor, Dr. Mazen Saleh, whose support and guidance have been of immense benefit in the success of this review essay. His encouragement, insightful feedback and comments were invaluable in shaping the success of this review essay. I am also grateful to my course supervisor, Dr. Sabine Montaut, for creating an excellent and conducive learning environment and providing the necessary resources that made it possible for me to complete this review essay. My sincere thanks also goes to my committee member, Dr. Guangdong Yang, whose valuable time and insightful comments were instrumental in the success of this research. I am truly grateful for all the assistance given to me throughout this academic journey.

Special thanks to my course mates and friends in the program for their cooperation, advice, intellectual discussions, support and motivation in all aspects of my journey. It is because of their company and friendship that this experience has been not only fruitful but also memorable.

Words do not suffice to express my love and gratitude to my family for supporting and staying with me at the most difficult time. Love, support, and trust from my family have been my biggest strength. To my dear parents, Chief & Lolo D.U. Ohalekwu, for whose strength and good qualities I live and because of whom I am grateful every day of my life, I hope that this achievement brings you joy and pride. I am especially grateful to my loving husband, Mr. Chinwendu Ikechukwu, for

his love and encouragement, which helped me to stay focused and grounded on my goals and have been the foundation of all my successes. Finally, my heartfelt gratitude to my friends turned family, for their love, support and motivation, which has been my strength in all aspects of my academic journey.

Declaration of AI Usage

I acknowledge the use of ChatGPT [<https://chatgpt.com/>] to organize my drafted outline and generate more outlines. I entered the following prompt: “Organize the following tentative outline and add more if needed for the topic on STb enterotoxins; History, Significance, Structure, Mechanism, Effects and Advancement of STb enterotoxins of E. Coli”. I developed more points from the output it provided. I also used Grammarly to rephrase and correct sentences and grammar.

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Abbreviations

- [Ca²⁺]_i - Intracellular Calcium Concentration
- 3'-SO₄-GalCer - 3'-Sulfogalactosylceramide (Sulfatide)
- 5-HT₅ - Hydroxytryptamine (Serotonin)
- CaCC - Calcium-Activated Chloride Channel
- CaMKII - Calcium/calmodulin-dependent protein kinase II
- CFAs/CFs - Colonization Factor Antigens/Colonization Factors
- CFTR - Cystic fibrosis transmembrane regulator
- cGMP - Cyclic guanosine monophosphate
- cPLA₂ - Calcium-dependent cytosolic phospholipase A₂
- CRP - cAMP Receptor Protein
- cryo-EM - Cryo-Electron Microscopy
- DAG - Diacylglycerol
- Dsb - Disulfide bond
- *E. coli* - *Escherichia coli*
- ELISA - Enzyme-Linked Immunosorbent Assay
- ER - Endoplasmic Reticulum
- *estA* - Gene encoding heat-stable enterotoxin a (STa)
- *estB* - Gene encoding heat-stable enterotoxin b (STb)
- ETEC - Enterotoxigenic *Escherichia coli*
- IL-6 / IL-8 - Interleukin-6 / Interleukin-8
- IM/OM - Inner membrane/Outer membrane
- IP₃ - Inositol Trisphosphate (Inositol 1,4,5-trisphosphate)

- LFA/LFIA - Lateral flow immunoassay
- LT - Heat-Labile Enterotoxin
- NMR - Nuclear Magnetic Resonance spectroscopy
- OmpR - Outer membrane protein R (transcriptional regulator)
- PCR / qPCR - Polymerase chain reaction / quantitative PCR
- PGE₂ - Prostaglandin E₂
- PIP₂ - Phosphatidylinositol 4,5-Bisphosphate
- PKA/PKC - Protein kinase A/ Protein Kinase C
- SCFAs - Short-Chain Fatty Acids
- scRNA-seq - Single-Cell RNA Sequencing
- SecA - Secretory pathway Sec translocon ATPase subunit
- Sta(I) / STb(II) - Heat-Stable Enterotoxin a (type I) / b (type II)
- TER/TEER - Transepithelial Electrical Resistance
- TJ/AJ - Tight Junction / Adherens Junction
- ZO-1/2/3 - Zonula occludens-1/2/3 (TJ scaffolding protein)

Chapter one: Introduction

1.1. Background on *Escherichia coli* as a Pathogen; Serotypes and Classification

Escherichia coli (*E. coli*) is one of the most well-studied model bacteria organisms in the field of microbiology and molecular biology research. *E. coli* is a common commensal bacterium that is part of the first bacteria to colonize the human gut after birth. However, in immunosuppressed patients or in healthy individuals whose physical, anatomical and physiological barriers have been compromised, *E. coli* can provoke severe systemic infections (Smith & Halls, 1967). Specific pathogenic variants, known as pathotypes, have evolved leading to several diseases ranging from intestinal infections to extraintestinal illnesses such as urinary tract infections, sepsis, and meningitis. Further, owing to genetic variability, some strains of *E. coli* differ from their commensal counterparts and encode specific virulence traits which render them capable of inducing disease in a variety of animals. Pathogenic *E. coli* are broadly divided into two groups, extraintestinal pathogenic *E. coli* (ExPEC) and intestinal pathogenic *E. coli* (InPEC). Depending on the presence of specific virulence factors, mechanisms of infection, tissue tropism, interactions with host cells and clinical symptoms, *E. coli* can be categorized into various pathotypes (Figure 1.1). These include: (i) Enteropathogenic *E. coli* (EPEC), a cause of acute and prolonged diarrhea in infants; (ii) Enterohemorrhagic *E. coli* (EHEC), which can cause hemorrhagic colitis and Hemolytic Uremic Syndrome; (iii) Enterotoxigenic *E. coli* (ETEC), a major cause of travelers' diarrhea; (iv) Enteroaggregative *E. coli* (EAEC), a cause of acute and chronic diarrhea; (v) Diffusely adherent *E. coli* (DAEC) which is associated with watery diarrhea in young children; (vi) Enteroinvasive *E. coli* (EIEC), a cause of dysentery and watery diarrhea; (vii) Adherent-Invasive *E. coli* (AIEC) which has been associated in the pathogenesis of Inflammatory Bowel Disease (IBD); (viii) Uropathogenic *E. coli* (UPEC), a common cause of urinary tract infections

(UTI); (ix) Neonatal meningitis *E. coli* (NMEC), a top cause of neonatal meningitis; (x) Septicemia-associated *E. coli* (SEPEC), which can cause bacteremia and sepsis; (xi) Avian pathogenic *E. coli* (APEC), which can cause severe respiratory and systemic infections in poultry (Pokharel et al., 2023).

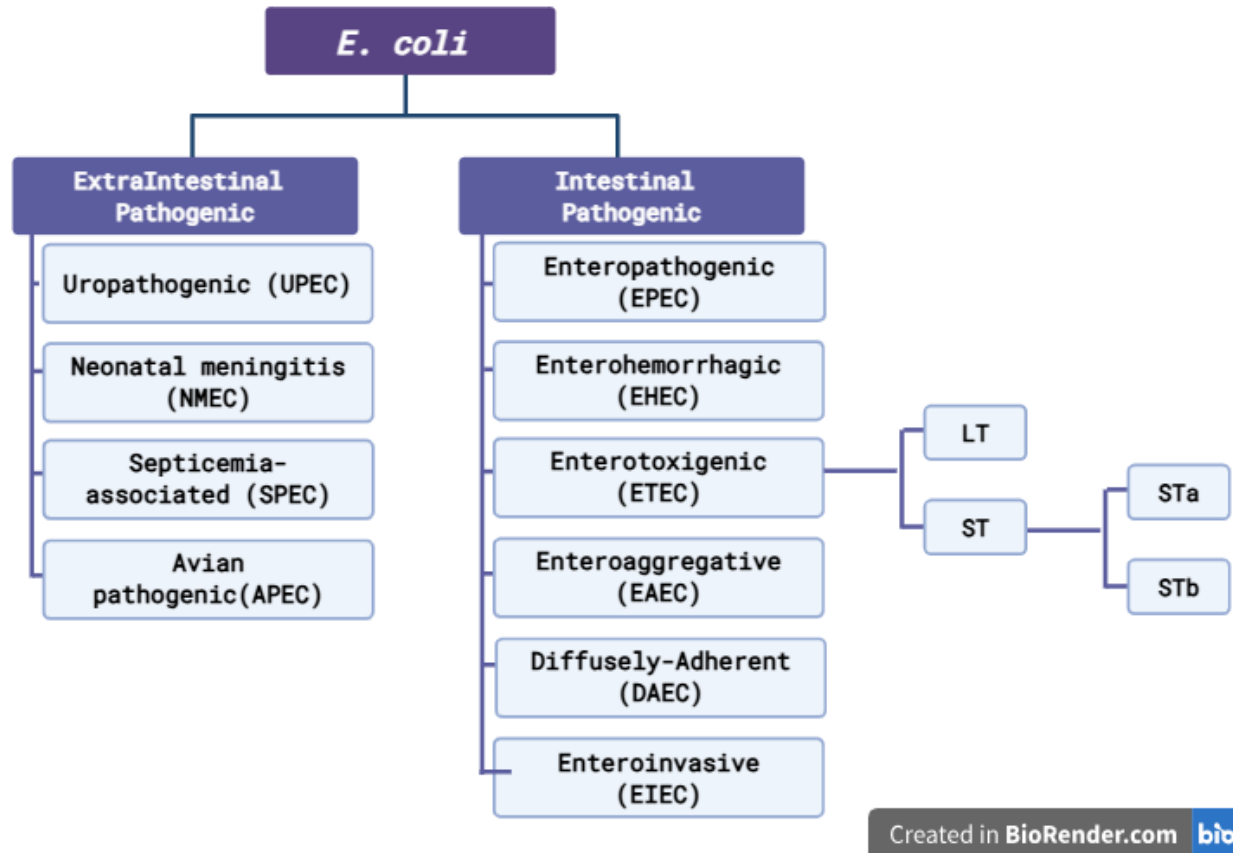


Figure 1.1: Pathotypes classification of *E. coli* (Created in <https://BioRender.com>)

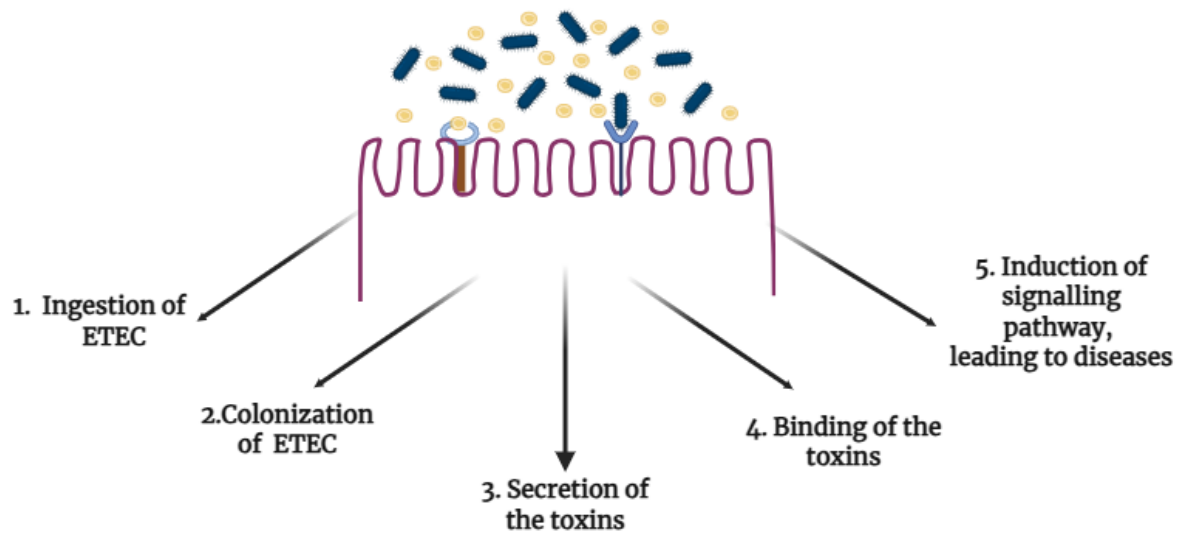
E. coli is one of the most genetically versatile microorganisms and can colonize and persist in primary (bird/animal/human host-associated) and secondary (open or non-host-associated) habitats. The high plasticity of the genome of this bacterial species gives it a tremendous capacity to evolve due to the gain and loss of genes through genetic changes, leading to the emergence of pathogenic strains from the commensal strains (Baumgart et al., 2021). Genomes of pathogenic *E.*

coli strains are generally larger, as the pathogenic strains require additional adaptive features, including virulence factors. Often, virulence genes are located on transmissible genetic elements such as pathogenicity islands (PAIs), bacteriophages, insertion sequences (ISs), integrons, plasmids, or transposons (Baumgart et al., 2021); hence, they can also be horizontally exchanged and may facilitate novel rearrangements among different bacteria. In contrast, commensal bacteria can also become pathogenic by the loss of genes. The horizontal transfer between different strains favors diversity and versatility, resulting in the creation of new pathogenic strains as well as the dissemination of acquired virulence genes with novel functions outside their clonal lineage. There are many identified PAIs in different *E. coli* pathotypes which were acquired via horizontal gene transfer and can contribute to fitness and the colonization of different niches (Pokharel et al., 2023).

1.2 Overview of Enterotoxigenic *E. coli* (ETEC) and Its Virulence Factors; Classification and Distinctions, Global Burden of ETEC Infections.

ETEC has a complex and heterogeneous nature with a genome range of 4.8 to 5.2 Mbp that encompasses a number of plasmids (Liu et al., 2016). It has long been considered a major cause of watery diarrhea in children under the age of five years for low- and middle-income countries and has become the predominant cause of traveler's diarrhea worldwide. The World Health Organization has estimated that about 220 million diarrhea episodes and 75,000 associated deaths are due to ETEC every year among children in the world, making its burden clear to everyone (Lamberti et al., 2014). It has virulence genes and genes that are theoretically associated with virulence that take an active role in various pathways of pathogenesis for diarrhea. The pathogenesis of the bacterium (Figure 1.2) involves bacterial attachment to specific receptors expressed on the intestinal epithelium, followed by the colonization of the small intestinal mucosal epithelium due to plasmid-borne colonization factor antigens (CFA) which are the proteins for the

fimbriae/pili for colonization, and then the secretion of enterotoxins that damage cellular signaling pathways of the host cells (Hazen et al., 2017). In order to cause diarrhea, ETEC express and produce either one or both of two well characterized plasmid-encoded enterotoxins; the heat labile enterotoxin (LT) and the heat stable enterotoxins (ST), together with more than 25 distinct plasmid-encoded colonization factors (CFs). LT is a multi-subunit AB₅ toxin related to cholera toxin, while ST_a and ST_b are small, cysteine-rich peptides. ST_a is further categorized into ST_p (porcine) and ST_h (human) variants. ST_b, the focus of this review, is less characterized than ST_a but is frequently identified in porcine and human ETEC isolates, particularly in association with porcine diarrhea and some cases of human illness (Labrie et al., 2001).



