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Shiga toxins

A Review of Structure, Mechanism, and Detection

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This work is dedicated to the memory
of Dr. William H. "Bill" Vensel.
He was a remarkable scientist,
an esteemed colleague, and an inspiration
who is sorely missed.

Foreword

Shiga toxins are a significant cause of human misery throughout the world. In the developing world, they are commonly produced by *Shigella dysenteriae* type 1 and in the developed world they are most frequently encountered when produced by strains of pathogenic *Escherichia coli* that sometimes contaminate food or water. The Shiga toxins produced by these bacteria have similar structures, binding sites on the target cell, and a common mechanism of cell toxicity, but they are produced in very different ways. A single Shiga toxin molecule suffices to kill a target cell, making the bacteria that produce them potent pathogens. There is currently no therapeutic intervention to prevent or ameliorate the effects of Shiga toxins.

Understanding Shiga toxins and their health effects requires a multidisciplinary approach that involves chemistry, proteomics, genomics, virology, microbiology, ecology, and medicine. Many of the examples of outbreaks in this book are drawn from the United States, but examples from Europe and Asia are included, and, together, they reflect the broader worldwide experience. This book also addresses the Shiga toxin-producing *E. coli* (STEC) and their mobile lambdoid phages that present a constantly varying threat, especially via our food supply. In brief, this book describes the properties of Shiga toxins and their threats to human health, and the authors hope that it will be useful to laboratory and clinical scientists from a variety of disciplines.

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Abbreviations

3D	Three-dimensional
Ab	Antibody
AE	Attaching and effacing
AKI	Acute kidney injury
AMS	Agricultural Marketing Service
Arg	Arginine
Arg-X-X-Arginine	Arginine-amino acid-amino acid-Arginine
Asn	Asparagine
att	Phage attachment site
*att*B	Site of phage attachment in the bacterial chromosome
attomol	1×10^{-18} mol
CAS	CRISPR-associated genes
CCD	Charge-coupled device
CD_{50}	Median cytotoxic dose
CFT	Cell-free translation
CFU	Colony-forming unit
CNS	Central nervous system
CRISPR	Clustered, regularly interspaced, short palindromic repeats
CT-SMAC	MacConkey agar with sorbitol, cefixime, and tellurite
Ctx	Cholera toxin
DAPI	4',6-Diamidino-2-phenylindole dihydrochloride
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E	Glutamic acid
eae	Gene that produces intimin
EAEC	Enteroaggregative *E. coli*
EAggEC	Enteroaggregative *E. coli*
EFSA	European Food Safety Authority
EHEC	Enterohemorrhagic *E. coli*
ELISA	Enzyme-linked immunosorbent assay
EMB	Eosin methylene blue

EPEC	Enteropathogenic *E. coli*
ER	Endoplasmic reticulum
ERT	Endoplasmic reticulum-routing protein toxins
ESI	Electrospray ionization
FDOSS	Foodborne Disease Outbreak Surveillance System
femtomol	1×10^{-15} mol
FOOD Tool	Foodborne Outbreak Online Database
FSMA	United States Food Safety Modernization Act
fur	Ferric uptake regulator
g	Gram
Gal	Galactose
GAP	Good agricultural practices
Gb3	Globotriaosylceramide
Gb4	Globotetraosylceramide
GFP	Green fluorescent protein
GHP	Good handling practices
Glu	Glutamic acid
GuCl	Guanidinium chloride
HACCP	Hazard analysis and critical control points
HC	Hemorrhagic colitis
HSP-90	Heat shock protein 90
HTH	Helix-turn-helix
HUS	Hemolytic uremic syndrome
HuSAP	Human serum amyloid protein P
IA	Iodoacetamide
IG	Intragastric
IgG	Immunoglobulin G
IgY	Immunoglobulin Y
IMS	Immunomagnetic separation
IP	Intraperitoneal
IS	Insertion sequence
ISt	Internal standard
IV	Intravenous
kDa	Kilodalton
LAMP	Loop-mediated isothermal amplification
LD_{50}	Median lethal dose
LEE	Locus of enterocyte effacement
LFA	Lateral flow assay
LFD	Lateral flow device
LOD	Limit of detection
LPS	Lipopolysaccharide
LT	Heat-labile enterotoxin
LT-I	Type I heat-stable enterotoxin
LT-II	Type II heat-stable enterotoxin
mAb	Monoclonal antibody

MAC	MacConkey agar
μL	Microliter
mL	Milliliter
MLD	Minimum lethal dose
MMWR	Morbidity and Mortality Weekly Report
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS1	First quadrupole of a triple quadrupole mass spectrometer
MS2	Third quadrupole of a triple quadrupole mass spectrometer
ng	Nanogram (1×10^{-9} g)
NORS	National Outbreak Reporting System
pAb	Polyclonal antibody
PCR	Polymerase chain reaction
PDB	Protein Data Bank
pg	Picogram (1×10^{-12} g)
Ptx	Pertussis toxin
Q	Glutamine
qPCR	Quantitative PCR
QSR	Quick service restaurant
RCSB	Research Collaboratory for Structural Bioinformatics
RELISA	Receptor/ELISA
RIP	Ribosome-inactivating proteins
RPLA	Reversed passive latex agglutination
rRNA	Ribosomal ribonucleic acid
SLT	Shiga-like toxin
SLT-I	Type I Shiga-like toxin (Stx1)
SLT-II	Type II Shiga-like toxin (Stx2)
SMAC	Sorbitol-MacConkey agar
ST	Heat-stable enterotoxin
STEC	Shiga toxin-producing *Escherichia coli*
Stx	Shiga toxin
stx	Genes in the Shiga toxin operon
Stx1	Type 1 Shiga toxin
stx1	Genes in the Shiga toxin type 1 operon
Stx2	Type 2 Shiga toxin
stx2	Genes in the Shiga toxin type 2 operon
stxA	Gene encoding the A subunit of Shiga toxin
stxB	Gene encoding the B subunit of Shiga toxin
SubAB	Subtilase cytotoxin
TBX	Tryptone bile X-glucuronide
TPP	Thrombotic-thrombocytopenic purpura
Trp	Tryptophan
Tyr	Tyrosine
USD	United States Dollar (currency)
USDA	United States Department of Agriculture

USDA-FSIS	U. S. Department of Agriculture - Food Safety and Inspection Service
VCT	Verocytotoxin
VT	Verotoxin
VTEC	Verotoxin-producing *Escherichia coli*
WBDOSS	Waterborne Disease Outbreak Surveillance System
WDOSR	Waterborne Disease & Outbreak Surveillance & Reporting
WGS	Whole genome sequencing
WHO	World Health Organization

About the Authors

David L. Brandon received an A.B. from Harvard College and Ph.D. in biochemistry from Harvard University (Cambridge, MA, USA). He retired in 2016, after 37 years as Research Chemist with the USDA Agricultural Research Service, and has served in leadership and editorial positions in the field of analytical food safety. He is recognized for his research in the areas of toxins and antinutrients in food and feed and immunoanalysis of foodborne contaminants, including bacterial pathogens and drug and pesticide residues. His work has involved significant international collaborations, with applications to food safety, crop improvement, and food defense. Dr. Brandon is author/inventor of over 80 publications and patents, with several technologies licensed to industry.

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Chapter 1
Introduction

Abstract Shiga toxin (Stx) constitutes the major virulence factor associated with Shiga toxin-producing *Escherichia coli* (STEC). The first reported STEC outbreak occurred in a quick service restaurant (QSR, an inexpensive restaurant with limited menu and no table service, where the food is pre-prepared and paid for prior to consumption) in the United States in the early 1980s. Although STEC is a comparatively rare cause of foodborne illness, it is disproportionately overrepresented in terms of cases requiring hospitalization. There are an estimated 175,000 cases of STEC infection per year in the US alone, including occasional major foodborne outbreaks. These STEC infections account for an estimated economic loss in excess of one billion USD per year. Monetary losses do not include the suffering and misery experienced by those infected with STEC or the toll taken by the average of about 100 deaths each year due to STEC infections in the US. STEC infections are a worldwide problem. The largest documented outbreak occurred in 1996 in Japan, and the most deadly, in Germany in 2011, with 53 fatalities. In short, STEC is a significant source of foodborne illness and will likely continue to be so in the future.

Keywords Shiga toxin • Vero toxin • Foodborne illness • Shiga toxin-producing *Escherichia coli* (STEC) • AB_5 holotoxins • *Escherichia coli* serotypes • O-antigen • H-antigen • Lambdoid phage • Retrograde transport

Shiga toxin (Stx) constitutes the major virulence factor associated with Shiga toxin-producing *Escherichia coli* (STEC). The first reported STEC outbreak occurred in a quick service restaurant (QSR, an inexpensive restaurant with limited menu and no table service, where the food is pre-prepared and paid for prior to consumption) in the United States in the early 1980s. Although STEC is a comparatively rare cause of foodborne illness, it is disproportionately overrepresented in terms of cases requiring hospitalization. There are an estimated 175,000 cases of STEC infections per year in the US alone (Scallan et al. 2011), including occasional major foodborne outbreaks. These STEC infections account for an estimated economic loss in excess of one billion USD per year (Frenzen et al. 2005; Hoffmann et al. 2015). Monetary losses do not include the suffering and misery experienced

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by those infected with STEC or the toll taken by the average of about 100 deaths each year due to STEC infections in the US. STEC infections are a worldwide problem. The largest documented outbreak occurred in 1996 in Japan (Michino et al. 1999), and the most deadly, in Germany in 2011 (Frank et al. 2011), with 53 fatalities. In short, STEC is a significant source of foodborne illness and will likely continue to be so in the future.

Shiga toxins are a set of protein toxins referred to as AB_5 holotoxins. Each holotoxin molecule consists of five identical B subunits non-covalently bound to a single A subunit, without any other "accessory" proteins. The B subunits bind to specific ganglioside lipids on the surface of target cells. The bound holotoxin is actively brought inside the target cell (endocytosis) where it is transported from the target cell membrane to its cytoplasm (i.e., retrogradely). During this retrograde transportation to the cytoplasm, a protease, furin, cleaves a characteristic motif in the A subunit, and a disulfide bond of the A subunit is reduced in the endoplasmic reticulum (ER) to free the catalytically active A1 domain of the A subunit. The A1 domain is then translocated from the ER to the cytoplasm, where it exerts its catalytic effect. The catalytic A1 domain is an N-glycosidase which cleaves adenine 4324 of the 28S ribosomal RNA (rRNA) of the 60S subunit of the ribosome, thereby halting protein synthesis. One active A1 domain suffices to kill a target cell.

Shiga toxins are associated with certain serotypes of *E. coli*, but toxin production is under the control of lambdoid phages that infect the host *E. coli*. The serotypes or serovars of *E. coli* are defined by the characteristic composition of their surface lipopolysaccharides (LPS, O antigens) and differences in their flagellae (H antigens) (Prager et al. 2003; Whitfield 1995). Each serotype (e.g., O157:H7) can have a number of genetic variants or strains that share common O and H antigens. In order to understand how Shiga toxins are produced, one must understand the life cycle of the lambdoid phages, how the host cell responds to the phage, and how other mobile genetic elements influence the genetic makeup of the phages. Although the STEC-borne phages are related to the phage lambda, they are phenotypically very different. For example more than one copy of the lambdoid phage can occupy the bacterial chromosome and more than one species of lambdoid phage can infect a single *E. coli* host. These diverse genetic components individually or in combination determine the type and amount of Stx produced by a given host.

In order for Shiga toxins to achieve their deadly effect, they must pass through a number of biological barriers. For a successful passage, each of these barriers requires a significant adaptation by the entity carrying the *stx* gene. The first barrier is entry into the human gut. *E. coli* O157:H7 has evolved a remarkable ability to tolerate human stomach acid and to thrive in the intestine. STEC-borne phages have adapted to infect *E. coli* that will bring them into the human intestine. Once in the intestine, the phages can replicate inside the host bacteria and direct their host to produce Shiga toxins. The structure of Shiga toxin has evolved to resist trypsin digestion and to bind specific types of gangliosides.

Thus, adaptations at the bacterial, phage and molecular levels have combined to produce and deliver these deadly toxins. In order to understand how Shiga toxins inflict their damage, one must understand the entire sequence of events that leads to their production and not merely focus on the specific features of the toxins themselves.

Currently there is no effective treatment for Shiga toxin poisoning. Treating a STEC infection with antibiotic is not recommended, since many antibiotics induce phage replication and the consequent production of Shiga toxins. Approaches that are being pursued by researchers include: (1) antibiotics that can kill STEC without inducing phages (Kimmitt et al. 2000); (2) reagents, probiotics and killed bacteria that can bind Shiga toxins before they cause cellular damage (Nishikawa 2011; Paton et al. 2006); (3) vaccines based on B subunit sequences to elicit strong immune responses; (4) nanobodies to Shiga toxins, which may prevent the toxins from binding to target cells; (5) molecules that interfere with the retrograde transport of Shiga toxins or the N-glycosidase activity of the A1 domain; (6) development of new knowledge about retrograde transport. Cancer cells, such as those involved in Burkitt lymphoma, express globotriaosylceramide (Gb3 or Gb3cer) on their surfaces (Mangeney et al. 1993). The B subunit pentamer of type 2 Shiga toxin has been shown to induce apoptosis in Burkitt lymphoma cells. Since Shiga toxins effectively use the retrograde transport system, they can be used to understand the details of how retrograde transport operates in target cells. These efforts will help treat patients in the future and perhaps harness the toxicity of Shiga toxins to treat, rather than cause disease.

Shiga toxins can be expected to remain a continuing threat to worldwide health for the following reasons:

- Shiga toxin-producing phages can infect a variety of non-pathogenic enteric bacterial species.
- Domestic cattle are not affected by Shiga toxins, so Shiga toxin-producing phages can infect the various uninfected *E. coli* serotypes present in these animals without causing Shiga toxin-related disease.
- Shiga toxin-producing phages may undergo recombination as they infect new hosts to produce new Shiga toxin producing phages.
- Shiga toxin-producing phages can be further modified by other mobile genetic elements present in the host cell such as transposons, so they may move antibiotic resistance or virulence factors to new hosts.
- Increasing consumption of fresh—i.e., raw, rather than processed or cooked—fruits and vegetables is a trend in modern industrial societies. Raw foods can be sources of foodborne outbreaks.
- There are currently >400 known STEC serotypes that have been isolated from humans (Tozzoli and Scheutz 2014), but even a previously innocuous STEC strain can suddenly emerge as a pathogen and cause a significant outbreak (Frank et al. 2011).

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Chapter 2
Outbreaks of Shiga Toxin-Related Poisoning

Abstract Foodborne outbreaks of Shiga toxin-producing bacteria occur with disturbing regularity. The two most common Shiga toxin-producing bacteria are *Shigella spp*. and the Shiga toxin-producing *Escherichia coli* (STEC). Among foodborne pathogens, they result in a disproportionately large share of hospitalizations, serious sequelae, and deaths. In 1982 the first reported outbreak of STEC was caused by an *E. coli* O157:H7 serotype in undercooked hamburger, but by 2008 it was estimated that most foodborne STEC disease was caused by other serotypes. As the food service industry has adopted more stringent cooking practices and as diets have changed, the sources of outbreaks have shifted as well. The two largest outbreaks on record were caused by consumption of uncooked radish and fenugreek sprouts. There are at least 200 different known strains of STEC found in farm animals, where they can propagate "below the radar" because farm animals lack the receptors that would make them vulnerable to Shiga toxins. Future outbreaks are likely to involve other uncooked foods and different strains of STEC, and they may be triggered by the agricultural practices, food processing and transportation conditions, and ecological factors that bring them together.

Keywords Shiga toxin poisoning • Quick serve restaurant • Effective dose • Pathogenic *Escherichia coli* • Shigellosis • *Escherichia coli* O157:H7 • *Escherichia coli* O104:H4 • *Shigella spp*. • Foodborne illness, Sakai, Japan • STEC outbreak, Germany

2.1 Human and Economic Impacts of STEC Outbreaks

The economic cost of Shiga toxin poisoning in humans is substantial. The situation in the United States can be used as an illustration of a worldwide problem. In 1996–1997, an estimated 110,000 people per year were infected with Stx-producing bacteria in the US (Mead et al. 1999). Of those, 2200 were hospitalized and 60 died. A subsequent analysis covering the years 2000–2008, estimates the number of STEC cases to be approximately 175,000 per year (Scallan et al. 2011). Between 2003 and

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2012 in the US, outbreaks of disease caused by *E. coli* O157:H7 had routes of transmission that were foodborne (65%), person-to-person (10%), animal contact (10%), waterborne (4%) or undetermined (11%) (Heiman et al. 2015). These more recent estimates suggest that approximately 64% of STEC infections are caused by non-O157:H7 strains and that the majority (68–82%) of cases are foodborne (Scallan et al. 2011). The direct economic costs of Shiga toxin infections in the United States are estimated to be at least 400 million USD and possibly in excess of one billion USD per year (Frenzen et al. 2005; Hoffmann et al. 2015). The indirect costs are more difficult to estimate, but are substantial. These sums indicate that STEC infections represent a significant cost to the United States in terms of both misery and money.