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Figure 1.2: The pathogenesis of enterotoxigenic Escherichia coli (ETEC) (Created in <https://BioRender.com>)

Some ETEC virulence-related plasmid and chromosomal-encoded genes have been involved in the outcome of infection. These non-classical virulence determinants are found to be located on the chromosome, pathogenicity islands and in plasmids. TibA is an example of chromosomally encoded adhesin that mediates adhesion to human cells and subsequently induces invasion. Another chromosomal gene, *clyA* encodes a pore-forming hemolytic protein and its expression leads to cytotoxic effects on mammalian cells (Ludwig et al, 2004). The *tia* and *leoA* genes, on the contrary, are harbored on pathogenicity islands. The plasmid gene *eatA*, encodes a serine protease that degrades the major protein on the mucosa layer of the small intestine and facilitates translocation of toxins. The EtpA extracellular adhesin is also a plasmid-encoded protein that is located on the tip of the flagella and is required for optimal delivery of LT to epithelial cells (Kumar et al, 2018).

1.3. Role of Heat-Stable Enterotoxins (STa and STb) in Diarrheal Disease

Heat-stable enterotoxins produced by ETEC are secreted peptides that can be divided into two types, STa and STb. While the latter is more virulent in animals and particularly in post-weaning pigs, the STa enterotoxin is more relevant in diarrhea induction in humans, newborn piglets and calves. These peptides are encoded by two genes, *estA* (STI) and *estB* (STII), which are located on plasmids, and can be distinguished from each other by their solubility in methanol and their protease sensitivity. Enterotoxin STa is methanol soluble and protease resistant, while STb is methanol insoluble and protease sensitive. According to the host species, STa is further classified into two subtypes, known as STp and STh, which were originally isolated from swine and human ETEC strains, respectively. While STp is widely found in porcine, bovine, and human ETEC strains, STh is only produced by human ETEC strains (Joffre et al, 2016).

The heat-stable toxins are the mediators of secretory diarrhea associated with an infection of the ETEC bacteria. Unlike the LT toxins, ST toxins are heat-stable and protease-resistant within the gut environment to enable them to function within the intestine. STa (ST-I) is a 18/19 amino acid peptide that acts by binding to membrane-bound guanylyl cyclase C from the intestinal epithelia to cause an increase in intracellular cyclic guanosine monophosphate (GMP) levels. The increase in cyclic GMP inhibits sodium chloride absorption and provokes Cystic Fibrosis Transmembrane Regulator-mediated chloride secretion to cause a subsequent loss of fluids (Butt et al., 2020). STb is a 48-amino acid peptide that does not interact with the signaling pathway of cyclic GMP. It only causes a pertussis-toxin-sensitive secretory response mediated by calcium influx to cause histopathological damage to intestinal mucosae to increase enterocyte permeability (Dreyfus et al., 1993). Although it is assumed that STa is the causative factor for human ETEC infection-induced diarrhea, STb is an important virulence factor for porcine ETEC infection, and it was isolated from human isolates, suggesting its role in its transmission and infection cases in humans as well (Labrie et al., 2001). The difference in mechanism between STa and STb illustrates the complexity of the system used by ETEC for the induction of diarrhea and underlines the relevance of identifying the individual mechanism of STb.

1.4. Focus on STb: Historical Discovery and Clinical Relevance

STb was originally detected in the latter half of the 1970s in porcine ETEC strains that produced diarrhea but not LT and the known ST, which came to be named STa (Burgess et al., 1978). Known as "ST-II" in early works, the factor remained different from STa in that the latter remained stable at temperatures of 100°C for 30 minutes and did not induce intestinal guanylyl cyclase (Burgess et al., 1978). In the 1980s, the *estB* gene for STb remained cloned and analyzed, yielding results that suggested the *estB* gene formed part of the plasmid operon that often carried other virulence

genes (Lee et al., 1983). STb is an important cause of post-weaning diarrhea in piglets and causes significant economic loss in the swine industry. In human medicine, its role in disease is more contentious; however, epidemiological studies have detected estB in 10–30% of human ETEC isolates, particularly from regions with high livestock exposure (Ngeleka et al., 1993). The toxin's capacity to compromise intestinal barrier function and provoke inflammation invites speculation that it may more notably contribute to the severity and duration of the infection under conditions of malnutrition or otherwise compromised host immunity. Consequently, STb is a relevant yet understudied virulence factor with implications for veterinary and human medicine.

1.5. Objectives and Scope: STb structure, receptor interactions, signaling, and pathology.

The central focus of this review is the integration of existing knowledge about the structural biology, mechanism of action, and physiological effects of STb. This essay will examine the genetic origin and biosynthesis of STb, its tridimensional structure, as well as the structural basis of the heat-stability and receptor-binding activities. In particular, it will describe the characteristics and function of the host cell receptors such as sulfatide and the subsequent signaling pathways that ultimately trigger ion secretion, disruption of the tight junctions, as well as the inflammatory reactions. In addition, the essay will review the significance of STb in the context of the overall mechanism of ETEC infection, where it may be in synergy with other virulence factors. Finally, it will review the existing diagnostic approaches, treatments, and vaccines aimed at STb. In doing so, it intends to present an integrated view of STb as a model biosystem which replicates the bacterial enterotoxin action through an outlook to its future research needs.

1.6 Significance: Understanding STb Informs Vaccine/Antitoxin Development and Gut Pathogenesis Models

A mechanistic insight into STb is of utmost importance for a multitude of reasons. Firstly, from a medicinal perspective, STb is an ideal target for the development of innovative anti-diarrheal therapies. In contrast to STa, which acts through mimicry of natural peptides, STb utilizes an independent receptor mechanism, thereby potentiating the development of specific antagonists with minimal interference with natural biological functions. On the other hand, development of STb toxoids or receptor antagonists would assist in the development of an ideal vaccine or medication for porcine strains of ETEC, with applications in human biomedicine as well. Secondly, STb is an excellent model to understand natural biological processes at the cellular level. Its binding mechanism with sulfatide, a glycosphingolipid concentrated in lipid rafts, helps comprehend the mechanism of lipid raft-dependent endocytosis and signaling in intestinal epithelia (Labrie et al., 2001). The calcium flux, protein kinase C activation, and disruption of tight junctions caused by the toxin make the model attractive for research related to the regulation of the epithelial barrier and inflammation signaling. Finally, as the world faces the growing issue of antimicrobial resistance, the possibility of using targeted virulence factors such as STb as an ‘alternating approach’ to treating ETEC infections using antibiotics opens promising avenues. Hence, the understanding of the biological mechanism of the action of STb not only enhances scientific knowledge but also heralds the beginning of an era of innovations.

Chapter two: Genetics and biosynthesis of STb

2.1. Genetic Determinants of STb: The estB Gene and Plasmid Localization

The estB gene, which is responsible for the production of the heat-stable enterotoxin STb, is an important virulence gene in enterotoxigenic *E. coli* (ETEC), especially in porcine ETEC. The estB gene is with an open reading frame of 171 base pairs in length and is responsible for the production of the precursor protein of the enterotoxin STb. The precursor protein is 71-72 amino acids long depending on the ETEC strain (Lee et al., 1983). The estB gene is mainly a plasmid-borne gene compared with the chromosomal location of the virulence genes of *E. coli*. The chromosomal location of the virulence genes in *E. coli* is a unique characteristic in the pathogenesis of the bacterium. The localization of the estB gene on the plasmid makes the spread of virulence and antibiotic resistance easier. The estB gene is mainly located on the heterogeneous large extrachromosomal plasmids. These plasmids vary in size from 70-120 kb in length. These plasmids are mainly from the IncF incompatibility complex (IncF_{Ic} replicons). The estB gene is mainly located on the same plasmid with other enterotoxin genes such as the heat labile. These plasmids often carry genes for fimbrial adhesins such as F18 or K88 (F4), which are essential for ETEC colonization in piglets (Smith & Linggood, 1971).

The defining feature of the estB gene's genetic environment is its association with a transposable element. The estB gene is part of a 9-kb transposon designated Tn4521 (Lee et al., 1983). Tn4521 is flanked by IS2-like sequences in an inverted repeat orientation. The right terminal repeat contains an open reading frame encoding a 159-amino acid protein that likely functions as the transposase required for the element's movement. Virulence plasmids carrying estB are frequently multidrug-resistant vehicles, facilitating the co-selection of virulence and resistance (Hu et

al.,1988). These plasmids usually code for resistance to tetracyclines, such as tetA and tetB, as well as ampicillin, spectinomycin, and kanamycin. The physical connection of estB and resistance genes suggests that the use of antimicrobial drugs in animal production could inadvertently select and maintain STb-producing ETEC in the environment (Ramírez-Bayard et al., 2023).

The structural gene for STb from different clinical isolates appears to be uniform in size, but the flanking sequences are heterogeneous, suggesting that estB could be found on different transposons (Hu et al., 1988). The plasmid carrying the estB gene appears to be quite stable, as laboratory strains can be maintained many years (>25 years) without loss of the genetic trait (Busque et al., 1995)

2.2 Regulation of STb Expression: Environmental and Host-Derived Signals

The estB gene is regulated by the environmental stimuli encountered in the host intestine. This ensures that the toxin production is done in an economical and timely manner. The expression of STb gene can be regulated by several factors. Notable among them is temperature. It is a major environmental cue for the regulation of STb. The expression of virulence genes in enteric pathogens is generally induced at 37°C, the body temperature of the mammalian host, compared with lower environmental temperatures. It has been found that the transcription of estB increases considerably at 37°C in comparison to 25°C. This regulation of estB in accordance with the temperature is achieved through the temperature sensitivity of the supercoiling of DNA and the action of proteins that play a regulatory role in this process, such as H-NS. H-NS is a repressor of virulence genes at lower temperatures, and its antagonism at host body temperature is thought to relieve estB repression, allowing its transcription (White-Ziegler et al., 2007).

Osmolarity is another important signal that ETEC encounters during its passage through the gastrointestinal tract. ETEC undergoes large changes in osmotic strength, ranging from hyperosmolarity in the postprandial small intestine to near-isotonic conditions in the fasting state (Kiers et al., 2006). Iron availability also affects the expression of STb. In the host, the availability of free iron is greatly reduced due to sequestration by lactoferrin and transferrin. ETEC, like other pathogens, has been shown to compensate for this iron limitation by increasing the expression of virulence genes. The Fur (ferric uptake regulator) protein is generally a repressor of target genes in iron-sufficient conditions and activates gene expression in iron-limited conditions. The general iron regulatory circuit may have an indirect effect on STb expression, which is dependent on growth state and metabolic regulation (Marcoleta et al., 2018).

Host-derived signals, especially those of the intestinal mucosa, may also be involved in the regulation of STb expression. Bile salts, for example, are known to be present in large concentrations in the small intestinal lumen, where ETEC adheres and colonizes. These compounds have been shown to influence the expression of virulence genes of other diarrheagenic pathogens (Hamner et al., 2013). The catabolite repression system of *E. coli* involves cyclic AMP and the catabolite activator protein. This system could be involved in the regulation of estB. It has been known that in *E. coli*, Cyclic adenosine monophosphate (cAMP)-protein complexes play a role in the activation of genes for carbon utilization and virulence. Glucose-mediated catabolite repression reduces the intracellular concentration of cAMP and would therefore decrease Catabolite Activator Protein (CAP)-dependent activation of any estB regulatory elements responsive to this regulatory system. Consequently, the ever-changing availability of glucose in the intestinal environment generates a complex regulatory environment in which estB expression is likely to be spatially and temporally heterogeneous during infection (Zubay et al., 1970).

2.3. Biosynthetic Pathway: Post-Translational Modifications and Maturation

2.3.1. Colonization of ETEC

ETEC infection of animals and humans results in diarrhea. The infection begins as the bacteria colonize the small intestine and then proceed to produce toxins that result in the secretion of fluids. The colonization of the small intestine by ETEC using colonization factors and the production of toxins (Figure 2.1) that result in the secretion of fluids are responsible for the infection of animals and humans (Wang et al., 2022). They are filamentous surface appendages, referred to as fimbriae, where adhesins, specific proteins that are involved in the adhesion of bacteria to cells, are found. These structures allow bacteria to attach to specific enterocyte receptors on the intestinal mucosa, as seen in Figure 2.1. After the bacteria are attached to the intestinal mucosa, they initiate the synthesis and secretion of STb. The host-derived epithelial factors trigger this process. Factors such as cAMP-protein complexes, biles, low oxygen levels, and metabolic changes, produced by the intestinal cells of the host orchestrate the production of STb and other toxins in the bacteria, highlighting the point that the bacteria detect the host environment once they arrive, leading to the activation of the gene that produces the toxins. The absence of colonization factors from the bacteria results in the inability of the bacteria to colonize and, as a result, to cause infection. Several types of fimbriae are found in ETEC that result in diarrhea in pigs. The types of fimbriae may include F4 (K88), F5 (K99) and F6 (987P) (Wang et al., 2022). The colonization of the small intestine by *E. coli* results in the localized action of enterotoxins.