STEC are a subset of pathogenic *Escherichia coli*, but they often possess virulence factors found in other pathogenic *E. coli* (Tozzoli and Scheutz 2014). Table 2.1 lists this diverse cast of characters and some of their most salient characteristics, including virulence factors such as intimin, fimbrae, heat-labile enterotoxin (LT), and heat-sta-

Table 2.1 Pathogenic *Escherichia coli* and associated pathologies (Alteri and Mobley 2012; Brzuszkiewicz et al. 2011; Nataro and Kaper 1998; Tozzoli and Scheutz 2014)

E. coli group name	Acronym	Properties
Attaching effacing	AEEC	Cause attaching and effacing lesions
Adherent-invasive	AIEC	Invade cells to cause their pathology
Avian pathogenic	APEC	Cause extraintestinal infections in birds
Diffusely adherent	DAEC	Cause diarrhea in children
Diarrheagenic	DEC	Cause the symptom of diarrhea, as part of pathology
Enteroaggregative	EAEC or EAggEC	Aggregate host cells, produce hemolysin, and heat-stable (ST) enterotoxin to cause pathology that leads to watery diarrhea without a fever
Enteroaggregative haemorrhagic	EAHEC	EAEC that also express Shiga toxin(s)
Enterohemorraghic	EHEC	Express Shiga toxin(s) and encode a locus of enterocyte effacement (LEE)
Enteroinvasive	EIEC	Invade cells, causing diarrhea and high fever.
Enteropathogenic	EPEC	Attach to host cells via intimin (int); effacement of cells; may invade various tissues
Enterotoxigenic	ETEC	Noninvasive; attach to enterocytes; produce heat-labile (LT) and/or heat-stable (ST) enterotoxins; cause traveler's diarrhea
Extraintestinal pathogenic	ExPEC	Leave the intestinal area to cause diseases such as septicemia and urinary tract infections
Intestinal pathogenic	IPEC	Cause pathology inside the intestine
Meningitis-associated	MNEC	ExPEC that cause meningitis and sepsis
Shiga toxin-producing	STEC/VTEC	Express Shiga toxin(s)
Uropathogenic	UPEC	ExPEC that cause uropathology

ble toxin (ST). The most serious of these pathogens are the Shiga toxin-producing *E. coli* (STEC), also known as Verotoxin-producing *E. coli* or Verocytotoxin-producing *E. coli* (VTEC). Although STEC may have the same virulence factors found in other pathogens, they also produce Shiga toxin(s), their major virulence factor. Delivery of a single Shiga toxin molecule to the cytoplasm of a target cell is sufficient to kill it.

The symptoms of a STEC infection are highly varied, but most patients recover in 5–7 days without specialized treatment. Most suffer only moderate to severe stomach cramps with or without diarrhea. For example, a survey of patients infected with STEC (O157:H7 serotype) between 2003 and 2012 in the US revealed that 83% had symptoms that were not serious enough to require hospitalization (Heiman et al. 2015). The initial symptoms were moderate to severe abdominal discomfort with diarrhea. Throughout the disease course, there is usually no fever, but if it is present, it is not very high (<101 °F; <38 °C). The clinical manifestations of STEC infections can then progress from diarrhea to hemorrhagic colitis (HC, bloody diarrhea). Among children under the age of 10, approximately 15% of cases progress to the more serious hemolytic uremic syndrome (HUS) (Tarr et al. 2005). The overall mortality rate for infected patients is approximately 0.2%; for hospitalized patients, approximately 3%; and for patients with HUS, about 11%.

STEC infections are not the most common cause of foodborne illness, but they account for a disproportionately large share of the hospitalizations and deaths. For example, between 2009–2010 *Salmonella* accounted for 54% of reported US foodborne illnesses and 66% of the hospitalizations (Anonymous 2013). During the same time frame STEC accounted for less than 5% of reported cases, but 21% of hospitalizations (Anonymous 2013). Between 1998 and 2014 STEC accounted for 2.5% of total cases of foodborne illness, 13% of hospitalizations, and nearly 11% of the deaths associated with foodborne illness (FOODTool). The hospitalization rate for diseases associated with STEC infection is much higher than that seen for more common foodborne illnesses, such as those caused by *Salmonella* or *Campylobacter* (Mead et al. 1999).

STEC infections incur costs beyond those directly associated with treating the infected patients. The hamburger-associated STEC outbreak that occurred in the US in 1992 cost the QSR chain $160 million in lost sales and a 30% loss of its stock value (1992 USD) (McGrath 2009). In addition, there were costs related to litigation in excess of $40 million (1992 USD). A 2002 outbreak of STEC affecting 28 people led to the recall of 8.6 million kg of ground beef (Anonymous 2002). The estimated loss of bagged spinach sales as a result of a 2006 outbreak was in excess of $200 million (2006 USD) (Arnade et al. 2009). Even larger outbreaks of STEC have occurred in Japan and Germany (Frank et al. 2011; Michino et al. 1999). The sources of an outbreak can be limited to a single ingredient from a single farm, such as occurred in Japan, where one farm was responsible for the entire outbreak (Michino et al. 1999). A single supplier of contaminated fenugreek seeds was responsible for the outbreak in Germany (Buchholz et al. 2011). Since foods are often mixtures of ingredients, contamination from a single source can affect suppliers of the other ingredients, even if they were not contaminated. In this way the indirect economic effects of an outbreak can greatly exceed the direct costs associated with treating the afflicted patients.

2.2 Effectiveness of STEC in Causing Severe Disease Outbreaks

E. coli O157:H7 serotype is an extraordinarily effective organism for delivering Shiga toxins to the human intestine. The O157:H7 serotype has a number of mechanisms that make it very acid resistant, enabling it to pass through the human digestive tract unscathed (Miszczycha et al. 2014). It is so effective at surviving the stomach that the number of bacteria that need to be ingested to cause disease in humans is estimated to be less than 50 and perhaps as few as two STEC cells (Tilden et al. 1996), compared to 1000 colony-forming units (CFU) for ETEC and 1 million CFU for EIEC. Other Shiga toxin-producing bacteria associated with foodborne illness, such as *Shigella dysenteriae* type 1, are also able to cause human infection with inocula as low as 10–100 CFU (DuPont et al. 1989). These bacteria are an efficient means of delivering the toxin to the intestine, where it inflicts its damage.

Shiga toxin itself is not well acquitted to survive the journey through the stomach when orally delivered by STEC. Other toxins, such as botulinum toxins, have both structural elements and accessory molecules that allow them to survive journey through the human stomach and intestine to the bloodstream (Miyata et al. 2009). The intraperitoneal (IP) median lethal dose (LD_{50}) for type 2 Shiga toxin is 50 ng/kg or approximately 1 ng/mouse (Fuller et al. 2011; Tesh et al. 1993). However, when Stx1 or Stx2 are transmitted orally, the LD_{50} for Stx2a is approximately 3 μg/mouse and greater than 150 μg/mouse for Stx1a (Russo et al. 2014). Thus, the LD_{50} of a Shiga toxin is increased 1000-fold when delivered through the stomach. This is to be expected, since Shiga toxins are not stable at the pH of the stomach (Skinner et al. 2013). Further along the digestive tract, Stx has structural features that protect it from proteolytic digestive enzymes that are secreted into the intestine. Overall, these complex processes ensure that Shiga toxins are able to cause maximal damage to a human patient.

2.3 History of STEC Outbreaks and Their Continuing Evolution

The earliest reports of STEC disease were sporadic cases reported in the early 1980s (Anonymous 1982; Karmali et al. 1983). The first outbreak of STEC food poisoning was reported in the US in 1982 and was linked to a QSR chain in the states of Oregon and Michigan (Riley et al. 1983; Wells et al. 1983). In 1993, a more substantial and highly publicized outbreak occurred in another QSR chain in the state of Washington and clearly demonstrated the significant consequences of STEC (Bell et al. 1994). The cause of these outbreaks was eventually traced back to undercooked hamburger. The magnitude of the outbreak led to mandatory reporting of the O157:H7 serotype of *E. coli* in the United States (CSTE). A more recent outbreak,

in 2015, occurred in a fast casual restaurant chain (QSRs with more varied menu, where the meal is assembled in a separate location, before the customer relocates it to a table). The outbreak was caused by the O26 serogroup (Anonymous 2016), but the source of the contaminated food was not determined. This series of episodes made the general public aware that the once obscure STEC pathogens can be transmitted by a changing variety of foods.

Worldwide awareness of foodborne STEC-related infection paralleled the experience in the US. In the United Kingdom, the first reported outbreak of STEC (O157:H7 serotype) occurred in 1982 (Taylor et al. 1986), and the first foodborne outbreak occurred in 1985. Surprisingly, the associated food was potatoes, not beef (Morgan et al. 1988). In England and Wales, STEC outbreaks have remained relatively constant; those caused by infected meat and dairy declined, while those associated with children's activities (nurseries, schools, petting farms) increased from 1983–2012 (Adams et al. 2016). The largest outbreak reported so far occurred in 1996, in Japan where more than 8300 school children were sickened by eating contaminated, uncooked white radish sprouts (Michino et al. 1999). In 2011, an outbreak of STEC sickened more than 3800 people in Germany (Frank et al. 2011). The source of the disease was traced back to fenugreek sprouts. STEC infection is now a major worldwide health concern (Adams et al. 2016; Caprioli et al. 2014; Heiman et al. 2015; Terajima et al. 2014), with sources of the outbreaks reflecting the distribution of STEC and changing national food preferences.

The strains of STEC responsible for outbreaks have also changed over time. Domestic cattle alone are known to harbor more than 200 strains of STEC, and there are a large number of potential outbreak strains (Hussein and Bollinger 2005). It is therefore not surprising that while O157:H7 remains a serious threat, strains from other serotypes have emerged to rival that threat. During the survey period of 1996–1997, approximately 2/3 of STEC cases in the United States were caused by strains of the O157:H7 serotype (Mead et al. 1999). In contrast, a survey spanning the years from 2000–2008 estimated that the total number of STEC-caused disease cases increased, mainly due to non-O157:H7 serotypes (Scallan et al. 2011). By 2010, the actual incidence of non-O157:H7 serotype STEC infections equaled that of O157:H7 STEC infections in the US for the first time (Gould et al. 2013). Regulators have responded by classifying strains from six non-O157:H7 serotypes, O26, O45, O103, O111, O121, and O145 (the "Big Six"), along with O157:H7, as adulterants in ground beef that need to be screened for (Almanza 2012). However, the 2011 STEC outbreak that sickened more than 3800 people in Germany was caused by an O104:H4 serotype of STEC (Frank et al. 2011), a serovar not included among the Big Six. The German experience suggests that future outbreaks are likely to be caused by strains that are not on current regulatory lists.

Outbreaks of STEC in the US are recorded and available to researchers in a variety of databases and publications. The CDC produces the Morbidity and Mortality Weekly Report (MMWR), a weekly publication devoted to providing "timely, reliable, authoritative, accurate, objective, and useful public health information and recommendations" (MMWR). In addition to the information contained in the weekly report, the CDC maintains other web-based resources devoted to gathering

and disseminating information about disease outbreaks. These include the Foodborne Disease Outbreak Surveillance System (FDOSS) and the Waterborne Disease Outbreak Surveillance System (WBDOSS), now known as the Waterborne Disease & Outbreak Surveillance & Reporting (WDOSR). The Foodborne Outbreak Online Database (FOOD Tool) also provides an historical view of outbreaks (FOODTool). The National Outbreak Reporting System (NORS) allows health officials to report "waterborne and foodborne disease outbreaks and enteric disease outbreaks transmitted by contact with environmental sources, infected persons or animals, or unknown modes of transmission to CDC" (NORS). These websites provide useful, accurate information about outbreaks and archive their data for a number of years.

In addition to foodborne outbreaks, Shiga toxin-related disease caused by ingestion of STEC or *Shigella sonnei* has been transmitted by water (Auld et al. 2004; Bopp et al. 2003; Dev et al. 1991; Hrudey et al. 2003; Olsen et al. 2002; Swerdlow et al. 1992; Yarze and Chase 2000). In the US, most waterborne outbreaks are associated with recreational water use (Friedman et al. 1999; Keene et al. 1994; Paunio et al. 1999; Samadpour et al. 2002; Verma et al. 2007), but three large outbreaks occurred when Stx-producing bacteria (*S. sonnei* or *E. coli* O157:H7) contaminated drinking water in Florida, Missouri, and New York (Bopp et al. 2003; Swerdlow et al. 1992; Weissman et al. 1976). The source of waterborne STEC is often well water contaminated with fecal material. In 2000, nearly half the population of Walkerton, Ontario, Canada was sickened by drinking municipal water contaminated with STEC O157:H7 (Anonymous 2000; Hrudey et al. 2002). Subsequent investigation determined that water was contaminated with runoff from fields fertilized with STEC-containing bovine manure and then inadequately chlorinated before being supplied to consumers (Auld et al. 2004; Hrudey et al. 2003). The extent of waterborne outbreaks often dwarfs foodborne incidents because thousands of people may be exposed to a single water source. A survey of untreated water in Brazil found STEC, but not the O157:H7 strain (Lascowski et al. 2013). Again in the US, STEC is rarely a source of waterborne illness, but when it does occur, the health impact is disproportionately large compared to that seen with other microorganisms (Hynds et al. 2014).

The two largest foodborne STEC disease outbreaks occurred in Japan and Germany and were caused by consumption of contaminated fresh produce. In the US, the first outbreak of STEC transmitted by the consumption of fresh produce occurred in 1991, when apple cider was contaminated with *E. coli* O157:H7 (Besser et al. 1993). A foodborne outbreak occurred in 1996 via processed, but unpasteurized apple juice contaminated with *E. coli* O157:H7 (Cody et al. 1999), and fruit juices have remained a periodic source of outbreaks (Vojdani et al. 2008). Even the flour in cookie dough has been implicated in an STEC outbreak (Neil et al. 2012). The first reported produce-related outbreak of STEC-related disease caused by the O157:H7 serotype (1995) was traced back to contaminated lettuce (Ackers et al. 1998). Other outbreaks caused by this infamous STEC serotype (some discussed above) have been attributed to contaminated freshly bagged spinach and other fresh leafy produce in the US (Anonymous 2006; Grant et al. 2008; US-FDA 2006), the Netherlands, Iceland (Ackers et al. 1998; Friesema et al. 2008; Hilborn et al. 1999)

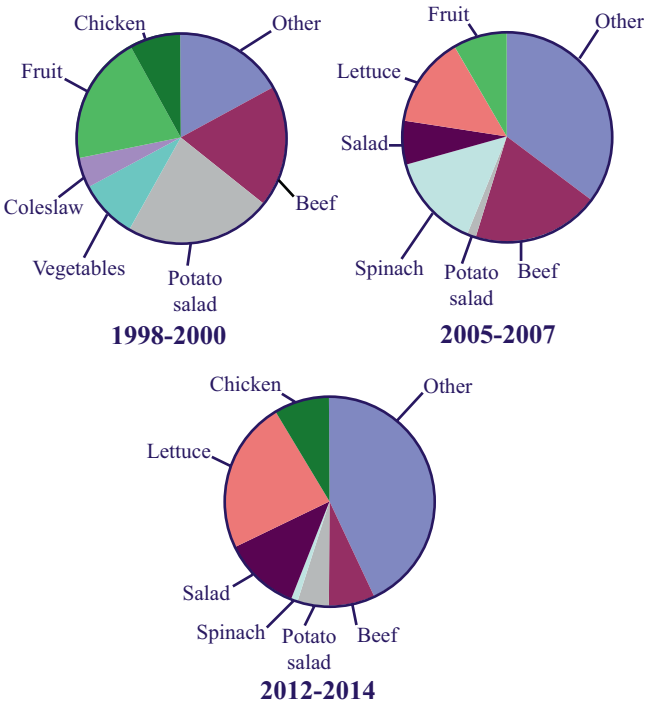

Fig. 2.1 Sources of foodborne STEC outbreaks. Types of food associated with outbreaks are shown as percentages of the total number of STEC-related illnesses reported in 1998–2000 (illnesses, 4111; outbreaks, 97), 2005–2007 (illnesses, 1647; outbreaks, 83), and 2012–2014 (illnesses, 1225; outbreaks, 86). Source: Foodborne Outbreak Online Database (FOODTool)

and Sweden (Soderstrom et al. 2008). The potential hazards of consuming contaminated raw sprouts is a worldwide problem (Taormina et al. 1999). In the US, STEC outbreaks were associated with contaminated alfalfa sprouts from several producers in California (1996–1998) and more recently (2003) in Colorado and Minnesota (Breuer et al. 2001; Ferguson et al. 2005; Mohle-Boetani et al. 2001). Fresh produce has been a source of a number of STEC outbreaks in Canada (Bolduc et al. 2004; Kozak et al. 2013).

In the United States, contaminated fresh produce has become an increasing source of foodborne outbreaks (Fig. 2.1) (Sivapalasingam et al. 2004). Perhaps not a coincidence, this trend comes at a time when the consumer demand for fresh fruits and vegetables has increased by 25% over the years 1961–2000 (Pollack 2001). In contrast, between 1971 and 2000 there was a 20% per capita reduction in the demand for beef in the United States (Haley 2001). This shift in consumer preferences mirrors a change in the properties of the foodborne STEC contaminants, particularly related to their ability to attach to surfaces. The pathogenicity markers for the O157:H7 strains are related to the attachment and effacement mechanism used by the O157:H7 serovar to bind to intestinal cells. These markers are genes that were absent in the

STEC outbreak serotype in Germany in 2011 (Bielaszewska et al. 2011). Instead, that serovar had genes that encoded factors enabling it to aggregate. These trends underscore the evolutionary potential of STEC, capable of finding a niche in edible plants as well as in its more familiar habitat, the mammalian colon. In summary, uncooked fruits and vegetables can efficiently transmit STEC to consumers.

As mentioned above, the 2006 outbreak in the United States caused by the O157:H7 serotype was associated with the consumption of bagged spinach (Anonymous 2006). The strain of O157:H7 associated with the outbreak was also isolated from animals on a nearby ranch and from feral swine (Jay et al. 2007). This represented the first time the O157:H7 strain had been isolated from a feral swine in the United States. A detailed examination of the watershed revealed that *E. coli* O157:H7 could have been present in some fields at various times during the 19-month study (Cooley et al. 2007). This suggests that the outbreak may have resulted from contamination by run-off surface water from a nearby dairy farm or contamination by feral swine. Although the specific source of the outbreak was never pinpointed, these results emphasize that produce is grown in an environment that is dynamic and difficult to control.

2.4 Less Common Sources of STEC Outbreaks

Person-to-person contact is a significant factor in STEC outbreaks because such a low number of STEC cells can cause an infection. Between 2008 and 2009 there were a number of STEC outbreaks transmitted person-to-person (Wikswo and Hall 2012). Most of these cases involved facilities serving populations that are most susceptible to Stx: the very young, in nursery or daycare centers, and the elderly, in senior and geriatric care facilities. Documented person-to-person transmission of STEC remains relatively rare outside highly vulnerable populations.