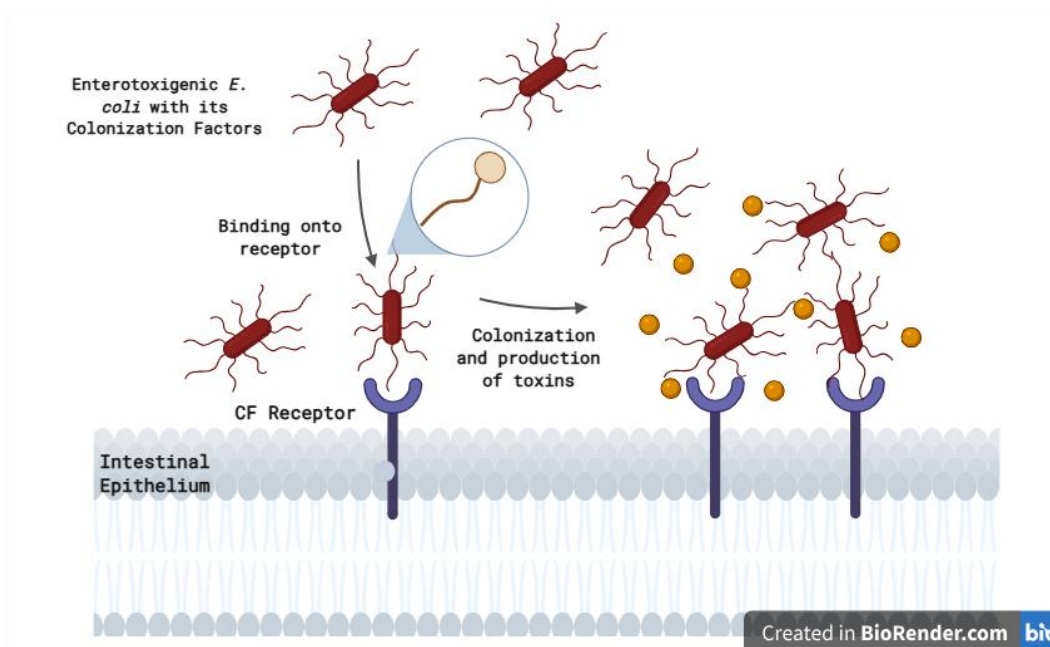


Figure 2.1: Colonization of the small intestine by ETEC using colonization factors (Created in <https://BioRender.com>)

2.3.2. Synthesis of STb

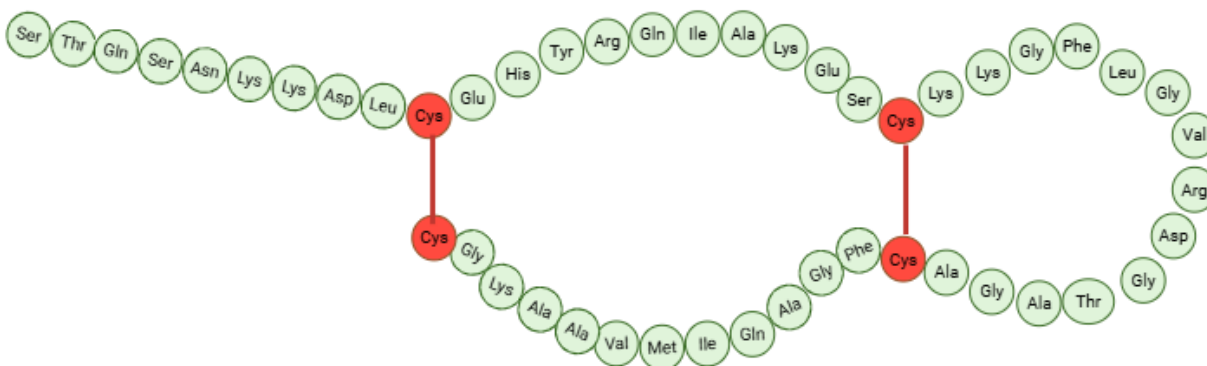
The STb is one of several toxins secreted by ETEC responsible for inducing diarrhea (Syed & Dubreuil, 2012). It has an initial precursor with higher length measurements as an inactive pre-pro-toxin. STb is made by ETEC as a pre-polypeptide of 71 amino acid peptides with a classical signal sequence of 23 amino acids.

2.3.3. Cleavage of the N-Terminal Signal Peptide

Once the STb is transported through the inner membrane of the bacterium, the signal peptide is removed by the enzyme signal peptidase I and is released in the periplasmic space as a mature peptide of 48 amino acids and a molecular weight of 5.2 kDa, starting from the N-terminal end (Kupersztoch et al., 1990). This mature STb peptide consists of two α -helices affixed by two

disulfide bridges shown in red in Figure 2.2. The integrity of the disulfide bond at Cys 21 and Cys 36 is necessary for the toxigenic region to be active.

The efficiency of signal peptide cleavage is a critical factor in determining the yield of toxin. In experiments using mutants of the signal peptide and SPase I inhibitors, it has been found that any interruption in this process of cleavage leads to a substantial reduction in the amount of correctly processed and secretion-competent STb (Sukumar et al., 1995). The efficiency of the cleavage of the signal peptide in wild-type ETEC strains is extremely high. This means that the protein precursor does not accumulate in the periplasm. It is quickly degraded by the signal peptides that are found in the inner membrane. The amino acids that are part of the degraded peptides are recycled back into the cell. The released pro-STb contains the mature toxin sequence of 48 amino acids (Figure 2.2) that is partially unstructured and ready for the process of maturation and secretion into the extracellular environment.



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Figure 2.2. Amino acid skeleton structure of the mature STb toxin. (Created in <https://BioRender.com>)

2.3.4. Maturation and Formation of the Active 48-Amino Acid Peptide

The maturation of the pro-STb into its biologically active 48-amino-acid peptide is a process in which two intramolecular disulfide bonds are formed from the four cysteine residues present in the peptide. These cysteine-containing amino acids are at positions 10, 21, 36 and 48 at the peptide chain. The formation of the disulfide bonds of the peptide is favored in an environment that is oxidizing in nature. This environment is found in the periplasm of the bacteria. The formation of the disulfide bonds of the peptide is catalyzed by bacterial Disulfide bond (Dsb) family oxidoreductases, particularly DsbA (Yamanaka et al., 1994). The maturation of STb occurs without the requirement for disulfide reduction and reoxidation in an A-B subunit structure.

The particular disulfide bond connection in the mature STb protein, which consists of two disulfide bonds that connect the N terminus and the C terminus domains, is an important component in the structural integrity and functional capability of the protein. Experiments were conducted whereby any of the four cysteine amino acids was replaced by serine or alanine, and the result was the complete lack of biological activity, even when the expression and secretion of the mutated protein were verified. This proves that the disulfide bonds are not just minor attachment in the protein structure but are definitely required for the receptor-binding in proteins and toxigenic activity (Dreyfus et al., 1992).

2.4. Mechanism of Secretion

2.4.1. Transport Across the Inner and Outer Bacterial Membranes

STb lacks an ATP-binding cassette transporter system, which is present in some toxins. The secretion of mature STb from the periplasm into the extracellular space necessarily involves a two-step secretion process (Figure 2.3). The first step, the crossing of the inner membrane, is mediated

by the Sec system through the Sec translocon, as suggested by the presence of its signal peptide. The second step, the crossing of the outer membrane, which may be controlled by Tolerance to outer membrane lipoprotein Colicin (TolC) (Foreman et al., 1995), is less clear and could be mediated by the type II secretion system.

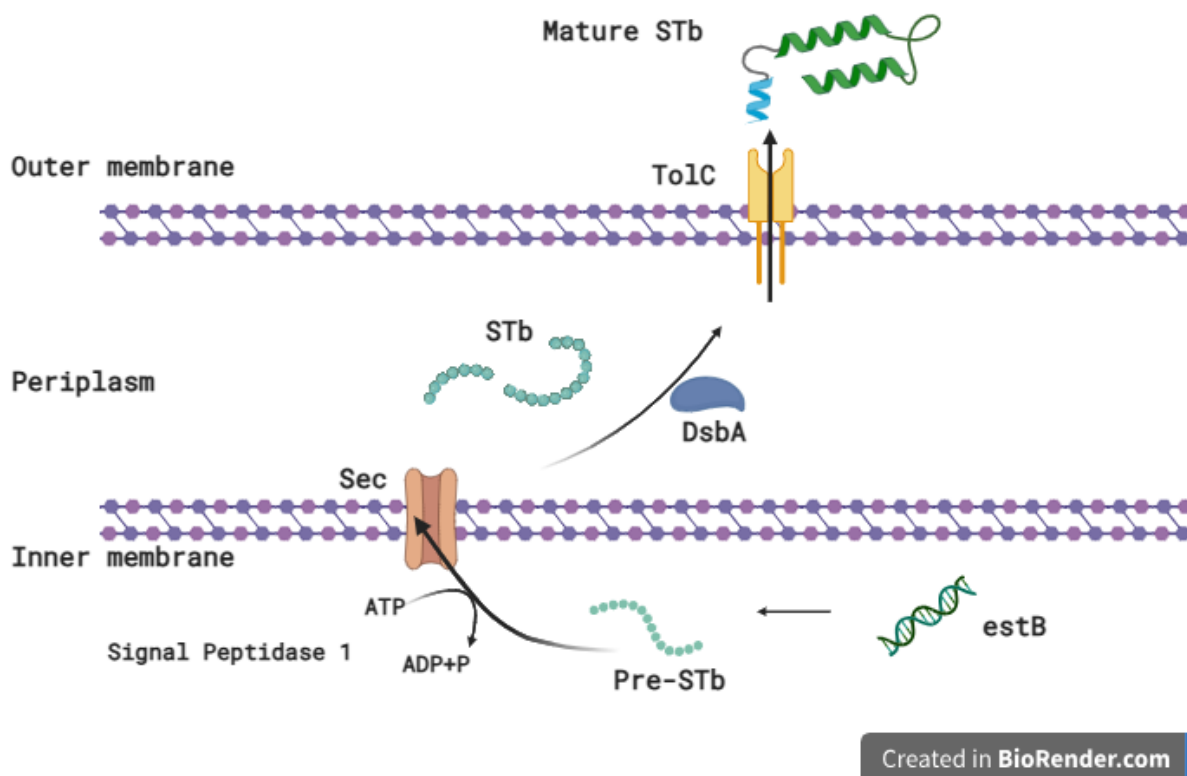


Figure 2.3: Mechanism of secretion of STb (Created in <https://BioRender.com>)

The process of the STb protein being secreted outside the bacterial membrane suggests that there is a process of Outer Membrane Vesicle (OMV)-mediated secretion and permeation of the outer membrane. Gram-negative bacteria, such as ETEC, are well known to secrete OMVs. OMVs are known for their spherical shape and bilayer composition. The diameter of OMVs varies between 20 and 300 nm. OMVs are formed when a part of the outer membrane of the bacteria detaches and forms a vesicle. OMVs contain periplasmic material such as proteins, lipopolysaccharides, and nucleic acids (Kesty et al., 2004). Various studies have established that STb is related to OMVs.

This suggests that a part of the periplasmic STb of the bacteria is included in OMVs and secreted in groups when the OMVs fuse with the targeted cell (Kesty et al., 2004). This secretion of STb via OMVs could be useful in the concentration of STb and its interaction with the sulfatide receptors located in the outer membrane of the bacterial cell.

In more recent research into the mechanisms of secretion of small ETEC toxins, it has been proposed that these mechanisms could possibly involve the translocation system from the outer membrane. The type II secretion system, also known as the general secretion system, is a complex molecular machine that secretes folded periplasmic proteins across the outer membrane using an ATP-dependent mechanism. The type II secretion system has been most well studied with respect to LT (heat-labile enterotoxin) secretion in ETEC. Emerging research has proposed that components of the type II secretion system could possibly be involved in STb secretion in ETEC (Kupersztoch et al., 1990).

2.4.2. Extracellular Folding and Stability in the Intestinal Lumen (Heat-Stability)

STb has to retain its stability and function in the intense biochemical environment of the small intestinal lumen, once it is secreted outside of the cell. This environment is characterized by an extremely low pH, varying from 4.0 in the stomach and rising to 7 or 8 in the small intestine, the presence of proteolytic enzymes like trypsin, chymotrypsin, and other serine proteases of pancreatic origin, bile salts, and the constant flux of the contents of the intestine. The thermostability of STb in the face of all this adversity, especially its resistance to boiling in water at 100°C for 30 minutes, is an important characteristic of this protein that enables it to survive in the lumen of the small intestine long after it has been produced by ETEC (Burgess et al., 1978).

2.5. Comparison of STb with STa (*estA*) and Other Enterotoxin Gene Clusters

A comparison of the sequence of the STb and its functional homolog STa (heat-stable enterotoxin a, *estA*) revealed a number of differences despite the fact that both are the heat-stable enterotoxins of ETEC, which induce intestinal secretion. The *estA* gene specifies a 72-amino-acid precursor from which the final toxin is composed of only 18-19 amino acids, which is much shorter than the 48 amino acids present in the final STb toxin (Aimoto et al., 1982). The final STa toxin also contains three disulfide bonds compared with the two in the final STb toxin. While STa takes a shape similar to the mammalian guanylin family, STb takes an α -helical structure with no homology to STa (Sukumar et al., 1995).

Genetically, *estA* and *estB* lack significant nucleotide homology. They are located on separate plasmidic regions, flanked by different mobile elements, and are controlled by overlapping but non-identical signals (Moseley & Falkow, 1980). Each of these strains may possess one or both of these genes, which are separately acquired through different horizontal gene transfer events facilitated by phylogenetic diversity. As far as the specificity of the receptors and the mechanisms involved in this process are concerned, the following may be noted: Unlike STa, STb fails to stimulate guanylyl cyclase receptor activity in the epithelial cells of the intestine, which in turn could stimulate an increase in cyclic GMP, cystic fibrosis transmembrane conductance regulator chloride channels, and hence stimulate fluid secretion. Contrary to this, STb stimulates sulfatide receptor activity, which in turn could stimulate prostaglandin E₂ and serotonin through a calcium-dependent mechanism (Gonçalves et al., 2007).

Comparison with other enterotoxins helps to further understand the special characteristics of STb. The heat labile enterotoxin (LT), which is encoded by the *eltA* and *eltB* genes on the Ent plasmids,

is an AB5 type enterotoxin that activates adenylyl cyclase through ADP-ribosylation of the Gs α subunit, thereby generating intracellular cAMP and activation of Cystic fibrosis transmembrane regulator. The simultaneous occurrence of LT and STb in ETEC strains is well known (Read et al., 2014).