E. coli O157:H7 has a high prevalence among domestic cattle and other domestic animals and is found in the feces, hides and hair of infected animals (Persad and LeJeune 2014). Consequently, gardening and visits to farms or petting zoos are also documented sources of STEC infection. The first case of STEC transmitted by animal contact was reported in 1992 (Renwick et al. 1993). STEC can be transmitted by touching the hair or hide of an infected animal (Elder et al. 2000). Two such outbreaks occurred in 2001, when children visiting farms in Pennsylvania and Washington state were infected with the O157:H7 serotype (Anonymous 2001). STEC outbreaks have frequently been associated with children and petting zoos in the US (Anonymous 2009; Goode et al. 2009; Heuvelink et al. 2002; Stirling et al. 2008). An outbreak in England involved children who came in contact with livestock during a “Lambing Live” event (Rowell et al. 2016), and petting zoos and farm tours remain a source of STEC infections in the UK (Stirling et al. 2008). Other outbreaks of *E. coli* O157:H7 infection resulted from farm visits by children, including one associated with a dairy farm in Japan (Anonymous 2001; Kassenborg et al. 2004; Muto et al. 2008).

The following discussion covers miscellaneous, but documented transmission of STEC-caused disease. The first case of STEC-caused disease being transmitted to a laboratory worker as an occupational hazard occurred in 2002. A garden fertilized with manure was another source of outbreak (Cieslak et al. 1993). Perhaps more startling was an outbreak of STEC O157:H7 that involved airborne dispersion and/or contaminated building surfaces at a County Fair, a traditional event in thousands of US counties in summertime (Varma et al. 2003). Studies have documented that many farm workers and their families have circulating antibodies to Shiga toxins and to the O and H antigens of *E. coli,* and some had apparently experienced infection with *E. coli* O157:H7, without clinical symptoms (Wilson et al. 1996).

Even though many STEC serotypes have been isolated from domestic cattle, not all of those isolated from humans are found in domestic cattle. A microbiological survey of the STEC present in domestic cattle revealed that approximately 261 STEC serotypes were present (Hussein and Bollinger 2005). By comparison, the number of different STEC serotypes found in human patients exceeds 400 (Tozzoli and Scheutz 2014). Domestic cattle are thought to be the major source of STEC outbreak serotypes (Karmali et al. 2010), but the STEC serotype (O104:H4) responsible for the outbreak in Germany has never been reported to be found in domestic cattle (Paddock et al. 2013; Shridhar et al. 2016). Even when researchers searched for it in domestic cattle in Germany after the 2011 outbreak, they were again unable to isolate the serotype (Wieler et al. 2011). It is not clear from which animal, if any, the O104:04 STEC serotype originated, but it does not appear to have been domestic cattle. Hence, identifying sources of origin for STEC, other than domestic cattle, is a pressing challenge.

In view of the low number of STEC cells necessary for infection, the source of an STEC outbreak can be quite prosaic, such as brief environmental exposure. Sources of future STEC outbreaks will undoubtedly include the infamous O157:H7 strain along with previously unrecognized strains. The ease of mobility and variability of the Shiga toxin-producing phages in domestic cattle will undoubtedly be the cause of many future outbreaks, but we can expect some surprises, too. It had been assumed that the attaching and effacing genes associated O157:H7 and other serotypes were required for an STEC serotype to cause serious disease. However, the 2011 outbreak in Germany was caused by an O104:H4 strain that does not have attaching and effacing genes (*vide infra*). Instead, the serotype possesses the enteroaggregative genes which are also known in other serotypes, but had never been associated with a large outbreak before (Bielaszewska et al. 2011). This experience suggests that future outbreaks may be caused by other serotypes that lack the genes that enable them to efficiently attach to intestinal cells. Since Shiga toxins do not cause disease in domestic cattle, Shiga toxin-producing phages can infect new *E. coli* serotypes and undergo recombination or modification by mobile genetic elements (discussed in Chapter 3), all without affecting bovine health. These facts suggest that domestic cattle will remain a reservoir for a constantly increasing number of STEC serotypes, whether they are passed to humans via consumption of plant- or animal-derived foods.

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Chapter 3
Structure of Shiga Toxins and Other AB_5 Toxins

Abstract Shiga toxins (Stx) are part of a general class of protein toxins referred to as AB_5 holotoxins, including pertussis toxin, cholera toxin, *E. coli* heat-labile enterotoxins, and subtilase cytotoxin. The structural differences between the types and subtypes of Shiga toxins subtly influence the specificity and strength of binding of these toxins. There are two types of Shiga toxins, Stx1 and Stx2, each of which has multiple genetic variants. Each Shiga toxin has five identical B subunits that bind to gangliosides, such as the globotriaosylceramide (Gb3) on the surface of the target eukaryotic cell. The B subunits of Stx1 and Stx2 differ substantially from each other (62% identical), but within each of the Stx1 or Sxt2 types, the B subunits are more than 80% identical. The A subunits of Stx1 and Stx2 have the same enzymatic activity, but are less than 60% identical. The A1 domain of the A subunit is released by an intracellular protease, and its specific N-glycosidase can then inactivate the 60S subunit of ribosomes. A single holotoxin molecule that is properly delivered and processed is sufficient to kill a target cell by inhibiting protein synthesis.

Keywords Shiga-like toxin • Type 1 Shiga toxin • Type 2 Shiga toxin Maybe • Cholera toxin • Ricin • Ribosome inactivation • Gb3 ganglioside • Retrograde transport • Human serum amyloid protein P (HuSAP) • *Shigella dysenteriae* type 1

3.1 Structure of Shiga Toxins

Shiga toxins were independently identified by different investigators several times in the twentieth century. Shiga toxins were initially identified in *Shigella dysenteriae* type 1 and described as neurotoxins (Trofa et al. 1999). Many years later toxins were found in strains of *E. coli* isolated from infants suffering from diarrhea (Konowalchuk et al. 1977, 1978). These toxins were referred to as Verotoxins or Verocytotoxins (VT or VCT), since they were toxic to Vero cells (derived from "verda reno;" Esperanto for "green kidney"), a cell line derived from African green monkey (*Cercopithecus aethiops*) kidney epithelial cells (Konowalchuk et al. 1977; Speirs et al. 1977; Yasumura and Kawakita 1988). Subsequent genetic analysis of Shiga toxin and type 1 Verotoxin showed they differed by only one amino acid. Analysis of type 2 Verotoxins revealed that they were also AB_5 toxins with similar

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protein sequences and identical mechanisms of action to that of Shiga toxins. As a result Shiga toxin (Stx), Shiga-like toxin (SLT), Verocytotoxin (VCT) and Verotoxin (VT) are all now referred to as Shiga toxin (Stx), independent of the bacterium of origin and sites of action (Scheutz et al. 2012).

Shiga toxins belong to a class of holotoxins referred to as AB_5 toxins (Fan et al. 2000) because the toxin has a single A subunit and five B subunits (Fig. 3.1). The enzymatic activity responsible for the observed toxicity resides in the A subunit.

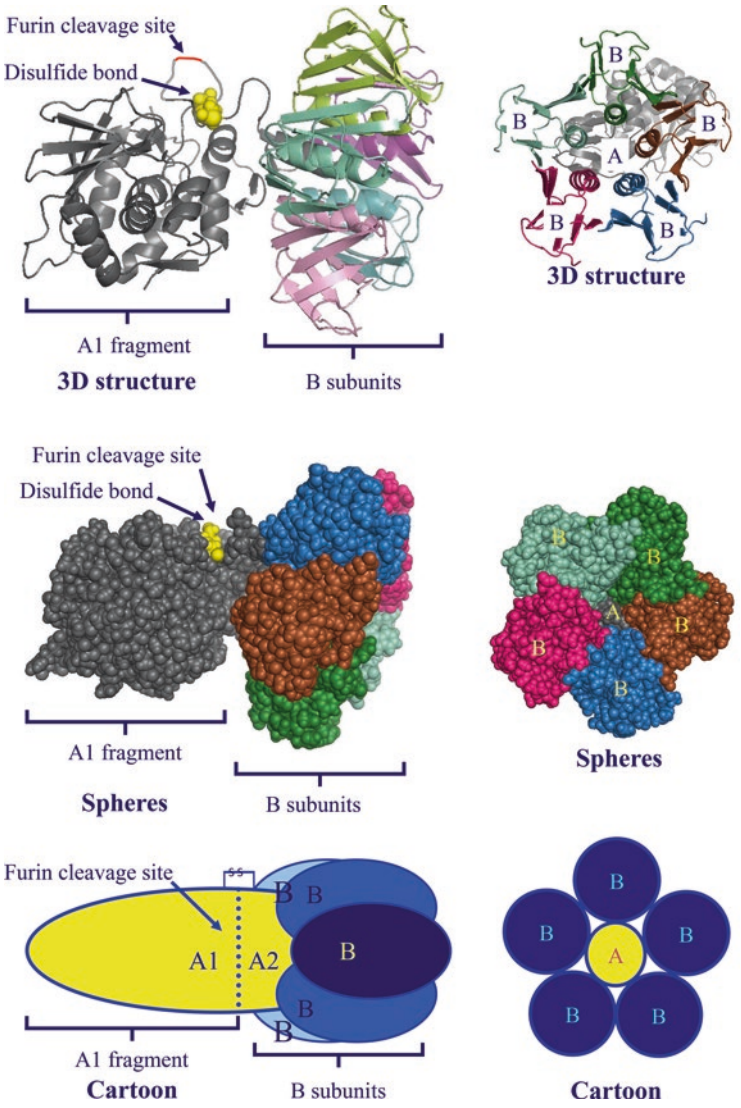

Fig. 3.1 Shiga toxin represented as a three-dimensional (3D) ribbon structure or rendered in spheres or as a cartoon. The 3D structure consists of the secondary structures (α-helix, β-sheet) and unstructured segments. Spheres are scaled according to atomic radii in the spherical representation

The A subunits are sometimes classified as Type II ribosome-inactivating proteins (RIP), based on their enzymatic activity. Based on other features, they have also been referred to as ER-routing protein toxins (ERTs). The pentameric B subunits bind the toxin to the surface of the target cell. The bound toxin is endocytosed into the cell, retrogradely transported to the ER and the A1 domain is then retro-translocated to the cytosol where it is able to cause the observed damage to cellular metabolism. In addition to Shiga toxins, four other kinds of AB_5 toxins have been identified: cholera toxin (Ctx), *E. coli* heat-labile enterotoxins (LT-I and LT-II), pertussis toxins (Ptx), and a more recently discovered subtilase cytotoxin (SubAB) (Paton et al. 2004). These toxins are grouped into four related families, based on structure and enzymatic activity. In addition to their structural and mechanistic differences, the production and expression of these AB_5 toxins are controlled by very different genetic elements.

The two types of Shiga toxins, type 1 (Stx1) and type 2 (Stx2), have similar structures, but different amino acid sequences (Scheutz et al. 2012). Type 1 Shiga toxins (Fig. 3.2) are closely related to the archetypal Shiga toxin isolated from *S. dysenteriae* type 1. The B subunits of the Stx1 subtypes are 84% identical, having 58 of 69 of the same amino acids at the same positions. The B subunits of the Stx2 subtypes are 80% identical. Stx2 toxins (Fig. 3.3) are structurally similar to Stx1 toxins, but the amino acid sequences of the A and B subunits are significantly different from the archetypal Stx1. The A subunits are only 55% identical, while the B subunits are only 62% identical. The B subunits of Stx1 and Stx2 contain either 69

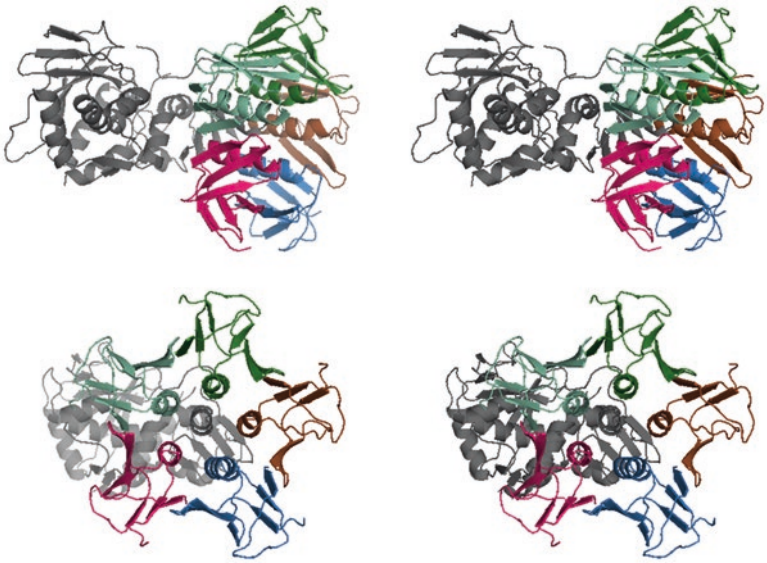

Fig. 3.2 3D stereoview of Shiga toxin type 1 (Stx1) from *E. coli* O157:H7. This rendering is derived from the 1DM0 crystal structure in the Research Collaboratory for Structural Bioinformatics (RCSB) Protein database (PDB) using PyMOL (PyMOL) software (Fraser et al. 1994)

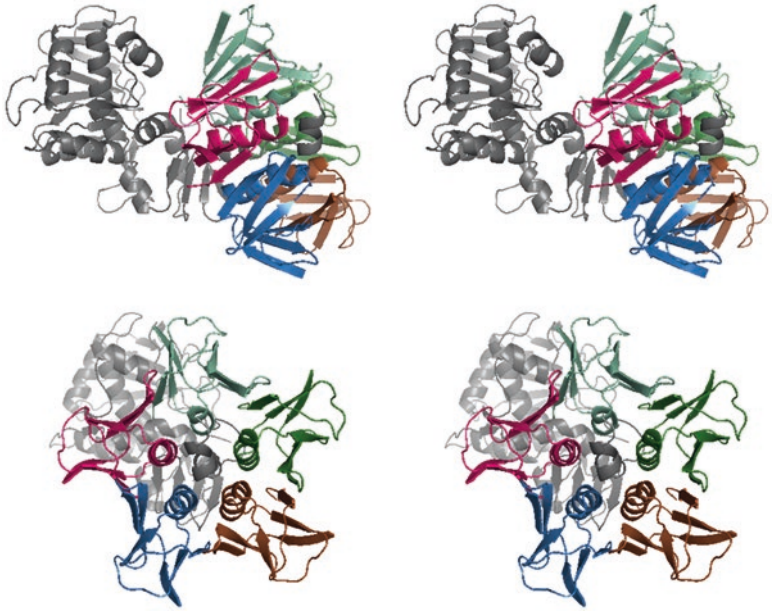

Fig. 3.3 3D stereoview of Shiga toxin type 2 (Stx2) from *E. coli* O157:H7. This image is derived from the 1R4Q crystal structure in the RCSB PDB using PyMOL software (Fraser et al. 2004)

or 70 amino acids, with a mass of about 7.7 kDa. The A subunits contain approximately 297 amino acids (~33 kDa). Thus, the common AB_5 structure of Stx1 and Stx2 permits substantial differences and many variations.

There are subtypes of both the type 1 and type 2 Shiga toxins (Scheutz et al. 2012). These subtypes are protein polymorphisms, proteins that differ because of amino acid substitutions in the primary sequence of their A subunit or B subunit (Fig. 3.4) or both. There are at least three subtypes of type 1 Shiga toxin. In addition to the protein difference, there are silent mutations in the gene sequence that do not alter the protein sequence. Seven known Stx2 subtypes, denoted Stx2a-Stx2g, and a total of 93 genetic variants of Stx2 have been identified thus far. The differences in structure among toxin subtypes often result in phenotypic differences in binding properties and toxicity.

3.2 Production, Activity, and Gene Structure

The control of Shiga toxin production varies with the bacterium. In *S. dysenteriae* type 1, the production of the archetypal Shiga toxin is under the control of the bacterium but appears to have evolved from a system controlled by a phage (McDonough and Butterton 1999; Mizutani et al. 1999; Strockbine et al. 1988). The production of Stx1 and/or Stx2 by STEC is controlled by an infecting lambdoid phage that

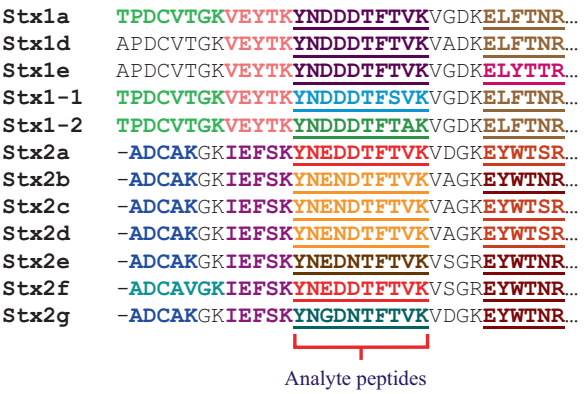

Fig. 3.4 Alignment of Shiga toxin B subunit sequences. The analyte peptides are underlined. Peptides used in the MS analysis (*vide infra*) are color coded to show common sequences

destroys the host bacterium in the process of producing and releasing toxin(s) (Huang et al. 1987; Newland et al. 1985; O'Brien et al. 1984). The production of Shiga toxins by *S. sonnei* and *S. flexneri* appears to be under the control of a converting lambdoid phage, one that can convert other bacteria into Shiga toxin producers (Beutin et al. 1999; Gray et al. 2014). Even though Stx1 and the archetypal Shiga toxin have nearly identical structures, their production is controlled by radically different methods. This difference in production between STEC has a significant impact on the treatment of Shiga toxin-related diseases (*vide infra*). It also means that, unlike in the case *S. dysenteriae* type 1, the genes necessary to produce Shiga toxins in STEC and other *Shigella* species can evolve and be transferred by the converting phage.