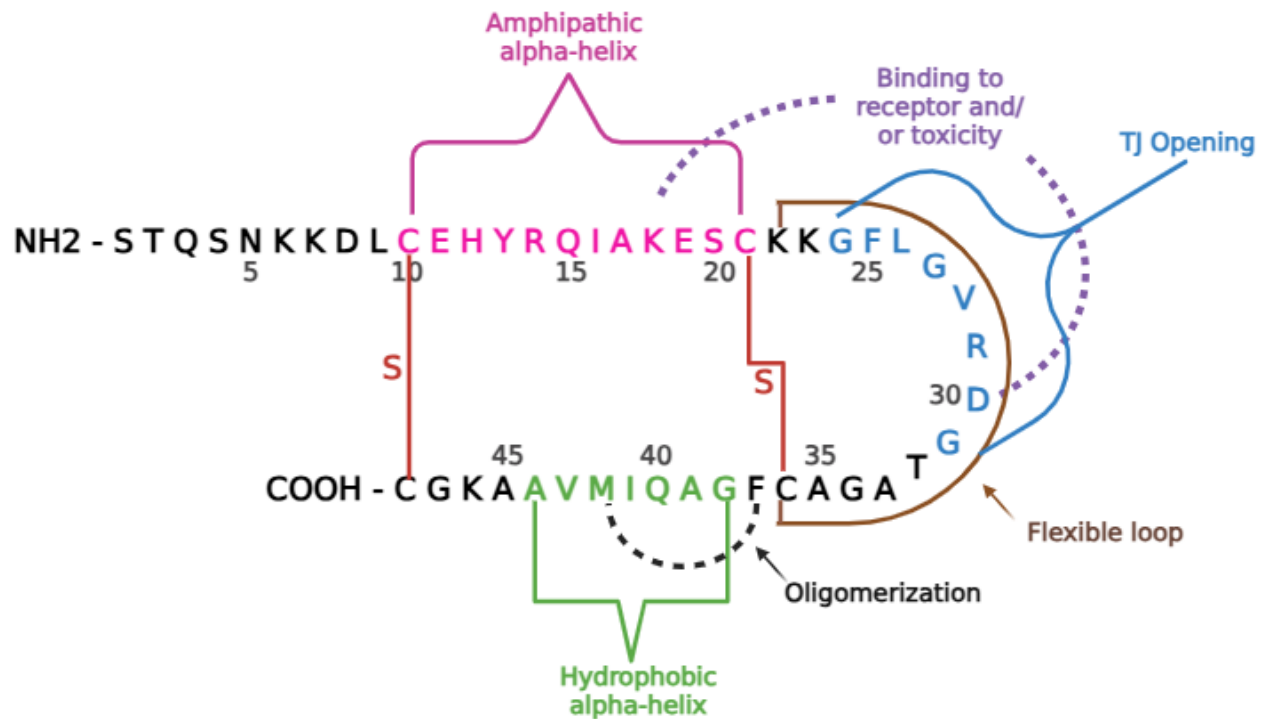
Chapter 3: Structural Biology of STb

3.1 Primary Structure: Amino Acid Sequence and Proteolytic Processing

The structure of STb refers to the sequence of amino acids that compose the toxin. This is the primary structure that gives rise to the secondary, tertiary, and quaternary structures of the enterotoxin. The 48 amino acids that compose the STb enterotoxin are derived from the C-terminal of the 71-72 amino acid precursor following the removal of the signal peptide and periplasmic processing of the precursor (Dreyfus et al., 1983; Picken et al., 1983). The entire sequence of STb has been elucidated using both chemical and DNA sequencing method, read as: Ser-Thr-Gln-Ser-Asn-Lys-Lys-Asp-Leu-Cys-Glu-His-Tyr-Arg-Gln-Ile-Ala-Lys-Glu-Ser-Cys-Lys-Lys-Gly-Phe-Leu-Gly-Val-Arg-Asp-Gly-Thr-Ala-Gly-Ala-Cys-Phe-Gly-Ala-Gln-Ile-Met-Val-Ala-Ala-Lys-Gly-Cys (residues 1–48) (Figure 2.2), though some variations have been reported for different strains of the bacterium (Sukumar et al., 1995).

The other characteristic of this primary structure is the presence of four cysteine residues that are absolutely conserved and are found at positions 10, 21, 36, and 48. These cysteine residues are involved in the formation of two essential disulfide bonds between cysteine residues at positions 10 and 48 and those at positions 21 and 36 as seen in Figure 3.1. These disulfide bonds are essential in maintaining the mature peptide in a conformation that contains two anti-parallel alpha helices (Cys 10-Lys 22 and Gly 38-Ala 44) separated by a loop (Dreyfus et al, 1992). The substitution of any of the cysteine residues in this protein subunit with a serine or an alanine residue has been shown to result in the loss of toxin activity. Apart from the cysteine residues, the sequence of the STb toxin is also characterized by its amphipathic nature, meaning it has both a face and a polar/hydrophilic face, where the N-terminal part of the sequence forms an amphipathic α -helix in

the primary structure, which is also seen in the secondary structure, whereas the C-terminal part of the sequence is rich in hydrophobic amino acids which acts as the hydrophobic, oligomerization-driving part of the toxin (Sukumar et al., 1995)



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Figure 3.1: Primary sequence of STb toxin. Light pink residues 10–21: amphipathic alpha-helix portion; residues 18–30 (purple dotted line): receptor binding/toxic region; light blue residues 24–31: tight junction manipulation region; residues 37–42: oligomerization region; light green residues 38–44: hydrophobic alpha-helix region (Created in <https://BioRender.com>)

3.2 Secondary and Tertiary Structure: NMR and X-ray Crystallography Insights

The three-dimensional structure of STb has been elucidated by nuclear magnetic resonance spectroscopy, as it is not possible to elucidate the three-dimensional structure of STb by X-ray crystallography due to its small size and low solubility. The first three-dimensional structural

analysis of STb by Nuclear Magnetic Resonance (NMR) spectroscopy, conducted by Sukumar et al. (1995) in aqueous solution with structure-stabilizing agents, revealed a compact disulfide-bonded globular fold comprising two amphipathic α -helical segments and a loop containing a turn.

The secondary structural elements of the protein are two helices. One is an N-terminal α -helix that spans residues 10 to 22, and the other is a C-terminal α -helix that spans residues 38 to 44. The amphipathic nature of the N-terminal α -helix is because of the fact that the side chains of Asp 8, His 12, Gln 15, Lys 18, Glu 19, Lys 22, and Lys 23 face the solvent. It is of interest to note that Lys 22 and Lys 23 have been shown to be of importance for toxic activity (Fujii et al., 1991). Some of the hydrophobic side chains of the N-terminal α -helix face those of the C-terminal α -helix. The C-terminal α -helix is relatively more hydrophobic, with Ile 41, Met 42, and Phe 37 forming a hydrophobic cluster. Ile 41 makes contact with the side chain of Arg 29 in the loop region (Sukumar et al., 1995). The loop region spans residues 21 to 36 and is connected by the disulfide bond.

The tertiary structure is a compact elongated structure with dimensions up to 30 Å in length. It is stabilized by the presence of two disulfide bonds between cysteine residues at positions 10 and 48, and 21 and 36. It also contains hydrophobic interactions between the two α -helices. The circular dichroism spectra confirm the presence of 40-50% α -helices with the characteristic two minima at 208 nm and 222 nm. The thermal denaturation study revealed the thermostability of the structure even at 100°C in the absence of reducing agents. However, the effect of reduction of disulfide bonds with dithiothreitol on the thermostability of the peptide is quite dramatic, which proves the role of disulfide bonds in the thermostability of the peptide (Sukumar et al., 1995).

3.3 Disulfide Bonding and Stability: Role in Heat Resistance

The high stability of STb is directly linked with its classification as a heat-stable toxin because of its two intra-molecular disulfide bridges (Okamoto et al., 1995). Disulfide bridges result in a cross-linked configuration of the peptide, which makes it stable against denaturation by heat and proteolysis. It has been found that the disulfide bridges of STb, analyzed by tryptic peptide analysis and Edman degradation, occur between Cys 10 and Cys 48, and Cys 21 and Cys 36 (Dreyfus et al., 1992). It seems that the disulfide bridge is crucial for stabilizing the toxin against proteolytic cleavage in the periplasm and that it is involved in determining the biological activity of extracellular STb. Evidence exists that indicates that formation of disulfide bonds is a periplasmic event (Kupersztoch et al., 1990). Reduction of these disulfide bonds by reducing agents like dithiothreitol, abolishes all activity and makes it more susceptible to proteases like trypsin (Fujii et al., 1991). The stability of STb is so great that it can survive temperatures as high as 100°C for 30 minutes as well as navigate through the proteolytic environment of the stomach and small intestine to reach the target cells in an active state. Site-directed mutagenesis confirms that both disulfide bonds are necessary for the stability and activity of STb. Altering either of the disulfide bonds between Cys10 and Cys48, Cys21 or Cys36 abolishes activity in the Chinese hamster ovary cell elongation assay and in infant mouse models of infection (Dreyfus et al., 1983). Inhibition of either of the disulfide bonds prevents activity. This demonstrates the requirement for both disulfide bonds in the formation of a functional receptor-binding conformation.

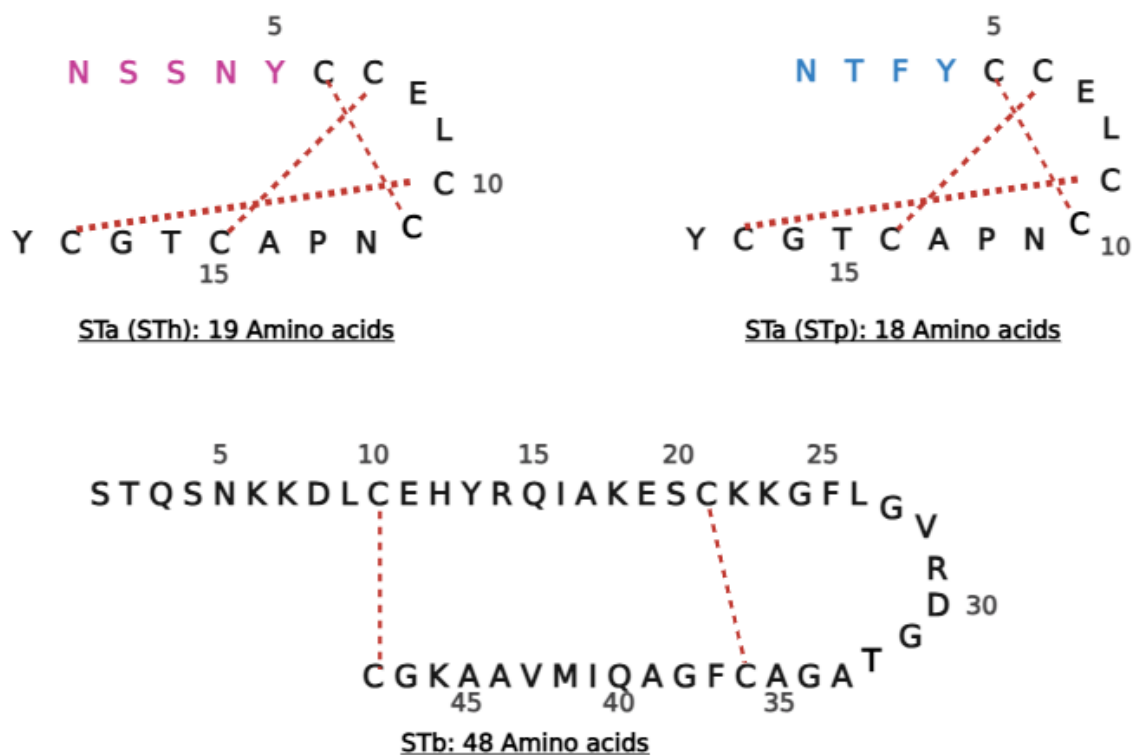
3.3.1. Heat stability: Role of Cysteine Residues and Structural Rigidity.

The molecular basis for the heat stability is not just the disulfide cross-links but also the geometry of the disulfide cross-links, which also constrains the entropy change in the peptide chain when it

is unfolded. Thermodynamically, the disulfide cross-links impose a constraint on the entropy change in the peptide chain when unfolded. It also stabilizes the hydrophobic core region. This is a result of the interacting faces of the two structures of the α -helix. As seen in mutagenesis studies, when serine replace cysteine, it results in a loss of heat stability as well as a disruption of the interacting faces, thus hindering membrane binding as well as toxicity (Okamoto et al., 1995). Thus, cysteines are not just structural components, as they also play a role in forming a specific three-dimensional structure for receptor interaction as well as disruption of membranes.

3.4. Comparative Structural Analysis with STa and Other Bacterial Toxins

Comparative structural analysis of STb with STa and other bacterial toxins revealed fundamental differences in the structural architecture of the toxins. Though both toxins are named heat-stable enterotoxins, structurally they are unrelated to one another. These are the result of independent evolutionary processes. STa is a much shorter peptide than STb. STa is a peptide of 18 or 19 amino acid residues in the toxin's mature form, whereas STb is a 48-amino-acid residue peptide. Three disulfide bridges in the structure of STa stabilize the peptide, whereas two disulfide bridges in the structure of STb stabilize the peptide. Another interesting homology of STa is its similarity in the overall tertiary structure to the mammalian guanylin/uroguanylin family of peptides. It is, therefore, expected that STa will bind with high affinity to the mammalian guanylyl cyclase receptor C. There is no homology in the structure of STb with the mammalian guanylin/uroguanylin family of peptides. Also, STb does not bind to the mammalian guanylyl cyclase receptor C and, therefore, cannot stimulate guanylyl cyclase or elicit cGMP-mediated effects (Weikel & Guerrant, 1985).



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Figure 3.2: Comparison of STb with STa (Created in <https://BioRender.com>)

The structure of STb is more similar to that of pore-forming toxins or membrane active peptides, like melittin, a component of bee venom, than that of STa. It has amphipathic alpha helices, like melittin, which are known to be capable of oligomerization. However, the mechanism of action of STb is receptor-dependent, as it first binds to sulfatide with high affinity, which is different from most pore-forming toxins. Its ability to bind to a receptor in a specific manner followed by non-specific membrane interaction makes STb a unique entity in both its structure and function, unlike the AB5 subunit of the LT and CT, large molecules with enzymatic activity against intracellular signaling proteins.

3.5 Conformational Dynamics and Functional Epitopes: Receptor-Binding vs. Pore-forming Regions

The conformational dynamics of STb in solution, in membranes, and upon receptor binding are crucial for an understanding of its mode of action. Nuclear magnetic resonance (NMR) relaxation measurements of ^{15}N -labeled STb were used to study the internal dynamics of different parts of the protein on the picosecond to nanosecond time scale (Sukumar et al., 1995). These measurements indicated that, while the two α -helices and the central disulfide-bonded loop display restricted internal motions, consistent with these structural elements being part of the structural core of STb, the N-terminal region and C-terminal tail are relatively flexible. This differential flexibility is of potential importance because it indicates that these N-terminal and C-terminal regions of STb are capable of induced fit-type conformational changes upon receptor interaction or membrane association.

The oligomerization potential of STb, which allows it to dimerize, trimerize, and even oligomerize to higher order species at concentrations above approximately $1\ \mu\text{M}$ in solution, indicates that this protein is capable of adopting at least two different conformations. One is its monomeric form, which is of potential importance at lower concentrations, and the other is its oligomeric form, which could be of potential importance at higher local concentrations of STb at the membrane surface or in membrane domains that are highly enriched with sulfatide. STb is known to be able to exist as an oligomer of predominantly dimer and tetramer types at concentrations of more than $1\text{-}5\ \mu\text{M}$, and that this is encouraged by conditions that mimic those of the membrane environment, such as detergent micelle or lipid vesicle systems (Dreyfus et al., 1993).

3.5.1. N-Terminal Region (Residues 10-21): Amphipathic α -Helix

This helix, held together by the Cys10-Cys48 bond, is essential for the initial cytotoxic effects. Its hydrophobic face is associated with the early interaction with the lipid bilayer after binding to the receptor and the induction of membrane permeabilization (González et al., 2007). Increasing the hydrophobicity of the face through mutation increases the toxicity, whereas the introduction of polar residues has the opposite effect. This helix is thought to be the main contributor to membrane insertion, potentially leading to the formation of ion-permeable pores or non-specific membrane disruption.

3.5.2. Central Domain: Receptor Binding and Toxigenic Region

The central domain of STb is the major receptor-binding and toxigenic region of the protein. This unstructured loop and the second helix contain important residues that participate in receptor interaction and signal transduction. Various studies have identified that these regions play an important role in the interaction of STb with the receptor and subsequent toxic response in intestinal cells. Moreover, the presence of positive charges in this region, particularly Lys22 and Lys23, may be important in the electrostatic interaction with the negatively charged sulfate group of the sulfatide receptor (Labrie et al., 2001). The electrostatic complementarity of the basic residues in the central domain of STb and the negatively charged sulfate group of the sulfatide receptor may be the first step in STb-receptor interaction. This interaction may be similar to the electrostatic steering that is important in lectin-type carbohydrate-binding interactions (Rousset et al., 1998). In addition, this region may contain important residues that participate in the intracellular calcium signal transduction.

3.5.3. C-Terminal Hydrophobic α -Helix and Oligomerization

The hydrophobicity of the C-terminal helix, consisting of residues 36 to 48, is important since the pattern of sequence resembles that found in the transmembrane domains. Such characteristics are primarily responsible for the membrane-insertion capability of the STb protein and its membrane integrity disruption (Okamoto et al., 1995). The C-terminal helix is also involved in the oligomerization of the STb protein. Oligomerization is a prerequisite for the activity of the protein in the form of an ion channel. The oligomerization activity is hypothesized to be facilitated by the C-terminal helix in the membrane upon the binding of the protein to the host cell receptors. Oligomerization in the form of a tetrameric or hexameric channel is a common activity of many membrane-destroying toxins and may explain the increase in membrane permeability. The C-terminal helix, with its large hydrophobic face, is the most plausible interface for helix–helix packing in the oligomeric assembly. The disulfide bridges and residues Lys22, Lys23, and Met42 that are essential for STb's enterotoxicity and oligomerization of STb are also indispensable for the ability of STb to permeabilize cellular membranes (Gonçalves et al, 2007).

Chapter4: Cellular Receptors and Binding Mechanisms

4.1. Identification of STb Receptors: Sulfatide and Other Glycolipids

The first and most important event in the pathogenesis of STb infection is its attachment to a specific receptor present on the surface of a host cell. STb appears to interact with various cell types and in different ways. Since the early 90's, extensive research has been conducted on what form of cell receptor STb actually binds to, upon the intestinal cell surface in various organisms. Rousset et al. (1998) proved that STb possessed an excellent binding affinity with sulfatide, also known as 3'-sulfogalactosylceramide (3'-SO₄-GalCer), which is a type of glycolipid obtained from porcine jejunum brush border membranes and is expressed on the apical membrane of intestinal epithelial cells and other cells. It is a glycosphingolipid containing a ceramide lipid chain that binds with the plasma membrane of the cell and a carbohydrate moiety consisting of a sulfated galactose sugar. Observing the binding effects to pure sulfatide, they found that the binding occurred in a dose-dependent, saturable fashion, suggesting that sulfatide could in fact mediate the binding of STb to target enterocytes. This receptor has been characterized through several lines of evidence: 1) inhibitor studies demonstrated that the binding of STb to the intestinal brush border membranes/cells was inhibited by pretreatment of the cells with the sulfated carbohydrate galactose-3-sulfate; 2) treatment of the cells with arylsulfatase, which removes the sulfate from the cell surface, inhibited the binding of STb and subsequent toxicity; and 3) liposomes containing sulfatide but not other glycolipids such as monosialotetrahexosylganglioside (GM1) were able to bind STb (Labrie et al., 2001).

Sulfatide is a sulfoglycolipid consisting of a ceramide lipid group and a glycosidic linkage to a galactose group with the 3'-hydroxyl group linked to a sulfate ester group. The sulfate gives the

sulfatide a strong negative charge at a low $pK_a < 1$ at physiological pH and makes the sulfatide a highly electronegative lipid. The electronegativity of the sulfatide head group is used to interact with the positively charged amino acid residues in the central receptor-binding domain of the STb protein. The ceramide backbone of the sulfatide also makes hydrophobic contact with the amphipathic helices of the STb protein upon insertion of the protein into the membrane environment upon initial interaction with the sulfatide (Gonçalves et al., 2007). Early report by Dreyfus et al. (1993) suggested that STb binds to a G protein-coupled cell-surface receptor within the intestinal cell membrane. The coupled receptor was then found to dissociate in order to activate a receptor-dependent ligand-gated calcium channel embedded within the membrane. The consequences of such interactions have been determined to be activation of a G-protein-coupled receptor and calcium ion influx into the cell (Dreyfus et al., 1993). Although it is the recognized high-affinity receptor, some studies indicate that STb can also interact, in a low-affinity manner, with other negatively charged glycolipids such as GM1a and GD1a, but with significantly lower potency (Fujii et al., 1991). This could be important in the presence of other cell populations where the level of sulfatide is low.

4.2. Localization of Receptors in Intestinal Epithelial Cells

The distribution of the sulfatide molecules is not uniform on the cell surface of the intestinal epithelial cells. The distribution pattern of the sulfatide molecules provides a clue to the possible location of the action of the STb. Immunohistochemical and biochemical studies have established the fact that the sulfatide molecules are enriched in the apical membrane of mature enterocytes of the intestinal epithelium cells, particularly in the upper part of the jejunum and the ileum, which are the main colonization sites of the ETEC pathogens and the secretory response to STb infection (Rousset et al., 1998). This indicates the fact that after entering the intestinal lumen following

secretion by the colonizing ETEC pathogens, the toxin binds to the cell surface receptor precisely at the cell surface maximally exposed to the action of the toxin and the receptor. The fact that the sulfatide molecules are the main lipid molecules in lipid rafts, which are functional microdomains of the plasma cell membrane enriched in cholesterol and sphingolipids and signaling molecules, provides evidence that the action of the toxin to the cell surface receptor could be mediated by lipid rafts.

STb's interaction with sulfatide is probably the first event in the aggregation of complexes containing the toxin and the receptor into these lipid rafts, a process which is likely to be crucial for the efficient progression of the following events such as oligomerization and endocytosis (Fujinaga et al., 2003). The density of sulfatide in the apical membrane is adequate for the occurrence of a multivalent binding event involving this lipid and STb. This is likely to be crucial for the efficient occurrence of events such as the oligomerization of the toxin at the membrane surface as well as the membrane perturbation events associated with the action of this virulence factor (Gonçalves et al., 2007). Toxin binds minimally to crypt cells as well as goblet cells, a phenomenon which is consistent with the occurrence of secretory diarrhea caused by this virulence factor without major mucosal invasion/destruction. The apical localization of this virulence factor is crucial since it is on the enterocytes of the villus where fluid and electrolyte absorption occurs.

4.3 Binding Affinity and Specificity: Structure–Function Relationships

Quantitative measurements of the binding affinity of STb for sulfatide have been carried out through a number of different, yet complementary, approaches. All studies conducted to date have demonstrated that the K_d value for STb-sulfatide interaction is between 1 and 10 nM. This indicates that STb binds to sulfatide with very high affinity, similar to many protein-carbohydrate

interactions. In addition, all studies have shown that binding of STb to sulfatide is mediated by a very high degree of affinity and specificity, due to the high degree of complementarity between the two molecules. The molecular basis of STb-sulfatide binding has been studied using mutational analysis of the STb molecule and structural modifications of sulfatide to identify the specific determinants of this binding event. For STb mutagenesis, the receptor binding site of the STb molecule has been shown to be located in the central region of STb, which contains the positively charged amino acids. Substitution of these residues, with their predicted side chains involved in electrostatic interactions with the negatively charged sulfate ester group of sulfatide ($R-O-SO_3^-$), with alanine and glutamic acid, leads to a significant reduction in binding of STb to sulfatide-containing liposomes and cells, and abolishes toxicity (Labrie et al., 2001). Using HPLC-purified toxin, sulfatide binding in ELISA and toxicity in the rat loop model were assessed. When a mutant showed reduced sulfatide binding in ELISA, this was accompanied by at least an equivalent reduction in toxicity (Lortie et al., 1991). This showed that sulfatide binding was a prerequisite for toxicity. Residues 22-30 were directly implicated in sulfatide binding and expression of toxicity.

On the other hand, the hydrophobic surfaces of the N- and C-terminal α -helix do not play a significant role in the initial stage of the receptor recognition process; however, they do play a vital role in the membrane insertion process. The specificity of the STb-glycolipid interaction is also supported by the fact that desulfated sulfatide (galactosylceramide) and other non-sulfated glycolipids do not support the binding (Labrie et al., 2001). The chemical modification studies on the structure and function of the glycolipid revealed the significance of the 3' sulfate group on the sulfatide molecule. The modification of the 2' hydroxyl group on the galactose moiety does not affect the binding of STb with the glycolipid; however, the removal of the 3' sulfate group by

treatment with arylsulfatase does not allow the binding of STb (Beausoleil et al., 2002). Through this process of mutagenesis, it was established that the hydrophobic α -helix structure was responsible for the oligomerization of the STb molecules. Interference with this process inhibited the expression of toxicity in the rat loop assay system because oligomerization is likely to be a prerequisite for interaction with susceptible cells (Labrie et al., 2001). These elements describe a model of binding specificity in which the sulfate ester in the 3'-position on the galactose ring is both necessary and sufficient for the initial binding process, with secondary hydrophobic membrane interactions playing a role in enhancing the overall binding affinity.

4.4.Receptor-Mediated Internalization Pathway

After the sulfatide binding and aggregation in the lipid rafts, some of the cell-bound STb is taken into the target cells through endocytic mechanisms, although the role of this process in the toxicity of STb remains to be fully elucidated. The evidence supports the involvement of a clathrin-independent, lipid raft-mediated endocytic pathway. Further studies have revealed that the endocytic pathway for the sulfatide-bound STb is mediated through the caveolae-mediated endocytosis pathway. This is a clathrin-independent endocytic pathway. This pathway is mediated through the formation of a complex through the integration of these substances into the membrane microdomains rich in cholesterol and sphingolipids, also referred to as lipid rafts. This may be the major pathway for STb entry into cells because of the preferential association of sulfatide with lipid raft/caveolar microdomains. Inhibitors of clathrin-coated pit formation, such as hypertonic sucrose and chlorpromazine, have no effect on STb action, whereas inhibitors of lipid raft integrity, such as cholesterol-depleting reagents like methyl- β -cyclodextrin, have been found to inhibit the entry of STb and its physiological effects (Fujinaga et al., 2003) in a manner that correlates with its effects on signal transduction. However, confocal microscopy showed the colocalization of STb

with autocrine motility factor receptor (AMFR), a marker for the smooth endoplasmic reticulum. The use of methyl- β -cyclodextrin, which interferes with the expression of caveolae, inhibited the colocalization of STb with AMFR and reduced the internalization of STb but did not stop the endocytosis of STb (Dubreuil et al., 2007).

The internalization of STb via caveolae colocalizes with smooth endoplasmic reticulum and is associated with mitochondria, as identified by a mitochondrial heat shock protein 70 marker. Inhibition of this pathway by acid treatment of the cells to interfere with the clathrin-dependent pathway does not stop the internalization of STb. Endocytosis of STb via vesicles colocalizes with fibronectin fibers. Sulfatide, which is the receptor for STb, colocalizes with STb and smooth endoplasmic reticulum tubules (Dubreuil et al., 2007). Once inside the cell, the endosomes containing STb move through the endolysosomal pathway, and a portion of the internalized toxin is located in the early endosomes and recycling endosomes. This is a slow endocytosis, and the toxin is located in vesicles minutes after binding. The fate of the internalized STb is not well understood. However, it is thought that this part of the toxin could be targeted to early endosomes and that there could be a partial degradation in lysosomes. This could be to control or modulate the activity of the toxin. However, a major part of the activity of the toxin, especially in the rapid induction of calcium influx, could occur from the plasma membrane immediately after binding and oligomerization. This process could have two roles: 1) to target a part of the toxin to an intracellular compartment that could potentially act on other targets, and 2) to modulate or control the activity or length of time that a signaling process continues. The part of the toxin that is internalized could potentially continue signaling from endosomal compartments. However, this part of the activity of STb is not well understood.

4.5. Species and Tissue Specificity of STb Binding

The biological activity of STb in the living body is species-specific, a phenomenon that has been explained on the basis of differences in sulfatide content and structure in different species and tissues. Initially, STb was identified as a veterinary enterotoxin that induced secretory diarrhea in neonatal pigs and to a lesser extent in calves and rabbits. Later on, it was also identified as a human diarrheal toxin. However, the sensitivity of different animal species to the effect of STb differs considerably. For example, pigs are very sensitive to the effect of STb, humans are also sensitive to this toxin, especially young children. On the other hand, adult mice and rats are less sensitive to the instillation of STb in their intestines (Kennedy et al., 1984). These differences in the sensitivity of different species to the effect of STb have also been explained on the basis of differences in the amount of sulfatide present in the brush border membrane of the intestines in different species. For example, the effect of STb is very potent in pigs, a phenomenon that could be explained on the basis of the high amount of sulfatide present in the small intestine of pigs (Rousset et al., 1998). The relevance of STb to human disease has been studied. Human intestinal cells express higher levels of sulfatide and are susceptible to STb. This confirmed the presence of the receptor (Labrie et al., 2001). However, the density and availability of the sulfatide in the human gastrointestinal tract *in vivo* may be lower than in pigs. This may explain why STb-positive ETEC strains produce less severe diarrhea in human volunteers than STa-positive strains.

Apart from the species variation, the expression of sulfatide in different organ systems also influences the potential effects of STb on different organ systems. Sulfatide is highly expressed in the nervous system. It is particularly abundant in the myelin sheath of peripheral and central nervous system nerves and a major component of the myelin lipid fraction. Sulfatide is also highly expressed in the kidney tubular cells, the cornea, and the pancreas. Thus, apart from the

gastrointestinal tract, the presence of STb in circulation has the potential to affect many other tissues. In terms of physiological context, the major site of STb activity is the small intestinal lumen, where ETEC-colonized bacteria secrete the toxin directly adjacent to the sulfatide-positive apical membranes of small intestinal enterocytes. Although the binding of STb to these tissues has been observed, the pathological significance of this event *in vivo*, particularly in the context of natural infections, is likely minimal due to the restricted site of toxin activity (Rousset et al., 1998). This specificity is a reflection of the evolutionary adaptation of STb to its natural niche, which is the sulfatide-positive apical surface of small intestinal enterocytes.

The developmentally regulated expression of sulfatides in the small intestine is also a factor in the age-specific incidence of STb-induced diarrhea. In terms of density, the small intestine of neonatal animals and children is more densely sulfatide-positive compared to adults. This is consistent with the well-recognized phenomenon that ETEC diarrhea, including that caused by STb, is more common in neonatal animals and children aged five years or less, while adults exposed to the same strains of ETEC experience milder and/or shorter duration of disease, possibly due to immunity and possibly due to lower density of sulfatides in the small intestine of adults (Kumar et al., 2018).