Despite sequence differences between the A subunits of Stx1 and Stx2, their enzymatic specificities are identical (Fig. 3.5). The A subunit of Shiga toxin is a specific N-glycosidase that has the same target as the structurally unrelated plant toxin, ricin (Obrig et al. 1985; Sandvig et al. 2004, 2010b). The A subunits of both Stx1 and Stx2 cleave a specific N-glycosidic bond (adenosine 4324) in the ribosomal RNA (28S) component of the 60S subunit of the ribosome leading to the inactivation of the 60S subunit (Endo et al. 1988; Igarashi et al. 1987; Ogasawara et al. 1988; Reisbig et al. 1981; Saxena et al. 1989). The A subunit of type 2 Shiga toxins binds to the ribosome more tightly and is more catalytically active than is the A subunit of type 1 Shiga toxin (Basu et al. 2016). Even though the A subunits of Stx1 and Stx2 have the same enzymatic specificity, their binding to the substrate differs and may account for their distinct toxicities.

The Shiga toxin gene clusters (operons) for Stx1 and Stx2 are very similar. Each is composed of two genes: one for the A subunit, the other for the B, and two promoters, one for the *stx* operon and the other for the B subunit gene (Habib and Jackson 1992). The promoters for the Stx1 operon are very different from those of the Stx2 operon. The promoters are so different that Stx1 can be produced by the host *E. coli* under iron-poor conditions, while Stx2 cannot (Calderwood and Mekalanos 1987). The *stxA* gene is upstream of the *stxB* gene for both the Stx1 and Stx2 operon. These genes also encode an N-terminal signal peptide containing an

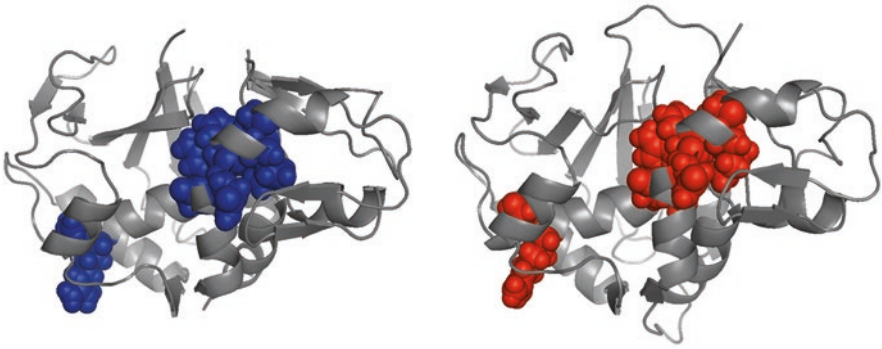

Fig. 3.5 Active sites of Stx1 and Stx2 in ribbon structures of the A1 domains. The active site of Stx1 is shown as *blue spheres*; that of Stx2 as *red spheres*. Structures are based on the RCSB PDB structures 1DM0 and 4M1U (Fraser et al. 1994; Jacobson et al. 2014)

extra ~22 amino acids for the A subunit and ~19 amino acids for the B subunit. Upon translation, these signal peptides direct the emerging peptide to the periplasmic space. The signal sequence is cleaved as a consequence of the translocation process. Stx1 generally remains in cells, while Stx2 is found in the medium (Strockbine et al. 1986). Type 2 Shiga toxins have a serine at position 31 of the B subunit that allows them to be secreted (Shimizu et al. 2007). The *stxB* gene has its own promoter and ribosome-binding site that result in the over-expression of the B subunit relative to the A subunit to yield the 5:1 stoichiometry observed in active AB_5 holotoxin (Habib and Jackson 1993). By these processes, the A and B subunits are produced in the correct proportion and translocated to the periplasmic space.

Spontaneous assembly is presumed to occur in the periplasmic space as well, when a sufficient number of appropriate subunits are present to assemble. Mixtures of compatible A and B subunits can spontaneously assemble in vitro (Ito et al. 1988). The C-terminal structure of the A subunit is inserted into the nonpolar pore formed by the five B subunits to complete the holotoxin (Jemal et al. 1995). Recent data suggest that some holotoxin may also assemble on the surface of the target cell (Pellino et al. 2016). The assembly of the subunits may become complicated if more than one Shiga toxin type or subtype is produced by the host cell.

Since STEC may produce more than one type and/or subtype of Shiga toxin, hybrid toxins can be formed (Ito et al. 1988; Weinstein et al. 1989). Hybrid toxins composed of the A subunit of Stx1 and the B subunits of Stx2 or the A subunit of Stx2 and the B subunits of Stx1 can be formed in vitro and possess a toxicity similar to that of the non-hybrid Stx1 or Stx2 progenitor toxins (Ito et al. 1988). In vivo experiments using cloned constructs showed that hybridizing the A subunit of Stx1 with the B subunits of Stx2 did not result in a functional toxin, but functional toxin hybrids could form from the A subunit of Stx2 and B subunits of Stx1 (Weinstein et al. 1989). When clinical isolates of the O157:H7 serovar expressing both Stx1 and Stx2 were analyzed, hybrid toxins were observed (Skinner et al. 2014). Shiga toxins are more structurally similar within a type (Stx1 or Stx2) than between types, so hybridized toxins with subunits from different subtypes of the same type

are fully functional (Weinstein et al. 1989). It is not clear what role, if any, hybrid toxins have in human disease.

The differences in the structures of Stx1 and Stx2 influence their biological properties, and amino acids in certain positions are particularly important. The AB_5 structure of the Stx1 holotoxin is more stable than the Stx2 holotoxin (Conrady et al. 2010; Kitova et al. 2005, 2009). Replacement of arginine with asparagine at position 70 of the A subunit appears to destabilize Stx2 relative to Stx1 (Kitova et al. 2009). The hydrophobic interactions between the A and B subunits are destabilized by the glutamine at position 40 in the Stx2 B subunit and stabilized by leucine in the same position in Stx1 (Conrady et al. 2010). Even though Stx1 and Stx2 share a common AB_5 structure, there are subtle differences that significantly influence the properties of these toxins.

Structural differences between Stx1 and Stx2 also influence the secretion of the toxins. *S. dysenteriae* type 1 has a secretion system to transport the active holotoxin (Stx1) out of the cell. In contrast Stx1 produced by *E. coli* is released only when the Stx-phages express the enzymes required to lyse the host cell, releasing the phages and the holotoxins (*vide infra*). However, *E. coli* does have the biochemical machinery to secrete Stx2 without requiring cell lysis (Shimizu et al. 2007). Once the Shiga toxin leaves the cell, it is free to diffuse until it binds to a eukaryotic target cell.

Shiga toxin has structural features that protect it from the enzymes found in the intestine (Fig. 3.6). Trypsin is active in the small intestine and would be expected to

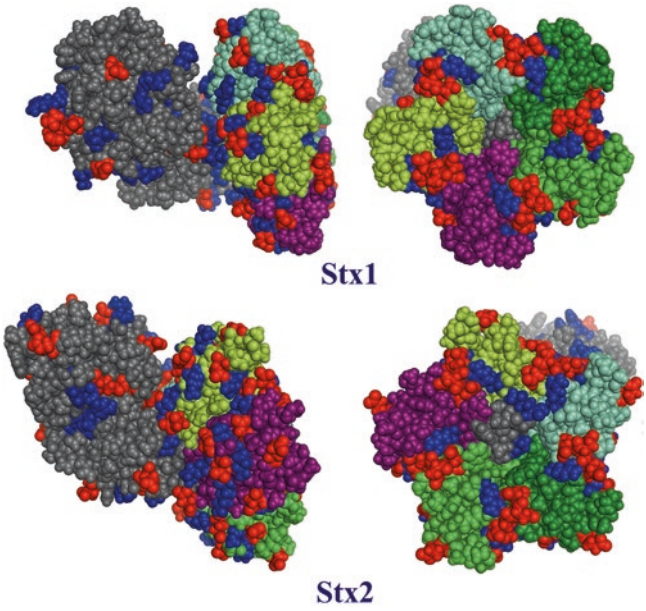

Fig. 3.6 The lysine, arginine, glutamic acid and aspartic acid residues present in Stx1 and Stx2. The basic lysine and arginine residues are shown in *blue*. The acidic aspartic and glutamic acid residues are shown in *red*. The structures are based on the RCSB PDB structures 1DM0 and 4M1U (Fraser et al. 1994; Jacobson et al. 2014)

digest Shiga toxins because they contain several lysine and arginine (Arg) residues in both the A and B subunits. The biological activity of type 1 Shiga toxin is not degraded by digestion with trypsin alone (Kongmuang et al. 1988; Reisbig et al. 1981), and trypsin digestion actually activates Stx2 by 30-fold (MacLeod et al. 1991). Activation is presumed to result from the cleavage at the site also recognized by the host cell-produced protease, furin (Garred et al. 1995b). The results suggest that the secondary structure of Shiga toxin is folded in a manner that shields all other potential trypsin cleavage sites from the action of trypsin.

3.3 Binding to Sugars of Gangliosides

As with other AB_5 holotoxins, it is the pentameric B subunits of Shiga toxins that bind to the sugar moieties of gangliosides on the surface membrane of eukaryotic target cells. Analysis of hybrid toxins revealed that the toxicity is related to the binding specificity of the B subunits (Head et al. 1991). Each B subunit of Shiga toxin has three identified ganglioside binding sites which means that there are 15 potential binding sites per holotoxin (Fig. 3.7) (Flagler et al. 2010; Ling et al. 1998; Soltyk et al. 2002). Engaging multiple binding sites provides optimal binding of the toxins. Most type 1 or type 2 Shiga toxin B subunits bind exclusively to globotriaosylceramide (Gb3) (Jacewicz et al. 1986; Lindberg et al. 1987; Lingwood et al. 1987). The Gb3 ganglioside is also referred to as CD77 or the P^k blood group antigen (Fig. 3.8). The B subunits of Stx2f and Stx2e can also bind a ganglioside with an extra sugar moiety, globotetraosylceramide (Gb4) (Fig. 3.8), in addition to Gb3

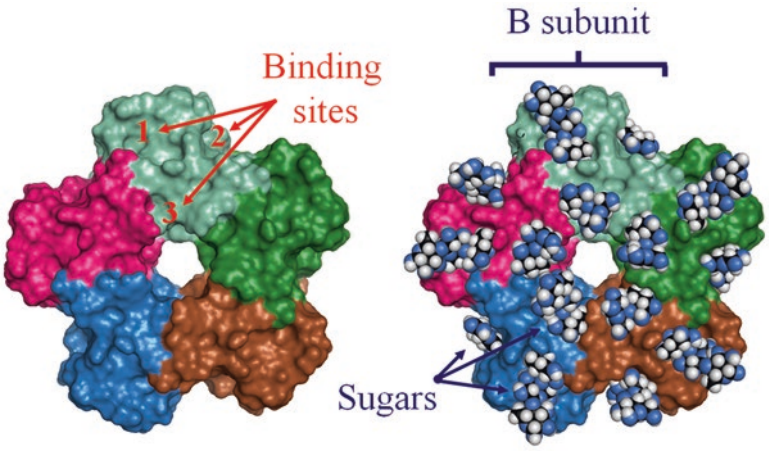

Fig. 3.7 Binding of disaccharide analogs of Gb3 gangliosides to B subunits of Stx1. *Left*, binding sites of one B subunit. *Right*, sugar residues (*blue, black* and *light grey spheres*) bound to the five B subunits. Structures derived from the 1BOS crystal structure in the RCSB PDB using PyMOL software (Ling et al. 1998). Each B subunit is shown with a different colored surface

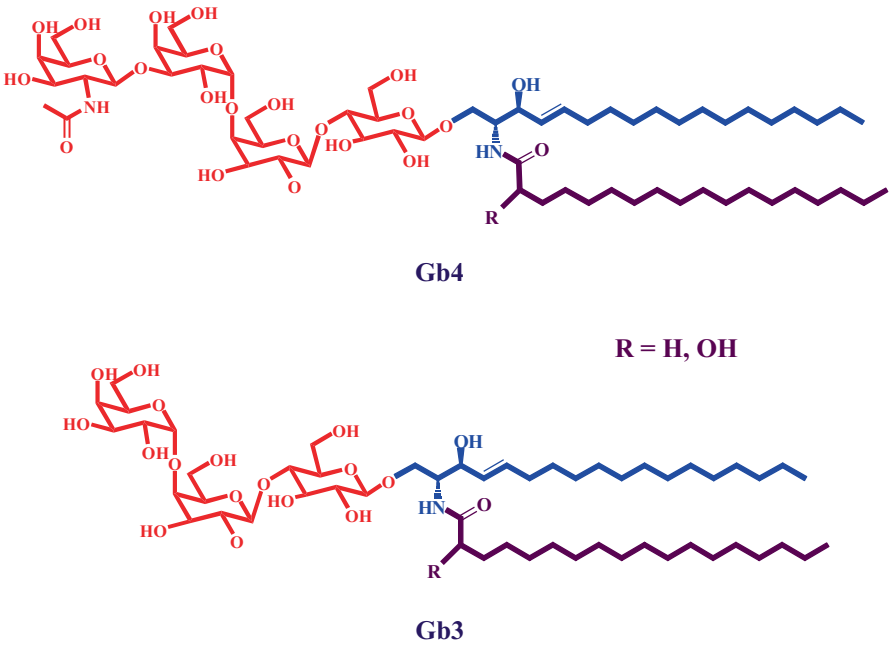

Fig. 3.8 Structures of Gb3 and Gb4 gangliosides. Sugar portions are shown in *red*, sphingosine in *blue*, and fatty acid (stearic acid 18:0) in *purple*. Gb3 and Gb4 in mouse intestinal epithelium contain varying amounts of hydroxylated or unhydroxylated palmitic (16:0), stearic (18:0), arachidic acid (20:0), behenic (22:0), tricosylic (23:0), lignoceric (24:0) and monounsaturated lignoceric (24:1) acids (Breimer et al. 1982)

(DeGrandis et al. 1989; Skinner et al. 2013). The subtle structural differences among Shiga toxins influence their binding to the gangliosides.

The binding of the gangliosides to B subunits of the Shiga toxins is complex and depends on structural features of the B subunit and the chemical structure of the ganglioside (Pellizzari et al. 1992). The Gb3 ganglioside is comprised of three covalently bound components: a Gal-α1–4-Gal-β1–4-glucosyl trisaccharide (sugar), a ceramide, and a variable fatty acid (Pellizzari et al. 1992). As noted previously, there are three sugar binding sites per B subunit. Stx1 will bind to the sugar portion of the ganglioside alone, while binding of Stx2 requires additional interactions with the ganglioside (Gallegos et al. 2012). The binding of Stx1 to Gb3 model membranes is more strongly dependent upon the ganglioside lipids than is the binding of Stx2 (Mahfoud et al. 2009). The composition of the fatty acid portion of Gb3 differentially influences the binding of Stx1 or Stx2 to Gb3 (Kiarash et al. 1994). Stx1 and Stx2 preferentially bind to α-hydroxyl fatty acid containing Gb3 versus the non-hydroxyl analog of Gb3 (Binnington et al. 2002). Although Stx1 and Stx2 bind Gb3, they bind in different ways. In an ELISA system with solid-phase Gb3, pretreatment with Stx1 blocks subsequent Stx2 binding, but not the converse (Itoh et al. 2001). This appears to be a result of the Stx1 binding more rapidly to Gb3, while Stx2

binds more slowly, but once bound is more difficult to remove (Nakajima et al. 2001). Thus, both the saccharide and lipid portions of the Gb3 ganglioside influence Shiga toxin binding.

The cell membrane milieu also affects the binding of the B subunits of the toxin to the gangliosides. The amounts of phosphatidylcholine and cholesterol in the membrane affect the extent of the binding of Shiga toxins to Gb3 in model systems (Arab and Lingwood 1996). For example, removal of cholesterol from a membrane prepared from human renal tissue conferred the ability to bind Shiga toxin (Chark et al. 2004). Furthermore, actual toxicity—not just binding—of the toxin was associated with the presence of lipid rafts in detergent-resistant membranes (Falguieres et al. 2001; Katagiri et al. 1999; Mori et al. 2000; Smith et al. 2006; Takenouchi et al. 2004). Lipid rafts, or detergent-resistant membranes, are microdomains of the lipid bilayer that are characterized by their lipid composition and the resistance to solubilization by non-ionic detergents at low temperatures (4 °C) (Levental and Veatch 2016). Even when Gb3 is present on a cell surface, the cell may still be impervious to Shiga toxin provided it is not part of a lipid raft (Hoey et al. 2003). Because Stx1 and Stx2 bind to different membrane microdomains of the cell they can be transported to the cytosol by different mechanisms (Tam et al. 2008). In sum, binding of Shiga toxins to Gb3 (and/or Gb4) is complex and as yet incompletely understood.

The holotoxin must bind to the appropriate ganglioside(s) to exert its effects. For example, if gangliosides are not present on a cell's surface, then the cell will be resistant to the toxin, as shown in the following two examples. Mice engineered not to express Gb3 could survive doses of Stx about 100-fold greater than sufficient to kill wild type mice (Okuda et al. 2006). In cellular models, when the production of the gangliosides is disrupted by the antibiotic tunicamycin, Stx is no longer toxic (Keusch et al. 1986). However, the mere presence of Shiga toxin-binding gangliosides on the surface of a target cell is not sufficient to make that cell vulnerable to the toxin. For example, domestic cattle are not susceptible to Stx poisoning, even though they express Gb3 on the surface of cells in multiple tissues: kidney, brain cells, intestinal epithelium (Pruimboom-Brees et al. 2000). They also suffer no significant pathology after experimental infection with STEC (O157:H7 serotype) (Brown et al. 1997; Cray and Moon 1995), and they can harbor a large number of STEC serotypes (Hussein and Bollinger 2005). In humans, Gb3 is expressed in relatively few cells. These include kidney epithelium and endothelium, endothelial cells in intestinal lamina propria, platelets, subsets of lymphocytes, monocytes, macrophages, dendritic cells, intestinal pericryptal myofibroblasts, neurons and endothelial cells in the CNS (Engedal et al. 2011; Obata et al. 2008; Ren et al. 1999). Presumably these results can be extrapolated to Gb4 gangliosides. The presence of Gb3 and/or Gb4 gangliosides is thus a necessary, but not sufficient requirement for intoxication by Shiga toxins.

3.4 Entry to Cells and Intracellular Trafficking

After Shiga toxins bind to a target cell surface, they are brought into that cell by one of several mechanisms (Fig. 3.9). Following the binding event, Shiga toxins induce the formation of narrow tubular membrane invaginations (Romer et al.