Chapter 5: STb–Cell Interactions and Signal Transduction

5.1 Early Events: Membrane Attachment and Oligomerization

After binding with high affinity to sulfatide on the surface of lipid rafts, STb rapidly sets off a series of rapid, coordinated actions on the apical surface of intestinal enterocyte membranes, converting the free diffusing toxin into a membrane-associated, biologically active species that disrupts the integrity of the plasma membranes. Upon encountering the apical surface membranes of enterocytes lining the small intestinal villus lumen, STb first engages in non-specific, electrostatic attraction to the negative charge of the glycocalyx and the head groups of the phospholipid components of the enterocyte membranes, owing to its overall basic character, as reflected by its isoelectric point (pI) of 9.6 (Handl et al., 1993). This initial interaction is expected to concentrate STb toxin on the surface membranes, thereby increasing its probability of encountering sulfatide molecules, which are organized into cholesterol-enriched lipid raft domains on the outer leaflet of the apical surface membranes, as suggested by Gonçalves et al. (2007).

Specific, high-affinity interaction with the sulfatide receptor occurs, with the central basic loop of STb making contact with the sulfate group of the sulfatide through electrostatic interactions, while the amphipathic C-terminal helix interacts with the outer leaflet of the lipid bilayer. This type of membrane association, which involves the dual anchor mechanism, results in the STb receptor being positioned at an angle to the plane of the lipid bilayer, with the receptor-binding loop exposed at the membrane-aqueous interface, while the C-terminal helix interacts with the lipid bilayer in the region of the acyl chains (Rousset et al., 1998). Insertion of the hydrophobic surface into the lipid bilayer is thermodynamically favorable due to the hydrophobic effect, which also provides the free energy for the subsequent structural changes, including oligomerization, that

occur. An important early event in the membrane insertion process is the oligomerization of the toxin. While the exact stoichiometry of the oligomerization event is unclear, studies have indicated that the STb toxin monomers form tetrameric or hexameric complexes in the target membrane (González et al., 2007). This is mediated by hydrophobic interactions between the structures of adjacent monomers, especially those at the C-terminus, which are stabilized by their interaction with their surrounding membranes. This is functionally important, as it is considered to be a prerequisite for the formation of a transmembrane pore or ion channel. This is seen to occur within minutes of binding, prior to any ion flux. Inhibiting oligomerization, by mutation of regions important for oligomerization, or by drugs that disrupt lipid raft structure, completely prevents toxin activity, highlighting its role in the mechanism of action (Labrie et al., 2001).

5.2 Impact on Membrane Integrity: Pore Formation vs. Receptor-Mediated Endocytosis

There are a number of toxins that, in order to create a pore in the plasma membrane of a target cell, undergo oligomerization. This is a key step in the internalization of the toxin, leading to the intoxication of the target cell. This oligomerized pore can be created before the membrane insertion of the oligomer or after the insertion of the monomeric peptides of the oligomer into the membrane (Van der et al., 1992). Having identified that STb is composed of an amphipathic (Cys10-Lys22) and a hydrophobic (Gly38-Ala44) α -helix, it has been suggested that it has the potential to insert itself into the plasma membrane, leading to pore formation via oligomerization (González et al., 2007). The first oligomerization characteristic of STb was identified by Labrie et al. 2001. The oligomerized STb complex directly compromises the plasma membrane. The mechanism whereby membrane-associated STb oligomers convert their structural state into a functional perturbation of membrane integrity has been the focus of significant research, and two models have been proposed, although they are not mutually exclusive. The models include the receptor-mediated

endocytosis pathway resulting in endosomal signaling (Dubreuil et al., 2007), and the model of direct pore formation in the lipid membrane by the oligomerized STb receptor complex itself. The evidence points to the former model of pore formation at or near the plasma membrane as the initiating event for ion flux and subsequent signaling.

The electrophysiological studies conducted on synthetic lipid bilayers and epithelial cells have demonstrated that the STb receptor causes a non-selective increase in ion permeability, suggesting the formation of small aqueous pores. The pores formed by the STb receptor complex are permeable to small, water-soluble ions such as Na⁺ and K⁺; this results in the rapid depolarization of the membrane potential (González et al., 2007). Though the internalization of STb is mediated through receptor-mediated endocytosis, this pathway is not the major mechanism for acute cytotoxicity. This is demonstrated through experiments involving inhibitors of the endocytosis pathway, whereby the inhibitors only partially inhibit the acute calcium influx and ion secretion, whereas the pore-forming activity is immediate (Dubreuil et al., 2007). Receptor-mediated endocytosis is also involved in the sustained signaling response from the endosomal compartments, and it is likely that the mechanisms occur in parallel, whereby the pore-forming activity is the initial mechanism for the acute ion dysregulation at the plasma membrane and membrane depolarization, and the sustained response is mediated through the receptor-mediated endocytosis pathway.

5.3 Activation of Second Messengers: Calcium Influx, IP₃, and DAG

The second messenger signaling cascade activated by STb is unique from that activated by STa via cGMP and LT/CT via cAMP, and it is mediated by a calcium-dependent signaling cascade involving inositol 1,4,5 trisphosphate (IP₃), diacylglycerol (DAG), and direct calcium uptake from

the extracellular space. This difference has important implications for the nature of downstream signaling, pharmacological inhibition, and secretory and inflammatory responses mediated by STb.

The first and primary way that STb acts as a signal to the cell is by raising the level of calcium in the cytoplasm ($[Ca^{2+}]_i$) dramatically and quickly. The rise in calcium levels results in a biphasic calcium response. The first phase is characterized by a quick increase in the levels of calcium from the external environment, due to the influx of calcium through STb-forming pores and/or due to the activation of store-operated channels due to STb-induced influx of calcium. This is then followed by a longer, plateau-like phase where calcium is released from the endoplasmic reticulum (ER) due to inositol trisphosphate (IP_3) (Dreyfus et al., 1993). The mechanism for STb to induce the elevation of cytosolic calcium levels is that STb activates phospholipase C (PLC). STb has been shown to activate a specific type of PLC; PLC β isoforms, which are found located within the cell membrane but intracellular to the plasma membrane. When Phosphatidylinositol 4,5-bisphosphate (PIP_2) is hydrolyzed in the intracellular membrane of the cell, this generates two second messengers, i.e., DAG and IP_3 that can then release Ca^{2+} from the ER when the IP_3 binds to the IP_3 receptor (Gonçalves et al, 2007).

DAG is the second messenger generated during hydrolysis of PIP_2 and is responsible for activating the conventional PKC isoforms (PKC α and PKC β) at the plasma membrane through a calcium (Ca^{2+})-dependent mechanism. This leads to the phosphorylation of many of the pathological effects observed in the epithelial barrier function. This second messenger pathway of IP_3 , DAG, and calcium is a highly amplified pathway in the cellular response to the binding of STb toxin to its receptor. This is because one mole of PIP_2 hydrolyzed produces one mole of IP_3 for the release

of calcium ions from the endoplasmic reticulum and one mole of DAG for the activation of PKC (Dreyfus et al., 1993).

5.4 Downstream Effects: Protein Kinase Activation and Cytoskeletal Rearrangements, Tight junction disruption

The increased $[Ca^{2+}]_i$ and DAG-mediated PKC activation caused by STb initiate a series of devastating events that collectively disrupt epithelial homeostasis and induce secretory diarrhea (Figure 5.1). Calcium/calmodulin-dependent protein kinase II (CaMKII) activation, Myosin Light Chain Kinase (MLCK) activation and reorganization of the perijunctional actomyosin ring and TJ complex create a closed paracellular space between enterocytes. Activation of PKC results in MLC and TJ proteins' phosphorylation (both cytoskeletal/junctional proteins phosphorylated). TJs are composed mainly of a protein complex including occludin and the claudin family of transmembrane proteins known to associate with the apical perijunction of the F-actin ring via cytoplasmic plaque proteins such as ZO-1, ZO-2, and ZO-3 (Puthenedam et al., 2007). TJs restrict the diffusion of solutes through the intercellular spaces between intestinal epithelial cells and function as a barrier against the entry of pathogens and harmful antigens (Roselli et al., 2007). Additionally, the formation and maintenance of TJs are important for the formation and function of the epithelia (Balda et al., 1993). The PKC-mediated phosphorylation of occludin and claudins, such as claudin-4, leads to their internalization from the membrane and subsequent degradation, thus increasing the permeability of the epithelium. At the same time, the STb signaling causes alterations in the cytoskeleton. The PKC and other kinases lead to the disruption of the perijunctional actomyosin ring, which holds the tight junction complex, thus causing the strands to contract and pull away from each other (Ngendahayo et al., 2013). The contraction causes the widening of the spaces between the cells, thus increasing the permeability and allowing the

unregulated movement of water, ions, and other molecules, which contributes to the leak diarrhea caused by STb. These two mechanisms are the major causes of diarrhea by STb, in addition to the direct action of the pathogen on the secretion of anions.

In addition to disrupting the tight junctions of the enterocyte, the increase in $[Ca^{2+}]_i$ and activation of PKC by the STb also stimulate the synthesis and secretion of prostaglandin E₂ from the enterocyte by a calcium-dependent phospholipase A₂ activation pathway (Figure 5.1), to free arachidonic acid from membrane phospholipids, causing cyclooxygenase-2 to upregulate (Harville & Dreyfus, 1995), resulting in an increase in intracellular cAMP by activation of EP₂/EP₄ receptors, thus initiating the cAMP/PKA signaling cascade, which also results in an amplification of Cystic fibrosis transmembrane regulator mediated Cl⁻ secretion by a secondary cAMP-mediated mechanism. This amplification cycle of signaling through PGE₂ is the reason that the secretion induced by STb is partially sensitive to the use of cyclooxygenase inhibitors such as indomethacin (Harville & Dreyfus, 1995).

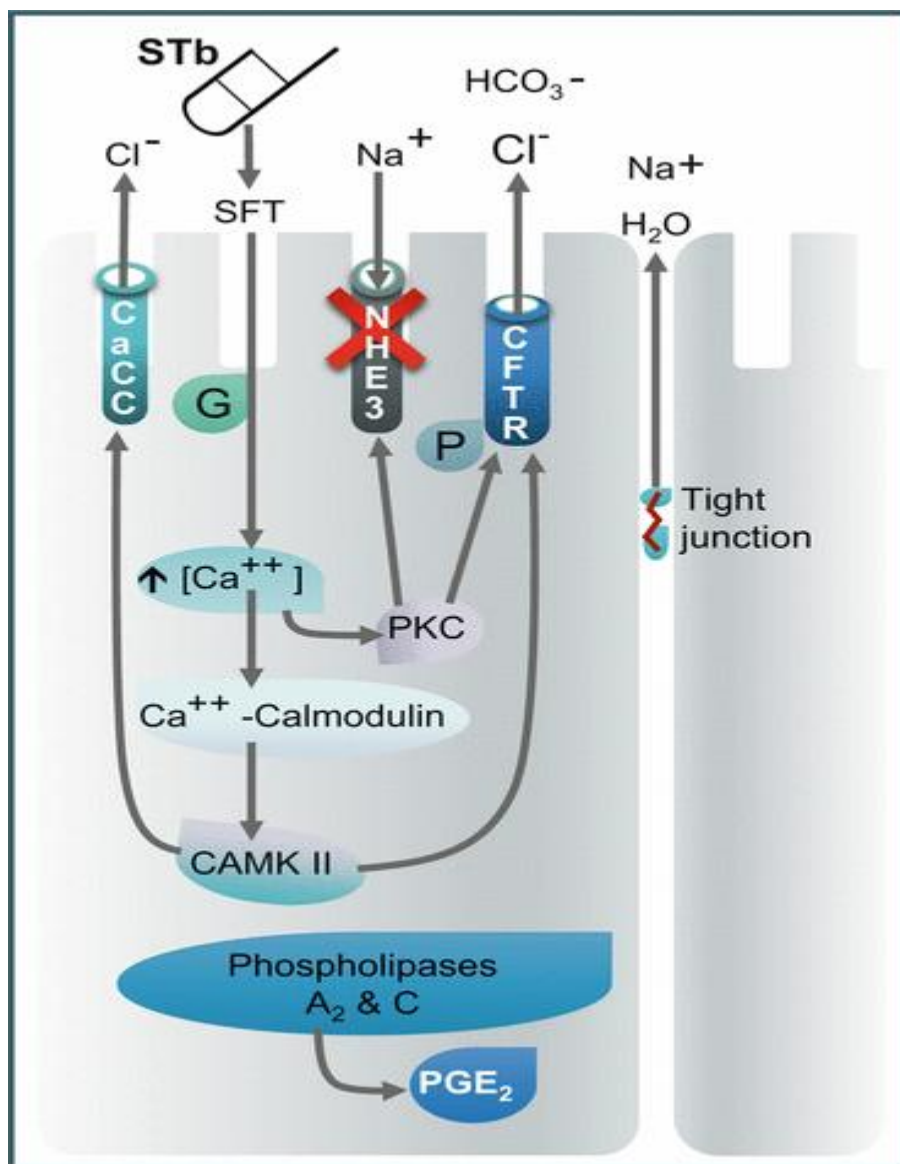


Figure 5.1: Mechanism of action proposed for STb induction of secretory diarrhea. Taken from Dubreuil, J.D. (2016). *Escherichia coli* STb Enterotoxin: A Multifaceted Molecule. In: Gopalakrishnakone, P., Stiles, B., Alape-Girón, A., Dubreuil, J., Mandal, M. (eds) Microbial Toxins. Toxinology. Springer, Dordrecht, with permission from Springer Nature (License no: 6239520694251)

5.5 Inflammatory Response: Cytokine Induction and Immune Cell Recruitment, Apoptotic and necrotic effects in enterocytes.

Besides its effects on the ion and water transport and barrier function of the intestinal epithelial cells, the STb also elicits a local inflammatory reaction in the intestine. The cellular stress and damage induced by the formation of pores, calcium overload, and disruption of the barrier function activate various inflammatory signal transduction cascades, which lead to the expression of pro-inflammatory cytokines, the recruitment of immune cells, apoptosis, and at higher doses, the necrosis of the enterocytes of the intestinal mucosa (Syed & Dubreuil, 2012). The cytotoxic and inflammatory effects of STb also increase the secretory response to STb and are responsible for the clinical manifestations and symptoms of the disease caused by ETEC infections. Both the NF-kappa B and Mitogen-Activated Protein Kinase (MAPK) signalling pathways in intestinal epithelium cells are activated by STb (Loos et al., 2012), which results in increased transcription of multiple chemokines and cytokines (for example, IL-8, Monocyte Chemoattractant Protein-1, Tumor Necrosis Factor-alpha, IL-1 β , IL-6). As a result of this activation of signalling through the convergence of the pathways activating NF-kappa B following activation of protein kinase C and calcium signalling; there is an increase in the transcriptional regulation and secretion of all inflammatory determinants (chemokines and cytokines).