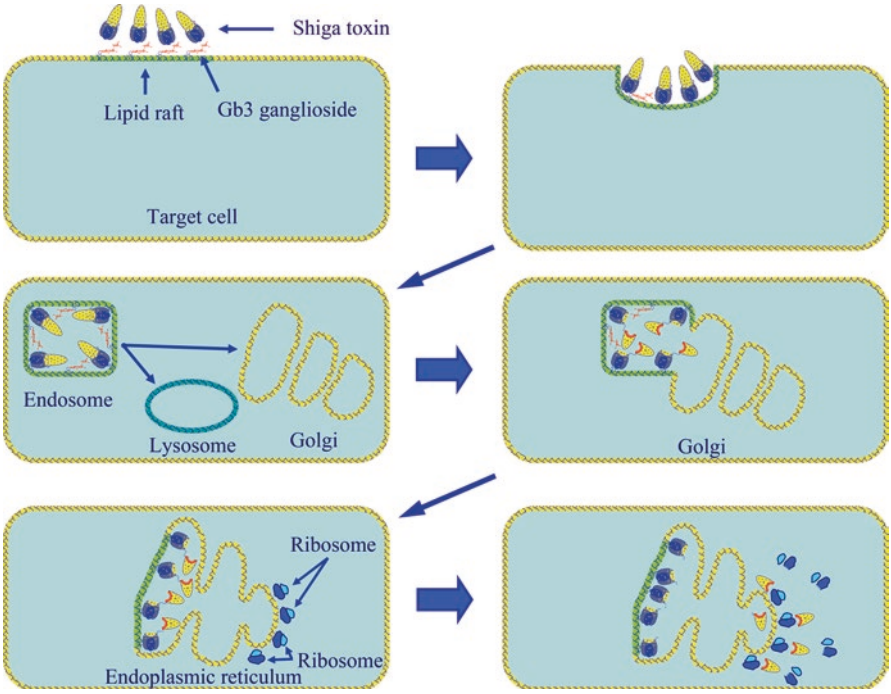

Fig. 3.9 Retrograde transport of Shiga toxin in target animal cell. (The process reverses the order of steps used in secretion of molecules, hence the designation "retrograde transport.") Shiga toxin binds to gangliosides on the cell surface, inducing endocytosis and formation of an endosome. The endosomes can be sorted to the lysosome for degradation or retrogradely transported to the Golgi complex (or network). The endosome moves to the Golgi and then to the endoplasmic reticulum. During retrograde transport to the Golgi, the A subunit is partially proteolyzed by the cellular enzyme, furin. A disulfide bond is then cleaved to release the catalytic A1 domain (red portion) in the endoplasmic reticulum. The A1 domain is translocated into the cytoplasm, where it deadenylates the 28S rRNA component of the 60S ribosomal subunit, irreversibly preventing further protein synthesis

2007). These invaginations result in endocytosis that occurs primarily by a mechanism that involves the membrane protein clathrin (clathrin-dependent endocytosis) (Lauvrak et al. 2006; Utskarpen et al. 2010; Walchli et al. 2009). To a lesser extent, Shiga toxins are endocytosed by other mechanisms that do not require clathrin (non-clathrin-dependent endocytosis) (Sandvig et al. 2010a, b, 2011). The cytoskeleton plays an essential role in endocytosis, and cytoskeletal changes are necessary to effect retrograde transport (Hehnly et al. 2006). In human kidney cells, Shiga toxin induces the cytoskeletal changes required to facilitate endocytosis (Takenouchi et al. 2004). One of the proteins involved is PKCδ , which helps transport the endosome to the Golgi complex (Torgersen et al. 2007). The protein P38a may also facilitate the transport of the endosomes to the Golgi (Walchli et al. 2008). Once inside the target cell, the holotoxin-containing endosomes are further processed.

After endocytosis, the toxin-containing endosomes are sorted to a lysosome or the Golgi, a critical step in Stx toxicity (Torgersen et al. 2010). Endosomes that are

part of a lipid raft undergo sorting for retrograde transport to the cytosol where the toxins can exact their effect. Non-lipid raft endosomes may be sorted into the lysosome where they are digested with proteases. Thus, the sorting process is central to pathogenesis. Shiga toxin bound to cell surface Gb3 on bovine intestinal crypt epithelial cells is endocytosed, but those resulting endosomes are transported to the lysosome where Stx is digested (Hoey et al. 2003). In contrast, Stx bound to Gb3 on a portion of the membrane that is retrogradely transported to the cytosol can be further processed and activated to exert its toxic effect. Cells lines defective in their ability to sort endosomes into the Golgi are immune to intoxication, even though they bind and endocytose Stx (Falguieres et al. 2001; Sandvig et al. 1992). Thus, retrograde transport takes the toxin from the cell surface into the lumen of the endoplasmic reticulum.

As summarized already, during retrograde transport from the cell membrane to the cytosol, the holotoxin is proteolytically cleaved and a disulfide bond reduced. Proteolysis is catalyzed by the cellular enzyme furin at the Arg-X-X-Arg motif found in the A subunit of the holotoxin (Garred et al. 1995b). This cleavage is believed to occur in the endosomes or Golgi (Garred et al. 1995a). After proteolysis, the catalytic (A1) domain remains attached to the rest of the holotoxin by a disulfide bond (Garred et al. 1995b; Olsnes et al. 1981) that must be cleaved before the A1 domain is free from the holotoxin (Garred et al. 1997). Reductive cleavage occurs in the endoplasmic reticulum (LaPointe et al. 2005), after which the activated A1 domain must be retro-translocated (different from retrograde transport). The C-terminal sequence (240–251) of the A1 domain is necessary for retro-translocation into the cytosol, but the features of this sequence that make it essential remain uncertain (LaPointe et al. 2005). Once retro-translocated, the A1 domain inactivates ribosomes, inhibiting protein synthesis. In addition to inactivating ribosomes, Shiga toxins can also exert their toxic effect by inducing apoptosis (Tesh 2010). It is estimated that only 4% of the toxin that binds to Gb3 receptors undergoes the entire translocation process and transfers an active A1 domain into the cytosol, but it takes just one A1 domain to kill the cell (Tam and Lingwood 2007).

The N-glycosidase catalytic sites of Stx1 and Stx2 are identical (Yamasaki et al. 1991), and nearly identical to that of ricin, an otherwise structurally unrelated plant toxin (Calderwood et al. 1987; DeGrandis et al. 1987). Five amino acids feature in the active sites of Stx1 and Stx2: Tyr77, Glu167, Arg170, Tyr114, and Trp203 (Cao et al. 1994; Deresiewicz et al. 1992, 1993; Di et al. 2011; Hovde et al. 1988; Ohmura et al. 1993; Suhan and Hovde 1998). Replacing the glutamic acid with aspartic acid at position 167 results in a 100-fold reduction in enzymatic activity (Jackson et al. 1990). Conversion of Glu167 to glutamine reduces the N-glycosidase activity by at least 1000-fold (Hovde et al. 1988). Using mutational studies to explore the importance of the active site amino acids of Stx1 and Stx2, researchers showed that Asn75 and Tyr77 were crucial for Stx2a toxicity, while Arg177 was essential for Stx1a toxicity (Di et al. 2011). The detailed mechanism of the rRNA N-glycosidase activity remains an area of active research.

3.5 Toxicity Differences Among Shiga Toxin Types and Subtypes

There is a complex relationship between the structure of Shiga toxins and the observed toxicities. The binding of Stx2 to the target cell ribosome is tighter than for Stx1, consistent with the observation that type 2 Shiga toxins are approximately 100-fold more toxic than type 1 Shiga toxins in mice (Head et al. 1991; Tesh et al. 1993). When the same comparison was made using a Vero cell assay, type 1 Shiga toxins were observed to be more toxic than type 2 Shiga toxins (Tesh et al. 1993). Differences in binding to Gb3 receptors may explain the relative toxicity in the cellular assay. Type 1 Shiga toxins bind to the surface Gb3 of Vero cells better than do type 2 Shiga toxins (Flagler et al. 2010; Head et al. 1991; Nakajima et al. 2001; Tesh et al. 1993). Stx2c is tenfold less toxic in vivo than Stx2d, yet the two subtypes differ by only two amino acids in the A subunit (Bunger et al. 2015). The binding/toxicity relationships in vitro may also pertain in the "parallel universe" of clinical observations. For example, STEC that produce only Stx1 are more commonly associated with uncomplicated diarrhea (Fuller et al. 2011). In contrast, STEC producing Stx2 are more regularly associated with severe human disease, such as HC and HUS (Bitzan et al. 1993b; Boerlin et al. 1999; Scotland et al. 1987; Werber et al. 2003).

Differences between the structures of type 1 and type 2 Shiga toxins also influence how they bind to a common human blood protein, human serum amyloid protein P (HuSAP) (Fig. 3.10) (Cox et al. 2014; Pepys et al. 1982). HuSAP differs from an analogous plasma protein in other mammalian species in that it binds to Stx2 and

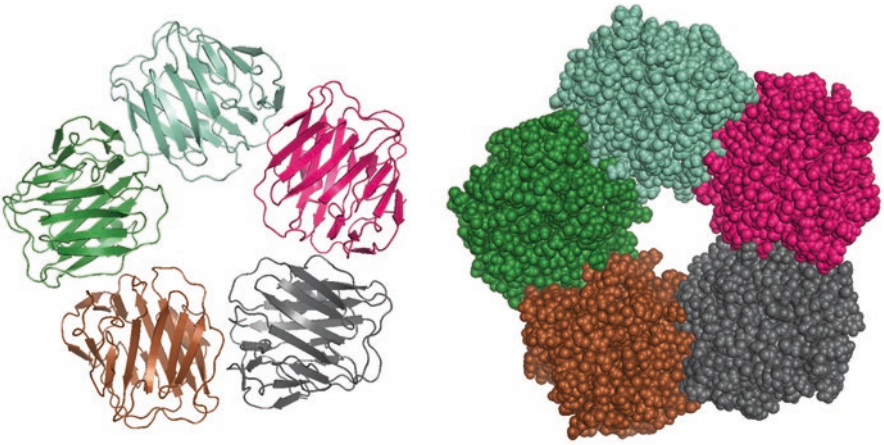

Fig. 3.10 Structure of human serum amyloid protein P (HuSAP). A 3D structure and spherical representation of the crystal structure of HuSAP are shown. These images are based on the 1SAC structure in the RCSB PDB and rendered in PyMOL (Emsley et al. 1994)

neutralizes the toxin (Bitzan et al. 1993a; Caprioli et al. 1994; Kimura et al. 2001). Purified HuSAP does not bind to Stx1 (Bitzan et al. 1993a), and its binding to Stx2 requires the presence of both the A and B subunits (Marcato et al. 2003). HuSAP interferes with the binding of Stx2 B subunits to Gb3 gangliosides. When wild type mice were injected with both HuSAP and twice the median lethal dose (LD_{50}) of type 1 or type 2 Shiga toxin, only those injected with Stx1 succumbed to toxin (Armstrong et al. 2006). Transgenic mice have been engineered to express HuSAP remained healthy after being dosed with twice the LD_{50} of Stx2 (Armstrong et al. 2006), but were not protected from ten LD_{50} (Kimura et al. 2003). The presence of HuSAP also interferes with the binding of Stx2 by antibodies that protect mice from Stx2 intoxication (Kimura et al. 2003). It has been suggested that HuSAP shields Stx2 from immune surveillance (Caprioli et al. 1992). This may explain why antibodies (Abs) to the lipopolysaccharides of the *E. coli* pathogen were observed in patients suffering from HUS, but not Abs to Shiga toxins. In another study, anti-Stx1, but not anti-Stx2 Abs were found in human serum (Ashkenazi et al. 1988; Morooka et al. 1996). This observation suggests that HuSAP may mask Stx2 toxins, preventing the development of antibodies to the toxin. Despite its ability to bind to Stx2, HuSAP in human serum does not seem to protect humans from the effects of Stx2, since STEC that produce Stx2 are associated with the more severe forms of Shiga toxin poisoning. One further effect of HuSAP on clinical studies is its interference with some assays of Stx2 (see Chap. 5), necessitating different approaches for detecting Stx1 and Stx2 in human plasma, serum, or blood (Kimura et al. 2001).

In summary, several structural features of the Shiga toxin molecule as well as the presence of Gb3 or Gb4 gangliosides in suitable cell membrane microdomains are involved in the observed toxicity. Subtle changes in B subunit tertiary structure seem to interfere with binding to gangliosides incorporated into a lipid raft. These changes ultimately cause the toxin-containing endosome to be sorted to a lysosome and degraded, rather than undergoing retrograde transport to the cytosol. Similar subtle structural changes could possibly alter the tissue preference of a toxin and, consequently, its pathological effects. The binding of HuSAP to type 2 Shiga toxins may interfere with the development of anti-Shiga toxin vaccines or anti-Shiga toxin therapies. Further elucidation of the interaction of Stx with target cells is needed to deduce structure-toxicity relationships completely.

Currently there is no effective therapy for Shiga toxin poisoning, but mapping the circuitous route of Stx from the cell surface to the cytosol identifies six points for therapeutic intervention. (1) Small molecules or antibodies could be developed to interfere with the binding of the toxins to Gb3 and Gb4. (2) Amphipathic compounds could be used to interfere with the initial membrane invagination of endosome formation. (3) Drugs could be developed to interfere with the initial sorting of the endosomes, by shunting them to lysosomes or to prevent their retrograde transport to the cytosol. (4) Small molecules could be developed to interfere with the enzymatic activity of furin. (5) Other molecules could be used to prevent the translocation of the A1 domain to the cytosol. (6) Lastly, drugs could be developed to interfere with the specific N-glycosidase activity of the A subunit.

3.6 Other AB_5 Protein Toxins

The generic AB_5 holotoxin is composed of a single A subunit and five B subunits that may be identical or different, depending on the toxin (Fan et al. 2000). The enzymatic activity responsible for the observed toxicity is contained in the A subunit. The role of pentameric B subunits is to bind the toxin to the surface of the target cell. As discussed above for Stx, bound toxin is endocytosed into the target cell and transported to a location in the cell where the A subunit is able to cause the observed damage to cellular metabolism. In addition to Shiga toxins, there are four other families of AB_5 toxins, grouped on the basis of structure and enzymatic activity: cholera toxin (Ctx), *E. coli* heat-labile enterotoxins (LT-I and LT-II), pertussis toxins (Ptx), and a recently discovered subtilase cytotoxin (SubAB) (Paton et al. 2004).

Cholera toxin (Ctx) and *E. coli* heat-labile enterotoxins (LT-I and LT-II) share both a common architecture and common mechanism of action (Figs. 3.11 and 3.12). The five B subunits of the Ctx are composed of an identical 103 amino acid protein (11 kDa). The A subunit is composed of 240 amino acids (28 kDa). The production of cholera toxin is controlled by a filamentous phage (CTXΦ), but the production of the toxin does not destroy the *Vibrio* host (Waldor and Mekalanos 1996). The *E. coli* heat-labile enterotoxin's B subunits are identical and comprise approximately 103 amino acids (12 kDa). Its A subunit (21 kDa) is composed of 185 amino acids. This enterotoxin is expressed by a multi-copy plasmid in the *E. coli* host. The B subunits of both the toxin and the enterotoxin bind to characteristic gangliosides on the surface of a target cell. The bound holotoxins are then endocytosed into the target cells and trafficked to the cytoplasm. The enzymatic activity of the toxin or enterotoxin is activated during this process. The ADP-ribosylase activity of the A subunit specifically modifies one of the target cell's G proteins and inactivates it (Cassel and Pfeuffer 1978; Cassel and Selinger 1977; Gill and Meren 1978; O'Keefe and Cuatecasas 1978; O'Neal et al. 2005; Pickett et al. 1987, 1989). This process leads to the eventual death of the cell.

Ptx is an AB_5 toxin of *Bordetella pertussis*, with the unusual feature of B subunits that are not identical (Fig. 3.13). The 26 kDa A subunit is composed of 235 amino acids. The proteins of the B subunits have three different sizes, approximately 199, 110, or 99 amino acids in length, corresponding to 22, 12, and 11.7 kDa. The two 110 amino acid proteins are identical. The two 199 amino acid proteins are different but share ~70% sequence identity. As with the other toxins discussed, the B subunits bind to the target cell surface gangliosides. The toxin is then endocytosed and trafficked to the cytoplasm, where the catalytic A subunit can cause its damage. The A subunit is an ADP-ribosylase that modifies a specific G protein that leads to target cell death (Antoine and Locht 1990; West et al. 1985).

Subtilase cytotoxin (SubAB) is a recently discovered toxin that is associated with Shiga toxin-producing *E. coli* (Fig. 3.14) (Paton et al. 2004). The A subunit is composed of 347 amino acids (37 kDa); the B subunits comprise five identical 120 amino acid proteins (13 kDa). Unlike the other AB_5 toxins, SubAB binds to a diet-acquired

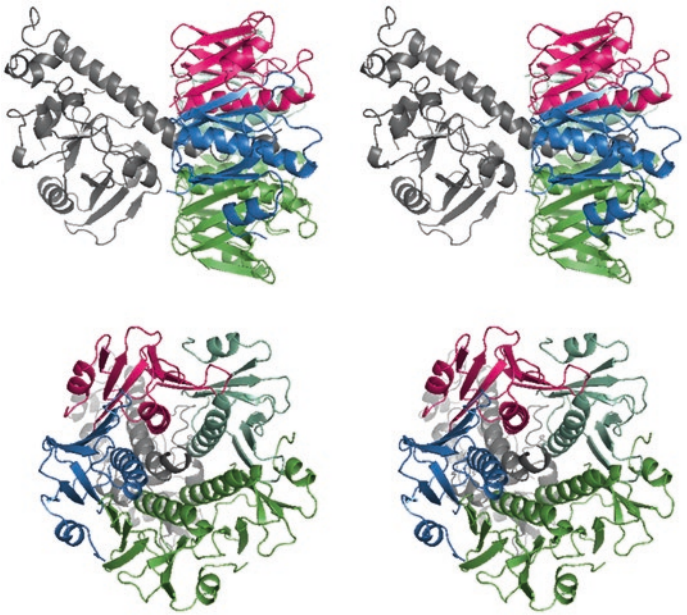

Fig. 3.11 3D structure of cholera toxin. The view of the cholera toxin structure is a PyMOL image derived from structure 1S5E (O'Neal et al. 2004) in the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB)

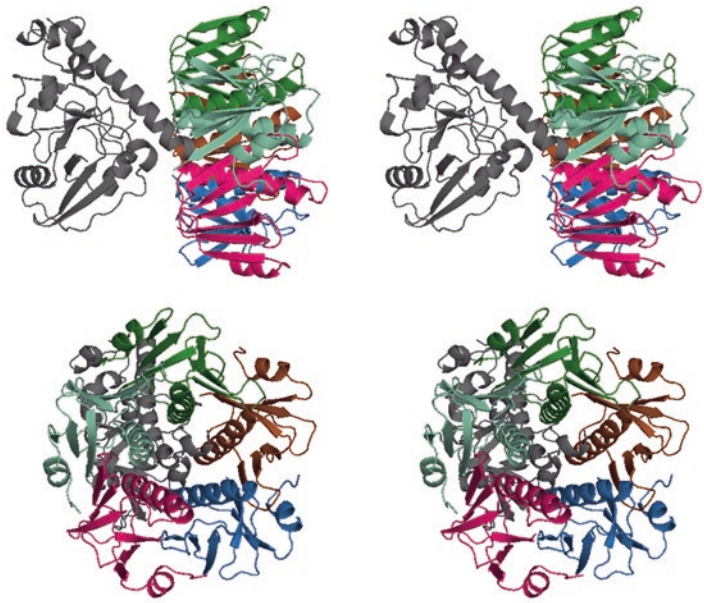

Fig. 3.12 3D stereoview of *E. coli* heat-labile enterotoxin (LT). This view is derived from the 1LTS crystal structure in the RCSB PDB using PyMOL software (Sixma et al. 1993)

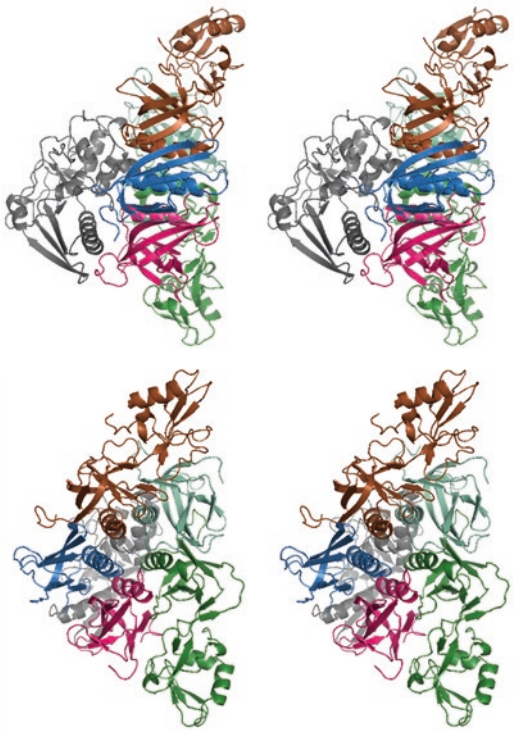

Fig. 3.13 3D stereoview of pertussis toxin. This view is based on a PyMOL rendering of the 1BCP crystal structure contained in the RCSB PDB (Hazes et al. 1996)

ganglioside with a distal N-glycolylneuraminic acid (Byres et al. 2008). SubAB is endocytosed and then transported to the ER. SubAB's A subunit is a serine subtilase protease that appears to cleave an HSP70 chaperone protein, referred to as a binding immunoglobulin protein (BiP) (Paton et al. 2006). The cleavage of BiP protein leads to the death of the target cell.

In addition to the structural and mechanistic differences among the AB_5 toxins, their production and expression are controlled by very different genetic elements. Shiga toxin production by STEC and some other bacteria is under the control of an infecting lambdoid phage that destroys the host bacterium in the process of producing the toxins (Huang et al. 1987; Newland et al. 1985; O'Brien et al. 1984). The cholera toxin is also controlled by a phage (CTXφ), but this filamentous bacteriophage does not destroy its host bacterium when it replicates. The enterotoxins produced by *E. coli* are under the control of a self-replicating plasmid (Paton et al. 2004). *E. coli* LT-I and LT-II are also under the control of a large plasmid. Production of Ptx is controlled by the bacterium (Gross and Rappuoli 1988). In *S. dysenteriae* type 1, Stx production is under the control of the bacterium but appears to have been originally controlled by a phage (McDonough and Butterton 1999; Mizutani et al. 1999; Strockbine et al. 1988). This is true of most other *Shigella* spp. as well (Nataro and Kaper 1998). Although these toxins share a common AB_5 structure and some common cell-surface binding sites, their methods of production are quite different.

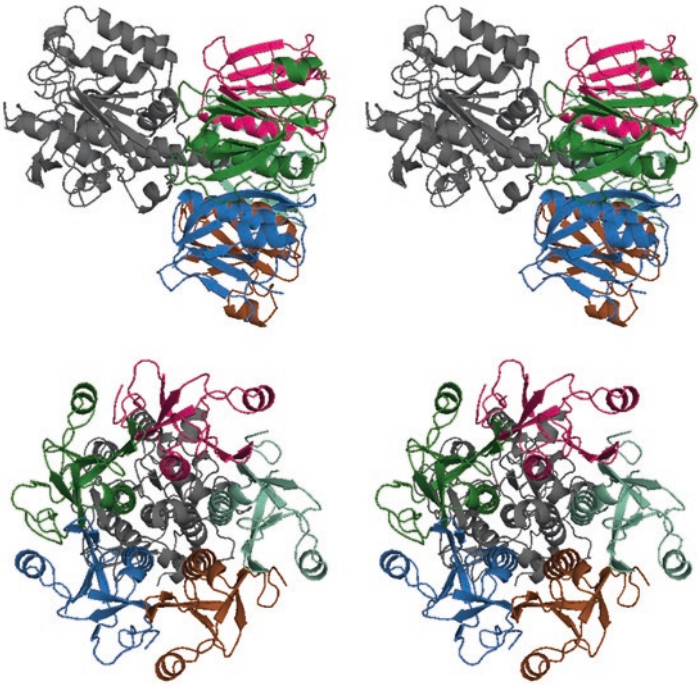

Fig. 3.14 3D structure of *E. coli* subtilase cytotoxin (SubAB). This view is a PyMOL derived rendering of the 4BWG crystal structure contained in the RCSB PDB (Le Nours et al. 2013)

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Chapter 4
Regulation of Shiga Toxin Production

Abstract Although Shiga toxins are produced by particular *E. coli* serotypes, the genes transcribed, as well as the timing and quantity of transcription, are determined by lambdoid phages (phages related to phage λ) that infect those serotypes. A lambdoid phage is referred to as temperate when it integrates into a host cell genome and replicates its DNA "lysogenically" (as the cell replicates its DNA). A stressed cell detects DNA damage and figuratively sends out an "SOS." The phages have a sophisticated mechanism that detects the host cell SOS response to DNA damage and responds by replicating "lytically" (with lysis of the host cell). Each "Stx-phage" has one Shiga toxin operon, composed of genes encoding the Shiga toxin StxA and StxB subunits. However, an individual Stx-phage may integrate into a bacterial chromosome more than once, and different species of Stx-phage can infect a bacterium simultaneously. Both the multiplicity of infection and recombination events permit a single host to produce more than one type or variant of Shiga toxin, each produced under the control of its own phage. Additional transposable elements (transposons) from the bacterial host add to this genetic diversity, and all levels of this diversity are transmissible to other bacterial hosts. To combat phages, bacterial hosts have a primitive adaptive immune system that employs clustered, regularly-interspaced, short palindromic repeats (CRISPR) and the CRISPR-associated genes (CAS). CRISPR/CAS can inactivate infecting Stx-phages.

Keywords Lytic replication • Shiga toxin-producing bacteriophages • SOS response • Transposons • Integrons • CRISPER/CAS • Lambdoid phages • Phage lambda • Superinfection • Pathogenicity islands

4.1 Lambdoid Phages

To put the control of Stx in context, we must first consider the biology of lambdoid phages, a group of bacterial viruses related to phage λ (Fig. 4.1) (Casjens and Hendrix 2015). The phage initiates an infection when its tail fibers bind to specific host cell surface proteins (porin, a LamB gene product that facilitates transmembrane transfers). The phage makes use of porin to transfer its chromosome as a linear DNA

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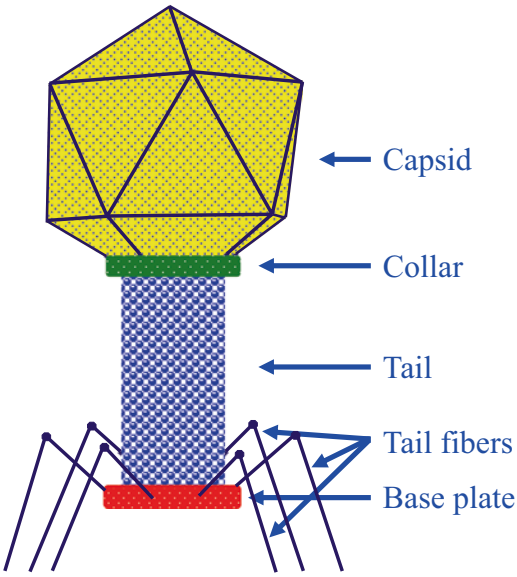

Fig. 4.1 Schematic structure of phage λ. The capsid or head contains the phage genome. The tail fibers bind to the host cell and the base plate attaches to porin protein on the host cell surface (Casjens and Hendrix 2015)

molecule into the cell. The DNA is rapidly circularized by *E. coli* ligases at the cohesive end sites (cos) of the linear phage chromosome. The circularized DNA may recombine with the host chromosome at a particular sequence, referred to as an attachment (*att*) site in the *E. coli* host (*att*B) chromosome. The *att* site for phage λ is between the host *gal* and *bio* genes. If the *att*B site is deleted, then phage λ can insert itself into other sites (*pro*B, *trp*C, *gal*T, *thr*A, or *rrn*B), albeit with 100–1000-fold lower efficiency. This recombination results in an integration of the phage genome into the *E. coli* chromosome.

Once integrated into the bacterial chromosome, the integrated phage chromosome is referred to as a prophage and passively replicates whenever the host bacterium replicates its chromosome. This process, lysogenic replication, does not damage the host cell. The prophage assesses its host cell health by monitoring for evidence of DNA damage. If the host DNA is damaged, the cell sends a figurative "SOS," (*vide infra*) that would normally arrest the cell cycle to facilitate repair of the damaged DNA (Radman 1975). Instead the prophage senses this signal and diverts the cell metabolism to the production of intact phages, leading to cell death in a process known as lytic replication (Fig. 4.2).

The maintenance of the lysogenic mode of prophage λ replication is controlled by the cI protein expressed by the phage. The 26 kDa cI protein is composed of 237 amino acids and belongs to a superfamily of repressor proteins that share a common structural motif, the helix-turn-helix (HTH). When two cI monomers dimerize, they can bind to adjacent promoter sites, P_L and P_R, to repress the expression of cro, a protein that controls lytic replication. The cro protein is a smaller monomeric protein comprised of 66 amino acids and a having a molecular weight of 7367 Da (Takeda et al. 1977). Like cI, it is a member of the HTH superfamily. The cI dimers can bind to the promoter P_{RM} that promote expression of cI. This is how cI, under

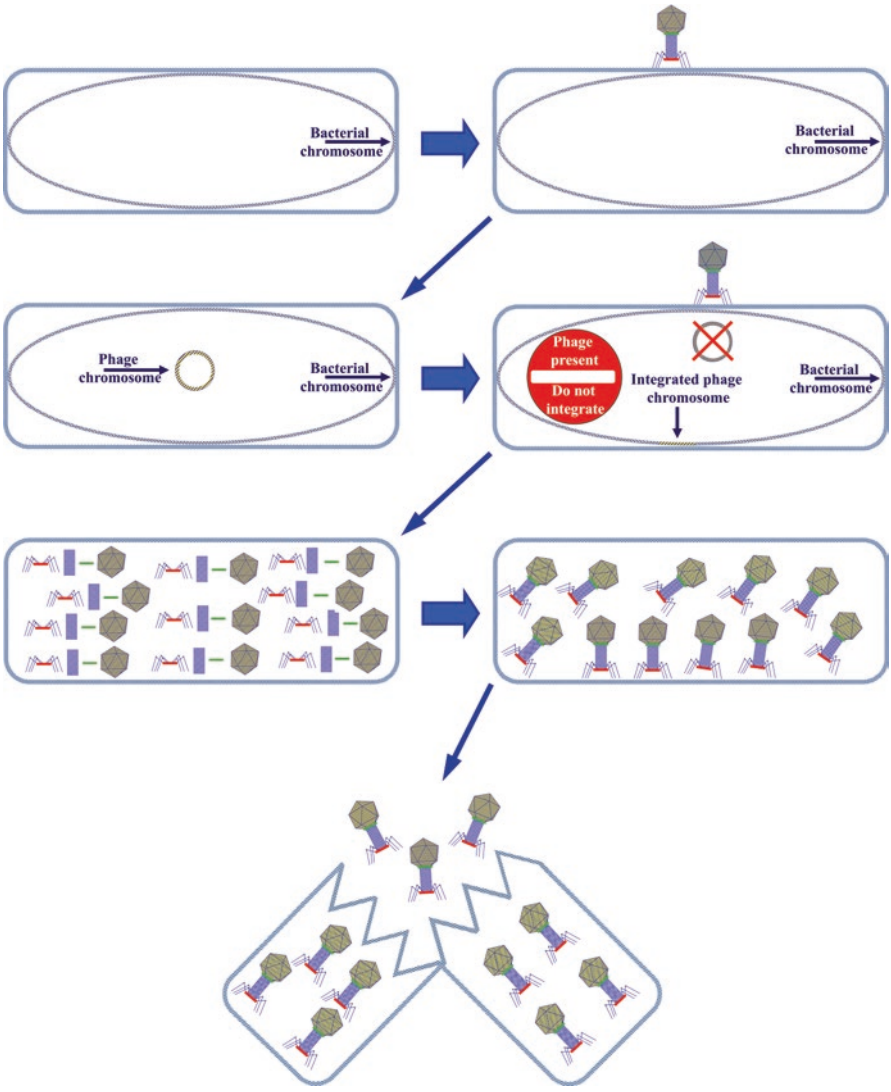

Fig. 4.2 Infection and replication of phage λ in an *E. coli* host cell. Phage λ inserts its genome into the host cell, a process that also prevents infection by other phages. The inserted DNA, now called a prophage, will replicate when the host genome replicates (lysogenic replication). If the host cell becomes stressed and induces its SOS response, then the phage will take over the host cell metabolism to produce intact phages (lytic replication). In the later stages of lytic replication, the phage expresses lytic enzymes, which break open the cell to release the new phages (Casjens and Hendrix 2015)

the control of the prophage, represses the induction of the phage and maintains its lysogenic replication. In addition, cI prevents superinfection of the host by another phage λ. The protein cI binds to P_L and P_R of the would-be superinfecting phage chromosome and prevents its integration. In this way a phage can both prevent superinfection and replicate passively without damaging its host.

The SOS response is initiated with the conversion of the monomeric protein RecA into an activated nucleoprotein filament, RecA* (Patel et al. 2010). RecA* binds to LexA, a repressor protein that suppresses the expression of genes that repair DNA, the SOS genes. The binding of RecA* triggers the autocatalytic degradation of LexA. In an infected cell, the prophage repressor cI, with a structure similar to LexA, also binds to RecA*. Binding induces the autocatalytic degradation of cI, thereby relieving repression of cro and allowing the lytic mode of replication to unfold.

At this point, the activated SOS response has prompted the prophage to assume control of cellular metabolism and divert it to the production of phage components, including phage DNA, capsid proteins, collars, tails, base plates, and tail fibers. The phage components are then assembled into intact phages. Near the end of this process, late-stage phage genes express the lysozymes holin and pinholin, which lyse the cell and release the newly produced phages. The number of phage λ produced per bacterium during lytic reproduction, or burst size, is approximately 150 (Shao and Wang 2009). The released phages have the capacity to infect other hosts and begin the lysogenic/lytic cycle anew.

In view of the destructive nature of temperate phages, it is unremarkable that *E. coli* has developed genetic tools to combat phage infection. *E. coli* possess a primitive acquired immune system that is designed to detect and eliminate phages (Barrangou et al. 2007; Ishino et al. 1987). Clustered, regularly-interspaced, short palindromic repeats (CRISPR) and the CRISPR-associated genes (CAS) combine to remove foreign DNA from bacteria (Sorek et al. 2008). This system can remove DNA from the *E. coli* chromosome or from extrachromosomal DNA within the bacterium, e.g., self-replicating plasmids. Although the system prevents phage replication, it does not excise the entire phage, and portions of the nonfunctional prophage remain in the *E. coli* chromosome after excision. Even though the prophage genes replicate simultaneously with the bacterial chromosome, expression of these genes remains under the control of the now nonfunctional progenitor phage, not the bacterial host.

4.2 Mobile Genetic Elements in *E. coli*: Transposons and Integrons

In addition to phages, *E. coli* possess smaller mobile genetic elements that can influence their genetic composition (Bennett 2004). These mobile genetic elements are generically referred to as transposable elements. The simplest transposable element, referred to as an insertion sequence (IS), consists of three genetic components. The terminal inverted repeats on the flanking ends of the IS will be inserted into the *E. coli* genome. In addition to the flanking inverted repeats, the IS contains a regulatory gene that controls the frequency of IS insertion by stimulating or inhibiting IS activity. The final component of an insertion sequence is a gene that codes for transposase, the enzyme that binds to the inverted repeats and facilitates the insertion of

the IS into another portion of the genome. Not surprisingly, IS are small, comprising 750–2500 base pairs (bp) of DNA. Insertion elements such as IS exist in only one copy in a genome, but the location varies due to the movement of the IS.