The immune system is alerted to recruit more neutrophils and macrophages to an area increasing tissue damage. Neutrophils that infiltrate the area release reactive oxygen species (ROS), pro-inflammatory cytokines, and proteolytic enzymes all of which contribute to the damaging of the epithelial barrier and more fluids lost. The presence of STb for longer periods of time can also initiate programmed cell death. High levels of intracellular calcium [Ca²⁺]_i and mitochondrial dysfunction can initiate the intrinsic apoptotic pathway characterized by (cytochrome c) releasing

into the cytoplasm and activating caspase-9/3 (Xia et al., 2018). The prolonged elevated concentration of $[Ca^{2+}]_i$ by STb results in mitochondrial overload of calcium and an irreversible loss in the mitochondrial membrane potential. Under conditions of extensive membrane injury the cell may undergo necrosis. This loss of enterocyte results in a decrease in absorptive and barrier function. The resulting inflammatory environment and cellular death establish the foundation for STb-mediated disease pathogenesis and also underlie the mechanisms responsible for inflammatory diarrhea (Syed & Dubreuil, 2012).

Chapter 6: Pathophysiological Outcomes/Consequences of STb Action

6.1 Impact on Intestinal Barrier Function: Tight Junction Disruption

Inside the intestines, the luminal surface is covered by a dynamic layer of epithelial cells. This surface maintains a barrier between the luminal substances and the underlying neuronal and immune systems. It also maintains the transport of nutrients, fluid, and ions. This barrier is sustained by the presence of junctions between adjacent enterocytes. These junctions are called adherens junctions (AJs) and tight junctions (TJs). AJs and TJs are specific types of cellular junctions composed of proteins and are found between adjacent cells, especially in epithelial and endothelial cells. They are part of the apical junctional complex. TJs form a complex that is a multi-protein ring that encircles the cell membrane at the apical and lateral membrane domains. This complex enables the transport of solutes and ions via the lateral intercellular space. TJs also prevent the passage and transfer of intestinal substances, antigens, microbes, and the harmful products that these substances generate (Gonzalez-Mariscal et al, 1990). TJs are made up of a complex of proteins that form a ring. This complex is made up of four unique groups of proteins: claudins, occludin, Zonula Occludens Protein (ZO), and junction adhesion molecules (JAMs) (Ngendahayo et al., 2013). Transmembrane claudins and occludin are anchored to the cell membrane via the ZO complex. The ZO complex gives structural support to the claudin and occludin complex from the intracellular surface (Nassour & Dubreuil 2014).

One of the most significant pathophysiological effects of the action of STb on intestinal epithelial cells is the disruption of this TJ complex, resulting in an increased paracellular permeability and the leaky gut phenotype observed in ETEC infection (Butt et al., 2020). The enteric pathogenic bacteria have developed different strategies to disrupt the TJ complex either by altering the cellular

cytoskeleton or by changing the composition of proteins in the TJ complex. The alteration in the composition of specific proteins in the TJ complex is either by degrading the proteins with bacterial proteases or by the biochemical pathway of phosphorylation and dephosphorylation (Yuhan et al., 1997).

The activation of PKC, CaMKII, and MLCK by STb results in the phosphorylation of myosin light chain (MLC) and the contraction of the actomyosin ring, which physically separates the TJ strands and increases the paracellular space. The TJ protein occludin is responsible for the continuity of the TJ strands and ion selectivity in the paracellular space. Occludin has multiple serine and threonine phosphorylation sites for PKC. Once occludin has been phosphorylated at those sites, the process leads to ubiquitination followed by an endocytic process resulting with occludin being degraded by lysosomal enzyme action (Ngendahayo et al., 2013). Changes in claudin/occludin levels may thereby increase the permeability of epithelial cells for small molecules and ions (Gonçalves et al. 2007). One means of determining the extent of this effect would be through measurement of the decline in transepithelial electrical resistance, as well as the increase in paracellular transport of inert molecules such as fluorescein isothiocyanate (FITC) dextran. In addition, transepithelial electrical resistance is often used as a measure of the epithelial ability to generate electrochemical gradients. It can also be a measure for the "tightness" of the TJs. Paracellular flux is a measure for transport efficiency over time. It can also be a measure for paracellular permeability (Ngendahayo et al., 2013). During active ETEC infection, ongoing disruption of the barrier may contribute to the translocation of luminal antigens, bacterial products such as lipopolysaccharide, and the toxin itself into the submucosa, thus creating a vicious cycle of barrier disruption, inflammation, and further barrier disruption.

6.2 Fluid and Electrolyte Secretion: Mechanisms of Diarrhea Induction

STb-induced diarrhea is an exemplary case of secretory diarrhea caused by the net efflux of ions and water into the lumen. The rise in $[Ca^{2+}]_i$ activates Calcium-activated Chloride Channel (CaCC) channels in intestinal cells, which are present on the apical membrane. The activation of CaCC channels enables the secretion of chloride from the cells into the lumen (Butt et al., 2020). The activation of protein kinase C and subsequent activation of cystic fibrosis transmembrane conductance regulator (CFTR) result in the secretion of H_2O , HCO_3^- and other electrolytes from the cells into the lumen (Figure 5.1). The activation of protein kinase C also inhibits sodium uptake by activating an unidentified sodium channel known as Sodium-Hydrogen Exchanger-3 (NHE₃), increasing the sodium ion concentration in the lumen (Rousset, et al, 1998). The secretion of chloride and sodium into the lumen generates an osmotic gradient that attracts water into the lumen passively. The water is attracted into the lumen through both transcellular and paracellular routes. Fluid produced in the lumen exceeds the absorptive capacity of the small intestine, resulting in excess fluid going into the large intestine. The excess fluid overwhelms the absorptive capacity of the colon and results in diarrhea (Weikel & Guerrant, 1985). The build-up of calcium ions within a cell could trigger phospholipase A₂ and phospholipase C activity to release arachidonic acid. Prostaglandin E₂ (PGE₂) and 5-hydroxytryptamine (5-HT), two secretory stimuli, would cause the movement of water and electrolytes into the space outside the cell; however, their mechanism of action is not yet known (Harville & Dreyfus 1995). The increased permeability of the paracellular space allows the unregulated movement of water and ions through the cell membrane in response to the osmotic and electrochemical forces. This results in the profuse and watery diarrhea associated with STb-positive ETEC.

6.3 Role in ETEC Colonization and Persistence

While colonization factor antigens (CFAs) (fimbrial and non-fimbrial adhesins) are the main factors for ETEC adhesion to the enterocyte, STb has a secondary role in altering the gut environment in ways that are conducive for colonization and pathogen fitness (Butt et al., 2020). The damaging effect of the toxin on the epithelial surface may expose new adhesin binding sites in the membrane or the lateral surfaces of the cell, which may facilitate the adhesion of bacteria that initially adhere to damaged surfaces of the villus tips (Dubreuil, 2013). ETEC bacteria adhere and colonize the mucosa of the small intestine, including the jejunum, the ileum, and the duodenum in smaller quantities. They have fimbriae or pili that adhere to specific receptor sites of the enterocytes without any lesions in the epithelial surface (Kennedy et al., 1984). Once ETEC bacteria have colonized the mucosal surface of the small intestine, they produce their enterotoxins, which have a local effect on the enterocytes. In an interesting twist, the resulting inflammation in the mucosa, characterized by an increase in mucus secretion, might actually aid in ETEC colonization. This is because mucins, in response to the infection in the mucosa, contain glycan chains that might aid in the adhesion of certain types of CFA to the mucus layer overlying the epithelial cells (Turner et al., 2006). However, more relevantly, the inflammation/cytokine response induced by STb might actually aid in the adhesion of ETEC by increasing the expression of certain receptors used by ETEC. Additionally, the neutrophil response might actually aid in the concentration of ETEC in the mucosa by trapping it in neutrophil extracellular traps (Loos et al., 2012). Fluid secretion might actually aid ETEC in persisting in the lumen by providing it with nutrients. These substances include electrolytes and amino acids that provide nutrients to support bacterial growth (Erume et al., 2010). The increased flow in the lumen caused by the secretory response might help in the spread of ETEC from the initial point of colonization to adjacent

surfaces. This would increase the surface area of the colonized epithelial cells. Other recent studies indicate that sublytic doses of pore-lytic toxins, like STb, trigger survival mechanisms in the epithelial cells, potentially creating an environment that supports bacterial survival without triggering rapid cell death that would remove the bacteria from the infected surface (Butt et al., 2020).

6.4 Synergistic Effects with Other ETEC Virulence Factors (e.g., LT, CFs)

STb is not usually expressed in isolation in natural ETEC infections. Synergistic activity is strong with other virulence factors, especially the heat-labile toxin (LT). The synergistic interactions between the co-expressed virulence factors increase the secretory activity, the colonization of the mucosa, and the range of host cell types and intestinal segments infected by the bacteria, which together result in a more severe and prolonged disease outcome than that which could be expected from the additive effects of the individual toxins.

The synergistic effect of STb and LT is possibly the most significant of all the ETEC virulence factors' synergies. Indeed, the activation of adenylyl cyclase by LT via ADP-ribosyltransferase activity leads to the activation of the cAMP/PKA pathway by increasing the cAMP level, which in turn leads to the activation of the CFTR by the cAMP/PKA pathway. STb, via its calcium-dependent pathway, concurrently activates the CaCC-dependent chloride secretion and the PGE₂-dependent secondary cAMP increase. Both of these toxins target the same effector, CFTR, via two separate pathways: the cAMP and the calcium/PGE₂ pathways. cAMP and Ca²⁺, the main second messenger for STb, are known to have the ability to cross-activate each other's pathways. cAMP can enhance the Ca²⁺-dependent chloride secretion, and Ca²⁺ can enhance the cAMP-dependent effects (Harville & Dreyfus, 1995).

The interaction between STb and CFs occurs at the level of intestinal colonization but not through the mechanism of toxin synergy. CFs facilitate the adherence of ETEC to the enterocyte surface, thereby placing the bacterium in close proximity to the apical membrane of the target cell. This close proximity maximizes the local concentration of STb at the surface of the enterocyte, thereby maximizing the tissue damage and fluid loss.

6.5 Animal Models of STb-Mediated Enteric Disease

ETEC infection is highly prevalent in livestock, resulting in considerable economic burdens for the livestock industry. The study of STb-induced intestinal pathology in an animal model has traditionally focused on three animal models: the ligated intestinal loop model (in pigs, rabbits, and rats), the infant mouse model of fluid secretion, and the organoid-based *ex vivo* model. The animal models have their own set of benefits and drawbacks in the study of the pathophysiology of STb infection. The animal models have helped validate the cellular and molecular mechanisms that are established in cell culture models.

In the ligated intestinal loop method, animals are anesthetized, and segments of the small intestine are surgically ligated. Purified STb or ETEC culture filtrates are instilled into the lumen of the intestines, and the amount of fluid accumulated in the lumen is measured after an incubation period. For pigs, the most sensitive animals to STb infection, instillation of purified STb into the intestinal lumen results in rapid fluid accumulation in the lumen (Whipp, 1987). Oral challenge of weaned piglets with STb-positive ETEC strains results in acute watery diarrhea, atrophy of the intestinal villi, inflammation in the intestines, and dehydration in 12-24 hours (Kennedy et al., 1984). This results in growth stunting or even death. With an increase in age in piglets beyond one

week, an increase in the number of STb-positive ETEC strains is observed. This model has also been used in the study of the effect of pure toxin in rodents (mice and rats) and rabbits.

The infant mouse model involves the intragastric inoculation of neonatal mice (less than 48 hours of age) with purified toxins and the measurement of the fluid-to-body weight ratio. Although adult mice are resistant to STb, neonatal mice show a significant secretory response when challenged with high doses of STb (Kennedy et al., 1984). This makes the infant mouse model suitable for the qualitative assessment of STb and the study of STb variants and inhibitors

Recently, intestinal organoid systems have been established as the next generation of systems for the study of the pathobiology of STb. Porcine and human intestinal organoid systems that express sulfatide on the luminal surface have been shown to respond to STb infection with lumen swelling (ballooning) and Transepithelial Electrical Resistance decrease, similar to the responses observed in the intact tissue (Saxena et al., 2015). The advantages of these systems lie in their specificity to either porcine or human systems, the absence of confounding factors such as those that exist in whole-animal systems, and the fact that the systems can be genetically manipulated. These 3D structures mimic the cell diversity and polarity observed in the normal human intestine and respond vigorously to STb infection with the hallmarks of tissue damage, including barrier disruption, the release of pro-inflammatory cytokines, and cell death. These systems provide a bridge between cell lines and whole-animal systems for the study of the pathobiology of STb.

Chapter 7: Diagnostic and Therapeutic Approaches

7.1. Detection Methods: Immunoassays, PCR, ELISA, Lateral Flow Assays and Biosensors

Accurate and timely detection of STb-producing ETEC is essential for clinical diagnosis, epidemiological surveillance, and food safety. Recent advances in ETEC detection technology employ a combination of nucleic acid detection and protein-based assays. A wide variety of ETEC detection technologies has been developed in the intervening years since STb was first identified, ranging from traditional bioassays to more recent immunological and molecular assays, each with its own relative merits in terms of sensitivity, specificity, speed, and convenience. The first ETEC detection technologies for STb involved biological activity assays that examined the *in vivo* secretory response to ETEC culture filtrates or toxins (Lortie et al., 1991). Although these traditional bioassays directly measure the biological activity of STb, they are time-consuming, require animals, are not well-suited to high-throughput surveillance studies, and are unable to distinguish between STb and other secretory agents present in culture filtrates.

The detection of these enterotoxins may also be achieved by the infant mouse assay or by the enzyme-linked immunosorbent assay (ELISA) technique (Ronnberg et al., 1985). Oligonucleotide and cloned polynucleotide DNA probes have been utilized for the detection of the genes of these enterotoxins (Sommerfelt, et al. 1988). The enzyme-linked immunosorbent assay technique is the most important method for the detection of the STb toxin. This technique utilizes polyclonal antisera, as well as purified STb, and, more recently, monoclonal antibodies (mAbs) of particular epitope specificities to the STb toxin (Urban et al., 1990). The specificity of mAb-based ELISA systems for STb, as opposed to STa, is very high, as there are no common epitopes between the two toxins. Recently, a polymerase chain reaction (PCR) assay, which is a quick, accurate, and

specific means of testing for the presence of toxin genes through the amplification of specific genes that code for bacterial toxins (Park et al. 2006), has been developed. In terms of detection of genes, PCR will remain the benchmark for the highest degree of sensitivity and specificity. Recent developments in PCR technology are focused on improving speed and portability. Lateral flow immunoassay (LFIA) or Immunochromatographic Strip are quick, 10-30 minute tests that are instrument-free, making them suitable for point-of-care use in veterinary clinics or in the field (Jung et al., 2005). LFIA is a paper-based technology that is often used in conjunction with traditional laboratory methodologies for the detection of pathogens in different samples. The latest technologies include biosensors, where electrochemical, optical, and piezoelectric technologies are employed, often using anti-STb antibodies or aptamers, and are thus proposed to have the ability for ultra-sensitive, real-time detection in complex samples such as stool or food samples (Lee et al., 2009). The choice of technology depends on the desired balance between sensitivity, specificity, cost, and speed/technical requirements.