Transposons and integrons are the names given to larger transposable elements that contain the genetic elements of IS plus other genes and gene clusters. The additional transposon genes often encode proteins such as β-lactamase that facilitate antibiotic resistance. When a transposon moves, the gene cluster moves with it. Some transposons encode a resolvase, which permits the transposon to make and then integrate a copy of itself into another portion of the genome. Integrons are still larger versions of transposons that may contain more than one gene cluster and have the capacity to capture new genes. Thus, both the transposon and the integron are transposable elements that can exist in multiple copies in a single genome and confer antibiotic resistance.

4.3 Phage Control of Shiga Toxin Production

Although the term STEC implies that Shiga toxin is produced by the various *E. coli* serotypes and strains, the actual expression of *stx* genes is largely controlled by the Shiga toxin-producing lambdoid phages (Stx-phages) that infect the *E. coli* host (Fig. 4.3) (Allison 2007; Kruger and Lucchesi 2015). When they infect a new *E. coli* host, Stx-phages convert a non-STEC *E. coli* into a STEC, so Stx-phages are also referred to as converting phages. These phages infect the host *E. coli* strain and direct the expression of the *stx* genes (O'Brien et al. 1984; Scotland et al. 1983; Smith et al. 1983; Tyler et al. 2005). As discussed in Chap. 2, Shiga toxin types (Stx1 and Stx2), subtypes, and variants represent variations in amino acid sequence, or polymorphisms of the toxin that display phenotypic differences in toxicity, binding, and target cell preference. Each Stx-phage contains a Shiga toxin operon (Stx-operon) composed of the two *stx* genes and the promoters necessary to produce a single Shiga toxin (Garcia-Aljaro et al. 2006; Koch et al. 2001; Muniesa et al. 2000; Ogura et al. 2007; Strauch et al. 2008; Strauch et al. 2004; Teel et al. 2002). The Stx operon is expressed during late-stage phage replication, assuring that Shiga toxins are produced after the phage has started its lytic replication cycle and released when the host cell is lysed (Plunkett et al. 1999). Many of the severe pathologies of STEC infections, those associated with Shiga toxins, result directly from the induction of the Shiga toxin-producing phage or phages (Tyler et al. 2013).

Under certain conditions type 1 Shiga toxin can be expressed without lytic phage replication, for example under low-iron conditions. The ferric uptake regulator (fur) binds to iron and represses the expression of the genes necessary to sequester iron. When iron levels in the environment are low, repression is relieved and *E. coli* responds by expressing genes necessary to obtain more iron. Under low iron conditions, if the *E. coli* is infected with a Stx1-producing phage, then some (fur-related) proteins that induce the genes needed for iron sequestration also induce type 1 Shiga

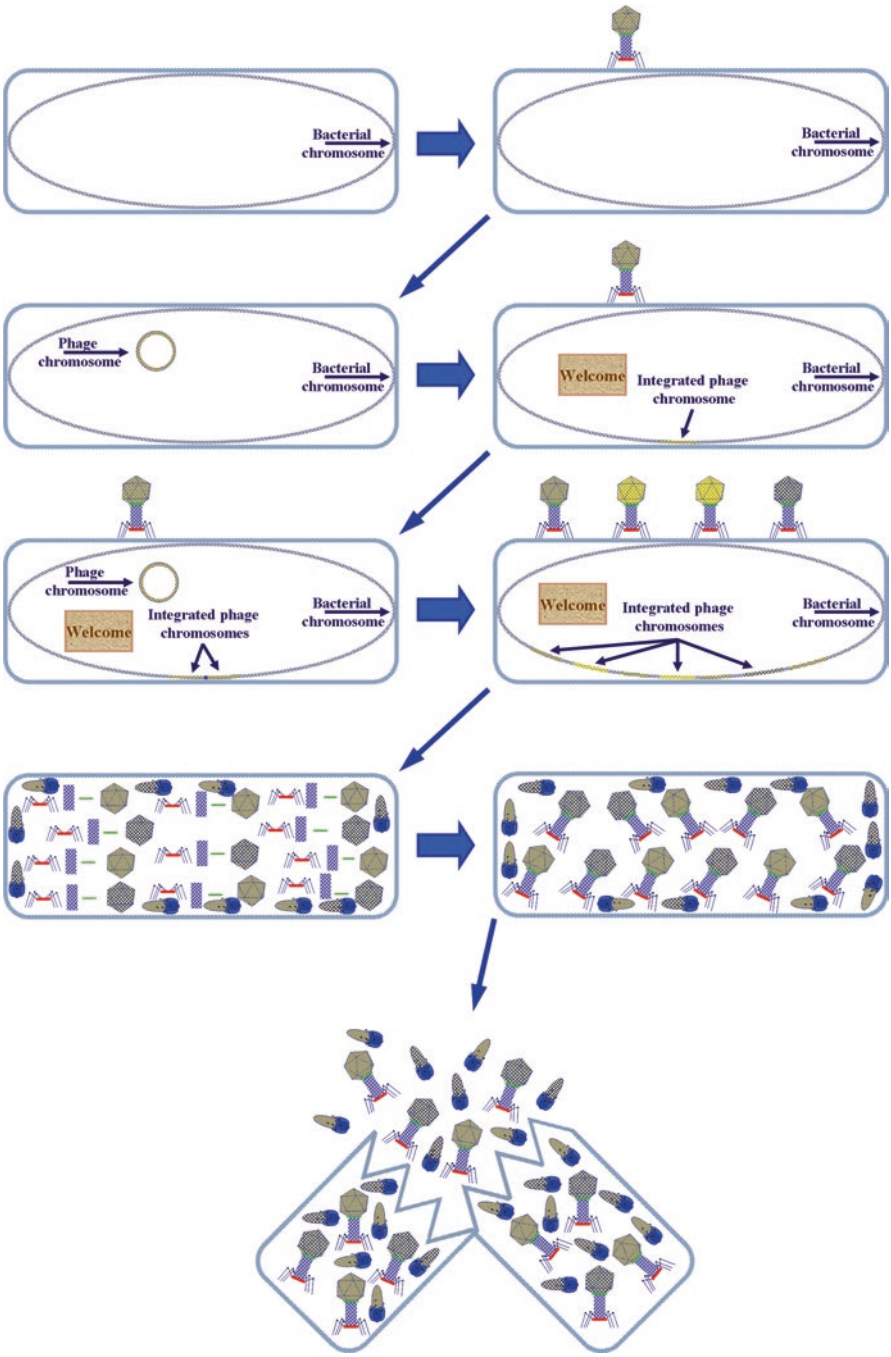

Fig. 4.3 Infection and replication of a lambdoid, Shiga toxin-producing phage. The phage infects a host cell and, integrates into the host cell chromosome. More than one species of Shiga toxin-producing phage may infect and integrate. The phage replicates lysogenically until induced to reproduce lytically, resulting in production of intact phages and Shiga toxins and the lysis of the host cell

toxin expression without inducing the prophage that contains the genes necessary to produce Stx1, the *stx1* operon, (Calderwood and Mekalanos 1987). This occurs because the sequence of the promoter regions of the *fur* and *stx1* are highly homologous and can be repressed by the same protein (Sung et al. 1990). When adequate iron is available, the expression of Stx1 remains under the control of the phage.

Although the Shiga toxin-producing phages are temperate lambdoid phages, their genotypes differ significantly from that of phage λ (Smith et al. 2012). The phage λ genome contains approximately 48 kb of DNA coding for 71 genes. In contrast, the size of the common $\Phi 24_B$ Stx2-producing phage is approximately 57 kb and codes for 88 identifiable genes (Smith et al. 2012). Phage P13374, responsible for the STEC (O104:H4 serovar) outbreak in Germany, has a genome with 61 kb of DNA and 79 open reading frames (Beutin et al. 2012). Although Stx-phages share the general morphology of phage λ (capsid, tail, tail fibers) (Ackermann 2001), they differ significantly in the size of various structural elements (Bonanno et al. 2016; Garcia-Aljaro et al. 2009). Based on sequence analysis, there are at least 31 other Stx-phages that infect STEC (Kruger and Lucchesi 2015). The size of the genomes for these phages varies from 29.7 to 68.7 kDa. The *E. coli* O157:H7 chromosome has at least five different *att* sites for Stx-phages to integrate. At least 12 different *att* sites have been identified in other STEC serotypes. The cI proteins of these phages can differ, which means that one kind of lambdoid phage may be unable to prevent an infection by a different kind of lambdoid phage (O'Brien et al. 1984). The genotypes of Stx-phages are different from that of phage lambda and so it is not surprising that there are phenotypic differences as well.

Stx-producing lambdoid phages share a common ability to recombine with phage λ, but they belong to different species of lambdoid phages. Each lambdoid phage has species-specific properties that are hybridized when two different phages recombine. These properties include host range, attachment (*att*) sites, and factors that affect the amount of Stx production. Thus, two or more phages may reside simultaneously in the same host and may inhabit different parts of the host chromosome. A single host may be infected with more than one copy of the same phage, or by one or more copies of different phage species. Each of these phages has its own single Stx operon, so an infected host may be capable of producing more than one type of Shiga toxin. Furthermore, multiplicity of infection permits recombination among the many infecting phages.

Multiplicity of infection is one area of difference between phage λ and lambdoid Stx-phages. Phage λ immunizes a host cell from superinfection: once it inserts itself into a host chromosome, it actively prevents subsequent infections. While there can be only one prophage λ per cell, Shiga toxin-producing lambdoid phages can infect the same cell multiple times (Allison et al. 2003). The first infection by certain phages facilitates subsequent infections with the same phage (Fogg et al. 2007). Such superinfections increase the production of the Shiga toxins expressed by that phage (Fogg et al. 2012). Since superinfected hosts contain multiple copies of a phage, there will more prophages in the host chromosome, resulting in more opportunities to genetically modify those prophages. There are many kinds of Stx-phage, and an *E. coli* host can be infected simultaneously by more than one kind (Kruger et al. 2011). Multiplicity of infection accounts for the large number of prophages

present in an STEC genome (Hayashi et al. 2001), and other genetic elements in the *E. coli* host can modify the prophages.

The location of the stx operon in a Stx-phage can dramatically affect the amount of toxin that a STEC can produce and even shut down production completely. Analysis of some of the strains of the O157 serogroup from Thailand revealed that these strains had an intact *stx* operon, including individual *stx*A and *stx*B promoters, but did not produce the Shiga toxin encoded by these genes (Koitabashi et al. 2006). However, when the Stx genes were cloned into *E. coli*, they could be induced to express the intact toxin. In these strains, the *stx* operon was moved outside the influence of the $P_{R'}$ promoter. The $P_{R'}$ promoter regulates expression of late-stage genes of these lambdoid phages and is much stronger than promoters for the *stx* operon (Plunkett et al. 1999; Wagner et al. 2001). These genetic arrangements thus created *E. coli* strains that had intact genes for the phage, the *stx* operon, and the O157 serogroup, but were nevertheless not STEC.

In addition to the effects of the specific genes they carry, transposable elements can disrupt the genome. Simply by insertion within a gene, a transposon can disrupt or ablate the expression of that gene. This is the basis of transposon mutagenesis, a widely used tool to knock out host genes in order to study their role. Transposons also have the capacity to introduce new genetic information into a phage infecting a host. The transposable elements can then move with the infecting phage to a new host genome. In this way a transposon can move to a new host. If the transposon inserts itself into the Stx operon, it can prevent expression of intact holotoxin. Furthermore, as discussed above, STEC also possess the CRISPR system (Yin et al. 2013), and prophages can be substantially altered by this adaptive immune system.

The interactions of bacterial defense mechanisms, coupled with the vagaries of phage replication, complicate the analysis of Shiga toxins. Host bacteria may be infected by 24 or more lambdoid phages, some of which are not capable of lytic replication and consequent release of toxins (Asadulghani et al. 2009; Hayashi et al. 2001). Thus, not all toxin genes will be expressed (Koitabashi et al. 2006). A further level of complexity is that intact phage can complement defective phages and permit their lytic induction or permit the expression of the *stx* genes (Allison 2007). Furthermore, the phages can infect different strains of *E. coli* and other bacterial species (James et al. 2001), conferring potential Stx production on these hosts [e.g., *E. coli* O104, *Enterobacter cloacae,* and *Citrobacter freundii.* (James et al. 2001; Paton and Paton 1996; Probert et al. 2014; Schmidt et al. 1993)]. The production of Shiga-like toxins and retention of the *stx* genes are sometimes transient (Paton and Paton 1997), presenting another complication in analysis of Stx.

The genes of lambdoid phages are highly conserved, making them a target of recombination with other phages. The recombination protein, RecA, is essential for the integration of lambdoid phages and can also facilitate recombination among homologous DNA sequences, such as those found in lambdoid phages. Phage genome sequencing shows that elements from different phages can be recombined into a single phage, said to have a mosaic character (Johansen et al. 2001). Although mosaic

phages contain genes from other phages, they contain only one Stx operon. This recombination results in phages with different host specificity, *att* sites, susceptibility to induction, and even other virulence factors, in addition to the Stx-encoding genes (Creuzburg et al. 2005). Thus, recombination is an important mechanism by which phages can change and thereby adapt to their hosts.

Other mobile genetic elements also influence the Stx-phages. The simplest modification of the phage is the insertion of a transposon or an insertion sequence into its genome. If this mobile element is inserted into a critical portion of the genome, it may prevent the phage from replicating lytically. It may insert into the promoter region of the *stx* operon and prevent the expression of the *stx* genes. If a transposon or IS is inserted into one of the *stx* genes, then it could prevent the expression of functional Shiga toxin. When any of these three events occurs, then the insertion effectively converts an STEC into a non-STEC.

Integrons are larger mobile genetic elements that influence the genetics of Stx-phages. Since integrons can insert into the genome of the phage, they have the capacity to cause the same sorts of disruptions that are observed with the smaller mobile genetic elements, transposons and IS. However, integrons have more DNA and they can pick up still more genetic information as they relocate in the host genome (Zhao et al. 2001). One of the most salient capabilities of integrons is introduction of antibiotic resistance to phages, giving them the ability to move that resistance to new hosts. Similarly, integrons have the capacity to move other virulence factors to new hosts (Zhao et al. 2001).

4.4 Impacts of Phages and Mobile Elements on Pathogenicity

Simply stated, Stx-phages drive the pathogenicity of STEC. They can convert a mild pathogen into a virulent one (Whittam et al. 1988). A case in point is the central role of domestic cattle in foodborne Stx intoxication. Much of the cattle population has antibodies to Shiga toxins, indicating that they were exposed to Shiga toxins at some time in their lives (Pirro et al. 1995). Domestic cattle are not affected by Shiga toxins, and they may therefore harbor STEC without substantial ill effects (Karmali 1989; Pruimboom-Brees et al. 2000). In the favorable environment of the bovine digestive system, Stx-phages that infect the various STEC are free to reproduce lytically and thereby convert other *E. coli* into STEC. This conjecture is supported by a microbiological survey of the *E. coli* present in domestic cattle which isolated 261 distinct STEC serotypes (Hussein and Bollinger 2005). It is also not surprising that many STEC with multiple Stx-phage infections have been isolated from domestic cattle (Kruger et al. 2011). The manufacture of Stx-phages is stimulated when antimicrobials are used for growth promotion in cattle, for these induce lytic phage replication and enhanced Shiga toxin production (Kimmitt et al. 1999; Kohler et al. 2000; Matsushiro et al. 1999; Zhang et al. 2000).

Once phages are free of a host *E. coli*, they are capable of converting *E. coli* into STEC in the gastrointestinal tract of animals as well as in the outside environment. Using mouse models, researchers showed that Stx-phages can infect non-*E. coli* bacteria in the mouse intestinal tract (Acheson et al. 1998). Even after phages have left the body of an animal, they can still convert a non-STEC *E. coli* host into STEC (Casas et al. 2006; Muniesa and Jofre 1998). Stx-phages present in the environment are significantly more resistant to some forms of inactivation than their *E. coli* hosts (Muniesa et al. 1999). In short, phages from environmental sources appear to play a significant role in converting *E. coli* serovars into STEC.

Genetic rearrangements of the Stx-prophages can substantially alter the production of Shiga toxins in the host organism, as exemplified by production of Shiga toxin in *Shigella dysenteriae* type 1. Although under the control of the bacterium (Greco et al. 2004; Unkmeir and Schmidt 2000), the *stx* genes were once part of a lambdoid phage. The present day *S. dysenteriae* type 1 originated when its progenitor rendered an infecting Stx-phage inert with an IS (Greco et al. 2004). Another example is based on genetic analysis of *E. coli* O157:H7. Some strains of this serovar contain *stx* genes that cannot be expressed, even though the phages containing them appear to be intact (Koitabashi et al. 2006). This situation resulted from a rearrangement of the genes that put them outside an important late-stage phage promoter. Thus, genetic rearrangements can lead to the transfer of the control of the Shiga toxin production from the infecting phage to the host bacterium or preclude the ability to express Shiga toxins. In either case the *stx* operon remains intact. Thus, the presence of *stx* genes does not mean that they are expressed. PCR-based inferential detection of Shiga toxins is therefore ambiguous, since PCR detects only the presence, not the expression, of the *stx* operon.

Nonpathogenic *E. coli* can amplify the amount of Shiga toxin produced if they are converted to STEC after being infected with Stx-producing phages. Since these *E. coli* are now STEC, they may begin to express Shiga toxin (Gamage et al. 2003). Gut bacteria other than *E. coli* can also be infected by Stx-phages, but the phages may not be able to integrate into their chromosomes. The infecting Stx-phages may replicate lytically and produce more viable Stx-phages, conversions and Shiga toxin (Cornick et al. 2006; Gamage et al. 2006; Gamage et al. 2003; Toth et al. 2003). In this way Stx-producing phages may create new Shiga toxin-producing bacteria (Allison 2007). As mentioned above, Stx-phages are stable in the environment for extended periods (Muniesa et al. 1999) and can infect new host bacteria long after they have passed through an animal host.