7.2. STb as a Vaccine Target: STb Toxoids, Fusion Proteins, Challenges and Opportunities

Considering the economic consequences of porcine colibacillosis and the worldwide prevalence of ETEC in humans, STb is an important vaccine target. A vaccine against ETECs must block the ability of these microbes to deliver their toxin payload to the correct receptor by directly neutralizing the enterotoxins, as well as indirectly by interacting with the virulence factors that are an integral part of the process. ST has poor immunogenicity, and in combination with the strong toxicity, this has hindered the development of vaccines against this ETEC pathogen, although toxoid vaccines have been explored as a method to combat the harmful effects of the toxin. Research that has been done to date has shown that it is possible, by either chemically conjugating peptides of the ST to carrier proteins (Klipstein et al., 1983) or by constructing fusion proteins

with the ST gene (Clements, 1990), to elicit an immune response against the ST. The approach that has been used is to develop toxoids, i.e., the detoxified forms of the toxin. STb toxoids or immunogenic domains have been constructed by genetic fusion to other ETEC antigens, e.g., LT toxoids or B subunits of fimbriae (F4, F18), to produce a single toxoid or immunogen, which provides broad protection against adhesions and various toxins (Klipstein et al., 1983). Other toxoiding procedures could involve the use of chemical inactivation agents, e.g., glutaraldehyde or formaldehyde, and a more refined approach using site-directed mutagenesis to produce genetically engineered toxoids by mutating key toxin regions identified by other studies (Xu et al., 2023). These, when expressed as recombinant toxoids, are non-toxic but induce the production of antibodies to neutralize the effects of the native toxin. However, the major limitation of the STb toxoid as a vaccine antigen lies in the immunogenic properties of the peptide, since the STb peptide is very small, toxic, and shares significant sequence homology with natural peptides.

7.3. Anti-STb Strategies: Neutralizing Antibodies and Receptor Blockers

In addition, passive and direct methods to inhibit STb activity are also under investigation. The use of neutralizing monoclonal antibodies (mAbs) that are normally part of our immune response to fight infections can be very effective. mAbs can be generated against the receptor-binding domain of STb, which physically inhibits the binding of STb to sulfatide. It does this by binding to STb in the lumen before it binds to the sulfatide receptor on the surface of the enterocytes, thereby neutralizing the toxin at the target site (Urban et al., 1991). Another direct method to inhibit STb activity is the use of receptor blockers, which are competitive antagonists that can bind to the sulfatide-binding site on STb or saturate the sulfatide in the membrane to inhibit the binding of STb. Compounds that mimic the sulfatide head group (e.g., synthetic sulfated galactose compounds) or that competitively inhibit STb (e.g., specific peptides or aptamers) could sequester

the toxin in the lumen prior to its arrival at the epithelium (Labrie et al., 2001). Although promising, the *in vitro* efficacy of these inhibitors in an *in vivo* setting is hindered by stability in the gut, cost of synthesis, and the need for high local concentration to effectively compete with the high affinity receptor-toxin interaction. These approaches are considered to be promising for the development of anti-virulence agents that can be used along with or instead of antibiotics, which will reduce the emergence of resistance.

7.4. Probiotic and Microbiome-Based Interventions to Counteract STb Effects

Probiotic and microbiome-based interventions are being used as effective means in counteracting the effects of ETEC and its enterotoxins. The main mechanism of action of these interventions is the maintenance of gut microbiota, improvement of immunity, and inhibition of pathogen adhesion. The effective interventions that have been identified for the prevention of the pathology of ETEC include probiotics such as *Lactobacillus* and *Bifidobacterium*. The advantages that are associated with the use of these interventions are that they have the ability to improve mucosal immunity, improve the intestinal barrier, and improve the gut microbiota, thereby preventing the adhesion of the pathogen and the production of the enterotoxin. The interventions are effective in the inhibition of the adhesion of the ETEC pathogen, thus preventing its colonization.

Additionally, these interventions are effective for the production of antimicrobial substances that inhibit the growth of ETEC. These interventions are effective for the enhancement of the epithelial barrier by increasing tight junction protein and mucin production. They are also effective for modulating the immune response to an anti-inflammatory state. Certain probiotics have the capacity to bind with STb toxins, thereby neutralizing the toxin (Paton et al., 2005). This has, however, not been substantiated as well as the other mechanisms. The postbiotics, which include

the microbial metabolites such as Short Chain Fatty Acids like butyrate, play an important role in the maintenance of the integrity of the epithelial cell layer. The postbiotics have the capability to arrest the action of STb (Xu et al., 2022). Prebiotics dietary fibers like fructooligosaccharides, galactooligosaccharides, and human milk oligosaccharides, may support the growth of good bacteria like Bifidobacteria, which may strengthen the intestinal barrier and inhibit the growth of ETEC (Morrow et al., 2004). Fecal microbiota transplantation, though extreme, has been used to treat recurring infections in animals. This illustrates the significance of the balance of the microbiota present within the human body to prevent toxin-induced diseases (Green et al., 2020). All of the mechanisms emphasize the importance of symbiotic relations between the human body and microbes to provide defense against diseases.

7.5. Future Directions: Small-molecule Inhibitors, Structure-based Drug Design and Personalized Medicine

The determination of the three-dimensional structure of STb and its mechanism of binding to its receptor and intracellular signaling cascade has paved the way for the rational design of new small molecule inhibitors targeting specific components of the STb intoxicating pathway. Unlike antibiotics, anti-virulence strategies such as small molecule inhibitors of toxin activity aim to disable the pathogen without killing it and thereby minimize the emergence of resistance while effectively controlling disease (Pettersen et al., 2026). This anti-virulence approach is particularly promising for ETEC.

Structure-based drug design is another powerful approach. With high-resolution structures of STb, e.g., by Nuclear Magnetic Resonance or Cryo-Electron Microscopy, and of its complex with sulfatide, computational methods can be used to screen virtual compound libraries to identify small

molecules that bind to the sulfatide-binding site on the central loop domain of STb, thereby inhibiting the binding to the receptor and the insertion into the membrane, or the oligomerization of the toxin (González et al., 2008). Apart from receptor blocking, other therapeutic approaches include the use of inhibitors of the signaling cascade activated by STb, such as PLC, PKC, CaMKII, MLCK, and NF- κ B. These offer additional therapeutic approaches which may reduce the diarrheal and inflammatory effects of STb infection without affecting the receptor binding process. However, the difficulty in achieving selective inhibition of the activated signaling cascade without affecting the same pathways in the physiological state remains a major problem to be addressed through careful validation of the therapeutic agent's tolerance (Pettersen et al., 2026). In addition, the trend in the treatment of various diseases is shifting to the use of personalized medicine. The differences in the composition of the microbiome in the gut may influence the susceptibility to various diseases and the efficacy of probiotics. In the future, it may therefore be possible to diagnose the composition of the individual's microbiome as well as their genetic predisposition to the production of sulfatide, thus determining the risk of acquiring the disease caused by STb. In addition, it may be possible to choose the appropriate therapeutic agent, which may include a probiotic mixture, antibodies, or inhibitors, to manage the infection caused by ETEC.

Chapter 8: Challenges and Future Directions

8.1 Gaps in STb Research: Unresolved Mechanisms and Unknown Receptors

Notwithstanding the extensive research efforts focused on STb over the past several decades, there remain important unanswered questions and critical knowledge gaps with respect to this toxin. Foremost among these is the issue of whether sulfatide represents the sole receptor responsible for mediating the biological effects of STb *in vivo*, or whether other co-receptors or secondary binding partners also play a role in mediating the complex cellular responses that have been ascribed to STb. While the evidence supporting sulfatide as the major receptor for STb is highly suggestive, there are certain findings, including the partial preservation of secretory function in sulfatide-depleted cell preparations and the induction of calcium responses in cell lines expressing only low levels of sulfatide, which suggest the possibility of secondary receptor interactions that have not yet been elucidated (Labrie et al., 2001). Modern approaches to receptor identification, including proximity ligation assays and genome-wide CRISPR knockout screens in sulfatide-expressing intestinal epithelial cells, would appear to offer a highly effective means of resolving this issue (Hazen et al., 2017). There remains a question about the exact atomic structure of the oligomer, although the structure of the monomer has been solved in solution. The structure of the functional oligomer within a lipid bilayer, including the stoichiometry, structure, and conductance properties, remains unknown. An exact structure, possibly by the use of cryo-electron microscopy of the oligomer embedded in nanodiscs, would be a major breakthrough, showing the exact mechanism of membrane disruption and allowing the exact design of pore-blocking inhibitors (González et al., 2008).

In the context of ETEC infection, Xia et al. (2018) showed the exact mechanisms by which ETEC infection *in vivo* resulted in the inhibition of the activation of caspase-9 mitochondrial-mediated intrinsic apoptosis. ETEC was shown to utilize and activate Caspase-8, a member of the extrinsic apoptosis pathway, instead. This implies that, during ETEC infection, intrinsic apoptosis is inhibited, whereas extrinsic apoptosis is activated and used for cell death, although the exact reasoning for this is unknown. The impact of STb on the secretion of GLP-1 and GLP-2, as well as the physiological changes to the endocrine cells, must be elucidated (Butt et al., 2020). It must also be considered that these cells are susceptible to intoxication, so it begs the question of the fate of these cells, as well as the fate of the regulation of gut hormones, during the course of an ETEC infection. Research has yet to shine its light on this area of study, as the deregulation of intestinal hormones has the potential to have underlying effects, aside from the effects of ETEC infection.

Another area that is not explored is the intracellular trafficking map and the possible targets within the cytosol. Do a proportion of the internalized STb reach the cytosol to have an effect, and how? Finally, the specific role of the human pathophysiology of STb remains to be elucidated. More studies are needed to understand the true role of STb in the pathogenesis of human traveler's diarrhea, as opposed to the established role in porcine diarrhea.

8.2 Technological Advances: Cryo-EM, Single-Cell Analysis, and Organoid Models

These gaps are likely to be filled by emerging technologies. One of the most efficient technologies in the field of structural biology has emerged as single-particle cryo-electron microscopy, which has the ability to reveal the high-resolution visualization of structures of membrane protein complexes and small oligomeric structures like the oligomeric pore complex of STb, at near-atomic resolution of 2-3 Å without the need for crystallization. Using this technology, the pore

structure of the complex of STb can be finally visualized. The structural details of the biological macromolecule at the atomic level are of immense value not only for understanding the fundamental mechanisms of myriad biological processes but also for developing drugs for the treatment of diseases caused by the dysfunctional biological macromolecules (Liang et al., 2022). Single-cell sequencing works like a microscope, differentiating between changes occurring at this microscopic level. It has the ability to examine the genome, transcriptome, and epigenome of the cell at the single-cell level, which is very important for the detailed examination of heterogeneity at the cellular level. Single-cell sequencing allows for a precise dissection of the differences in gene expression, metabolism, cell function, and cell cycle of the intestinal epithelium to STb, and it is expected to provide a comprehensive and in-depth understanding of the microscopic mechanisms of disease development (McNulty et al., 2023). This single-cell resolution is also expected to clarify which specific cell types are responsible for the serotonin release, cytokine induction, and barrier disruption seen in STb. Intestinal organoids and enteroids derived from human or porcine stem cells are a paradigm shift. Organoids are 3D multicellular culture systems that offer biomechanical support and biochemical cues. They are a good *in vitro* model of cellular heterogeneity because they accurately reproduce the complex interactions that exist in human physiology (Lancaster, et al, 2013). They reproduce the complex physiological structures of natural tissues or organs, and they offer a more accurate reflection of the *in vivo* environment than traditional 2D cell culture. These 3D self-organizing cultures include the complete set of epithelial cell types (enterocytes, goblet cells, enteroendocrine cells), and they can be maintained as monolayers or as complex 3D structures. They enable the study of STb action in a genetically controlled, species-specific, and physiologically relevant manner without the use of animals. They

can also be used for live imaging of toxin binding, trafficking, and barrier function/secretion in real-time (Lancaster, et al, 2013). This technology has taken *in vitro* research to new heights.

8.3 Integrating STb Research into Broader Enterotoxin and Microbiome Studies

STb research should not be conducted in isolation. Comparative research involving other enterotoxins like STa, LT, and Shiga toxin will provide a complete map of the general host molecular mechanisms and specific vulnerabilities. For example, the reasons why the host responds differently to cAMP (LT), cGMP (STa), and Ca²⁺ (STb) signals will provide new insights into the general principles of enterocyte signaling integration. By integrating this research, it will provide a clearer understanding of how STb, in conjunction with STa and Heat-Labile toxin (LT), impacts the gastrointestinal tract and the enteric nervous system. In addition, the pathogenesis of STb must also be integrated into the general microbiome. It is clear that the composition of the microbiota in the gastrointestinal tract plays a critical role in the severity of ETEC infection and the extent of the damage caused by the toxins. Future studies could investigate the role of particular microbial communities or metabolites (such as bile acids or Short Chain Fatty Acids) in regulating sulfatide expression on enterocytes, influencing STb stability or function, or tight junction integrity, or host signaling pathway responsiveness (Paton et al., 2005). On the other hand, inflammation and secretion mediated by STb will create a different microbial environment, possibly leading to dysbiosis. Analyzing STb as a disturbance in the environment of the gut will provide a more holistic perspective of disease and healing.

Conclusion: STb as a Model for Understanding Bacterial Toxin–Host Dynamics

In conclusion, STb is not just a diarrheal toxin, but a sophisticated molecular tool and paradigm for host-pathogen interactions. STb of *E. coli* has a unique and interesting position in the broad field of toxinology. As one of the few well-studied bacterial toxins, STb holds a paradigmatically different place within the larger field of enterotoxin research. Its small, disulfide-bonded structure illustrates a successful virulence factor designed for stability within the challenging gut environment. Its two-step mechanism, targeting specific receptors and then causing generalized membrane disruption, integrates the approaches of traditional A-B toxins and pore-forming cytolytins. The signal transduction pathway it initiates—with a focus on calcium and PKC—offers insight into the regulation of the epithelial cell barrier and inflammatory responses. The challenges it poses, ranging from structural analysis to therapeutic inhibition, are typical of those posed by many bacterial toxins and will provide general insights into the development of anti-toxin drugs and vaccines. By harnessing the power of emerging technologies, which promise to fill remaining gaps in STb biology and translate this knowledge into clinically and agriculturally relevant applications, future studies of STb will not only improve our capacity to prevent and treat ETEC infections but also yield broader insights into the complex interplay between microbial virulence factors and host intestinal homeostasis. This knowledge is critical for the development of the next generation of anti-infective agents in the face of increasing antibiotic resistance. STb, as a biologically interesting and tractable virulence factor, will continue to be a productive model system at the interface of microbiology, cell biology, structural biology, and public health for years to come.

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