As also noted above, DNA-based approaches for detecting Shiga toxins infer their production from the presence of *stx* genes. However, the *E. coli* host response to infection by a Stx-phage can inactivate phages and prevent expression, even though there is an intact *stx* operon. The CRISPR/CAS system, a recombination event, or insertion of mobile genetic elements, can ablate lytic phage replication.

For example, recombination can move the *stx* operon out of the late-stage portion of the phage genome, so *stx* are not expressed, even though the *stx* operon remains intact. In short, detecting the presence of *stx* or even an intact *stx* operon does not equate to Shiga toxin detection.

Since more than one phage type can infect a host, predicting the amount of toxin produced by each requires a detailed genetic analysis. For example, *S. dysenteriae* type1 produces the prototypical Shiga toxin and causes HUS (Butler 2012). STEC, despite producing the nearly identical type 1 Shiga toxin, do not causes HUS unless also they produce type 2 Shiga toxin (Ethelberg et al. 2004). Shiga toxin-producing phages induce to a varying extent, depending on both the inductant and phage (Los et al. 2009). Furthermore, different phages may express the same Shiga toxin (Yin et al. 2015). There is competition among phages infecting a single host that impacts phage replication and expression of Shiga toxins. For all of these reasons, a simple PCR-based approach to infer the expression of the *stx* operon can be misleading.

In addition to differences in the promoters for *stx1* and *stx2* genes, there are differences in amino acid sequences of Stx1 and Stx2 that allow the host cell to secrete Stx2, but not Stx1. Already mentioned above, under low-iron conditions, STEC can be induced to expression Stx1 in the absence of lytic replication by the Stx-prophage. Even though Stx1 is expressed, it remains inside the host cell because the host cell lacks the biochemical machinery for toxin secretion (Shimizu et al. 2009). STEC have the biochemical machinery to secrete type 2 Shiga toxins (Shimizu et al. 2009), but the promoters for *stx2* genes do not respond to low-iron induction. Instead, Stx2 is expressed only when the prophage undergoes lytic replication. Although Stx1 and Stx2 share many common genetic and structural features, there are important differences in the control of expression of the two toxins, depending on their host cell and environmental factors.

As noted previously, control of Shiga toxin production is not the same among all Shiga toxin-producing bacteria and may be under environmental influence in some cases. Whereas Shiga toxin production is controlled by phages that infect the STEC, in *S. dysenteriae* type 1 production of Stx1 is controlled exclusively by the bacterium itself. Based on sequence analysis, the Shiga toxin operon in *S. dysenteriae* type 1 was once part of a phage, but is now a permanent part of the bacterial genome (McDonough and Butterton 1999; Unkmeir and Schmidt 2000). The control of Stx1 production under iron-poor conditions reverts to the *E. coli* host, even though the genes reside in the prophage. This is not true for Stx2 production due to the differences in the promoters for the two operons. Stx1-expressing STEC can induce the expression of Stx1 under low-iron conditions, without inducing the Stx-phage to reproduce lytically, but they do not have the ability to secrete it. Stx2-expressing STEC have the capacity to secrete Stx2, but lack the ability to do so independently without lytic replication by the Stx-phage. Although it is possible that some STEC have the genetic potential to control the expression and secretion of Stx2, all known examples of STEC that can express Stx2 do so only by inducing the Stx2-prophage.

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Chapter 5
Significant Threats to Human Health

Abstract Shiga toxins represent a significant and evolving threat to human health. Shiga toxin-related illness is not a common foodborne illness, but it accounts for a disproportionately large share of hospitalizations and deaths. The phages that control expression of Shiga toxins can readily undergo recombination and incorporate new genetic information that can be passed on to their progeny. Because Shiga toxin-producing phages can infect bacteria other than *E. coli*, it is likely that new Shiga toxin-producing bacteria will emerge, such as the *Enterobacter* strain associated with a recent outbreak. Previous assumptions about pathogenicity have changed and will undoubtedly continue to change in the future. Based on prior research, the ability to attach and efface was considered to be essential for STEC pathogenicity, but the 2011 STEC outbreak in Germany demonstrated that a new strain (O104:H4), without attaching and effacing ability, could cause a major outbreak. New types of Shiga toxins are emerging, such as subtypes Stx2e and Stx2f that have a preference to bind Gb4. Changes in dietary patterns also influence the source and extent of outbreaks. Thus, the nature of the Shiga toxin threat is evolving on four levels: toxin structure, Stx-phages, bacterial serotypes/strains, and patterns of food production and consumption.

Keywords *Enterobacter* • Hemolytic uremia syndrome (HUS) • Thrombotic-thrombocytopenic purpura (TPP) • Shigellosis • Hemorrhagic colitis (HC) • Intimin • Attaching/effacing • Enterohemorrhagic *Escherichia coli* (EHEC) • Enteropathogenic *Escherichia coli* (EPEC) • Antibiotic induction of SOS response

5.1 Diverse Health Threats: Bacterial Species and Toxin Types, Subtypes, and Variants

Shiga toxin represents a distinct threat to human health that differs from that of other bacterial toxins. Exposure is commonly oral, and Shiga toxin relies on a bacterium to escape the harshly acid stomach and facilitate its delivery to the human intestine. As noted previously, the production of Shiga toxin by *S. dysenteriae* type 1 is under bacterial control and the stx-prophage is no longer mobile (McDonough and Butterton 1999; Unkmeir and Schmidt 2000). In contrast, the Shiga toxins

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produced by STEC and most other Shiga toxin-producing bacteria are not under the control of the host bacterium, but instead under the control of a temperate converting phage. Since these phages are mobile, they are able to convert other serotypes and strains of *E. coli* into STEC. Furthermore, these mobile phages can convert other species of bacteria into Shiga toxin-producers. Shiga toxin-related diseases are, therefore, both an ongoing and evolving threat to human health.

Shiga toxins may be produced by many species of bacteria and serotypes of *E. coli*, resulting in a great variety of potential Shiga toxin-producing pathogens. More than 400 STEC serotypes have been isolated from human patients, but only a comparatively small number of these are responsible for the foodborne, waterborne, or person-to-person outbreaks described previously. Among *Shigella*, the species *dysenteriae*, *sonnei*, *flexneri*, and *boydii* are all capable of producing Shiga toxin and generating even more severe sequelae. Other bacteria, such as *Enterobacter cloacae,* and *Citrobacter freundii,* are also capable of producing Shiga toxins. Since Shiga toxins are produced by converting phages, the ultimate limit to the development of Shiga toxin-producing bacteria is the host range of the converting Stx-phages.

The most severe worldwide Shiga toxin-related health threat is *S. dysenteriae* type 1 (Kotloff et al. 1999), often the cause of epidemics. The worldwide annual incidence of *S. dysenteriae* type 1 infections or shigellosis is estimated to be 165 million cases. Of those infected, as many as 1,100,000 die. In the industrialized world there are an estimated 1,500,000 cases of shigellosis cause by *S. dysenteriae* type 1. In some patients, shigellosis is characterized by severe diarrhea, while in others the symptoms are mild or non-existent. *S. dysenteriae* type 1 can cause HC when it colonizes the intestinal epithelium (Hale 1991; Sansonetti 1992), but occasionally it also causes HUS (Butler 2012). The role of Shiga toxin in shigellosis is important, but other bacterial virulence factors also play a significant role. Like some STEC, *S. dysenteriae* type 1 can infect a patient with an inoculum of as few as 10 bacteria. Shigellosis is mostly associated with poor hygiene and sanitation and is often transmitted by person-to-person contact. Unlike with STEC (Wong et al. 2000), treating a *S. dysenteriae* type 1 infection with antibiotics does not increase the chance of developing HUS (Bennish et al. 2006). The genes responsible for Shiga toxin production in *S. dysenteriae* type 1 are part of a defective phage and not mobile as they are in STEC.

Shigella infections also represent a significant problem in the United States, where approximately 500,000 people are infected by *Shigella* species each year. Most of these infections are cased by *S. sonnei* and *S. boydii*. In the US, shigellosis caused by *S. flexneri* and *S. dysenteriae* is sometimes encountered, but these infections are most often caused by travel to another country or person-to-person contact with someone exposed abroad. The Shiga toxins produced by some *S. sonnei* and *S. flexneri* strains appear to be under the control of a temperate converting phage (Beutin et al. 1999; Gray et al. 2014). New strains of *S. dysenteriae* type 4 that had not previously been described as producing Shiga toxins have been found to express Shiga toxins (Gupta et al. 2007), and the research suggests that converting phages

may have infected this strain. The unexpected mobility of Shiga toxin production in *Shigella* species indicates that other previously non-Shiga toxin-producing *Shigella* serotypes may have already or may in the future acquire Shiga toxin production capability.

In the developed world, with a clean water supply and functional waste water systems, the misery of Shiga toxin-related disease is most often caused by Shiga toxin-producing *E. coli*. These bacteria represent a distinct threat to human health primarily due to the mobility of the phages encoding the production of Shiga toxin and the facile spread of STEC via animal manure. Since domestic cattle are immune to the effects of Shiga toxins, STEC can grow and exchange Shiga toxin-producing genes. The myriad of different STEC serotypes that have been identified in domestic cattle is a testament to this diversity. This diversity is also reflected in the variety of STEC serotypes that have been isolated from human patients. Without Shiga toxins, these pathogens would cause mostly mild illness. With Shiga toxins, these bacteria may become highly pathogenic. Even though STEC are responsible for a minority of foodborne illness, they account for 90% of the cases of acquired childhood HUS (Keir et al. 2012) and a disproportionately large share of hospitalizations and deaths compared to the more common sources of bacterial foodborne illness. This difference is due almost entirely to the production of Shiga toxins.

5.2 Other Virulence Factors

Shiga toxin is the primary, but not the only virulence factor for STEC. It is responsible for the most severe symptoms associated with STEC infection. STEC possess other genes encoding proteins such as intimin, Paa, ToxB, Efa-1/LifA, and OmpA that cause the observed attaching and effacing (AE) lesions (Kaper et al. 2004). These proteins enable the adherence of *E. coli* O157:H7 and other serotypes to the intestinal epithelium. The AE lesion genes are also referred to as locus of enterocyte effacement (LEE) genes and are found on a large self-replicating plasmid (Perna et al. 1998). Once attached, the intimin-producing strains of *E. coli* damage the epithelium and cause enteropathogenic or enterohaemorrhagic diarrhea; they are referred to as EPEC or EHEC, respectively. Intimin is a virulence factor found in *E. coli* O157:H7 and other important serotypes (Pierard et al. 2012). As noted previously, these pathogenic genes are part of mobile elements within the bacterial genome. This means that a Shiga toxin-producing phage may convert an *E. coli* to a STEC or that other pathogenic genes may be moved to a STEC to make it more dangerous. In either case the mobility of these virulence factor genes can convert a mild pathogen into a much more serious threat.

Based on the pathology of the O157:H7 serotype, intimin was assumed to be essential for the pathogenesis of STEC. This reasoning made sense, since intimin can help the STEC attach securely to the epithelium. The proximity resulting from the attachment process was thought to be important for Shiga toxins to enter the

target cells. However, the recent STEC outbreak in Germany showed that a STEC could cause a serious outbreak, yet not possess intimin or the ability to attach and efface target endothelial cells.

The O104:H4 serotype was responsible for the 2011 outbreak in Germany, but it had not been previously associated with an outbreak. It had been only identified in two cases—in Korea and Italy (Bae et al. 2006; Scavia et al. 2011). Both of these patients suffered from HUS, but survived, albeit with long-term sequelae in the Italian case. Unlike most outbreak serotypes, the O104:H4 serotype had genes characteristic of an enteroaggregative serovar (*attA, aggR, aap, aggA, and aggC*), but not those (*eae* and *ehx*) associated with attaching and effacing (Frank et al. 2011; Pierard et al. 2012). These genes produce the aggregative adherence fimbriae which are responsible for the enteroaggregative pathogenicity. The German experience indicates that other virulence factors may prove to be more important than currently thought.

The O104:H4 serovar differed from the more common O157:H7 serovar, in that it was not found in domestic cattle. Enteroaggregative *E. coli* (EAEC or EAggEC) are not found in domestic cattle (Cassar et al. 2004; Uber et al. 2006; Veilleux and Dubreuil 2006). In contrast, O157:H7 are commonly found in domestic cattle. EAEC are often isolated from humans, but not animals, a situation that makes the source of the German outbreak perplexing (Pierard et al. 2012; Wieler et al. 2011). Although the O104:H4 strain is not the only EAEC that has been associated with outbreaks (Newton et al. 2009), it is the one responsible for the most severe outbreaks based on HUS cases. The source of the O104:H4 serovar remains disconcertedly enigmatic.

5.3 Shiga Toxin Binding Sites: Host Cell Gangliosides

A cell can suffer Shiga toxin intoxication only if it expresses a sufficient amount of specific gangliosides in an appropriate membrane substructure. Tissues that express Gb3 or Gb4 glycolipids can bind Stx and suffer consequent damage. Those tissues include kidney epithelium and endothelium, endothelial cells in intestinal lamina propria, platelets, subsets of lymphocytes, monocytes, macrophages, dendritic cells, intestinal pericryptal myofibroblasts, neurons and endothelial cells in the central nervous system (CNS) (Engedal et al. 2011; Obata et al. 2008; Ren et al. 1999). In humans the most obvious manifestation of STEC infection is the associated HC that occurs when the Shiga toxins damage the endothelial cells of the intestinal wall. The damaged intestine permits toxin to enter the blood stream, itself a target tissue, and move to other susceptible tissues, such as kidney and CNS. The most serious form of kidney damage is HUS, which occurs when damaged blood cells clog the kidney filtration system and cause severe and often life-threatening damage (Obrig and Karpman 2012). As has been mentioned, nerve cells are another target for Stx binding, and neurological symptoms were observed among patients afflicted with HUS from a STEC infection (Trachtman et al. 2012). Thus, the observed clinical symptoms are related to the binding of Shiga toxin to the known target receptors.

The binding of Stx to glycolipid receptors is absolutely necessary for the toxin to affect the cell, but the process is complex and not yet fully understood. In cellular models, the toxic effect of Stx is not observed if production of the glycolipids is disrupted by the antibiotic tunicamycin, presumably because there are no surface glycolipids to bind the toxin (Keusch et al. 1986). As noted previously, the binding by the individual sites of the B subunits is relatively weak, but binding affinity is substantially increased when multiple sites are engaged. Multi-site binding depends on the distribution of the glycolipids, the covalently attached lipids, and the amount of cholesterol in the membrane region. The complexity of multi-site binding probably accounts for much of the variation in animal host specificity and toxicity of Stx subtypes and variants.

5.4 Development of Serious Sequelae of STEC Infection

The initial symptoms of a STEC infection are indistinguishable from other, more common gastroenterological infections. Most STEC infections are limited to gastroenteritis, without more serious consequences. The more serious symptoms of the disease are associated with the expression of Shiga toxins and are characteristic of a STEC infection: (1) HC, which occurs without a fever; and (2) HUS (Melton-Celsa et al. 2012; Trachtman et al. 2012). HC occurs when the Shiga toxins kill intestinal endothelial cells, provoking bleeding in the intestine. Patients suffering from HC are often afflicted with severe abdominal cramps, but usually have no fever or only a mild fever, and most eventually recover.

As mentioned above, the intestinal damage caused by Shiga toxins may also permit the passage of those toxins from the gut through the blood stream to the kidneys. The most common serious sequela is HUS, which is characterized by three symptoms: loss of red blood cells (anemia), loss of platelets (thrombocytopenia), and acute kidney injury (AKI) (Trachtman et al. 2012). As the name "hemolytic uremic syndrome" implies, symptoms include hemolysis (lysed red blood cells) and uremia (an increase of urea in the blood), indicating that the kidneys are not functioning properly. HUS is most often found in children, but adults may also suffer from HUS or a related disease, thrombotic-thrombocytopenic purpura (TPP). TPP results from a reduction of platelets (thrombocytopenia) that leads to bleeding into tissues which produces clotted blood (thrombosis) in tissues and noticeable purple spots under the skin (purpura). Patients may recover from HUS or TPP completely or with varying degrees of permanent kidney damage. When Shiga toxins damage the blood vessels of the brain, neurological damage may occur. Although HC and HUS are the most common of the serious sequelae, neurological damage is the most devastating and (fortunately) the rarest (*vide infra*).

Both type 1 and type 2 Shiga toxins have been detected by immunoassay in the kidney tissue from patients who succumbed to HUS (Uchida et al. 1999). *S. dysenteriae* type 1 and the archetypal Shiga toxin it produces are occasionally associated with HUS (Butler 2012), yet type 2 Shiga toxins are consistently found in

STEC infections that progress to HUS (Boerlin et al. 1999; Fuller et al. 2011; Manning et al. 2008). However, only some type 2 Shiga toxins are associated with HUS. These results may reflect the fact that both types of Shiga toxins can bind to the Gb3 glycolipids on the surface of kidney cells, though their toxicities differ. Two characteristics of the A subunit of Stx2 provide an explanation for the general greater toxicity of Stx2: The A subunit of Stx2 binds more tightly to the ribosome and has a higher catalytic activity than does the A subunit of Stx1 (Basu et al. 2016).

In addition to HC and HUS, neurological problems are associated with STEC infections (Trachtman et al. 2012). The archetypal Shiga toxin was, in fact, first described as a neurotoxin. Neurons also express Gb3 glycolipids on their surface and thus provide the means for Stx to bind. Neurological problems are one of the most frequent causes of acute patient mortality (Trachtman et al. 2012) and the primary cause of sudden death in patients infected with STEC (Magnus et al. 2012; Nathanson et al. 2010). A re-examination of patients after the O104:H4 outbreak in Germany in 2011 revealed that many had suffered neurological problems. Though most patients recovered within a year, other patients continued to suffer neurological problems several years after the outbreak (Kleimann et al. 2014; Schuppner et al. 2016; Simova et al. 2014). Significant neurological sequelae were noted in only one of the two previous cases of STEC infection by the O104:H4 serotype (Bae et al. 2006; Scavia et al. 2011). It is not clear if these neurological problems are related to the specific Shiga toxin produced, the expression levels of the toxin, or some other characteristic of the STEC.

5.5 Antibiotics in Treatment of Shiga Toxin-Associated Disease

The control of Stx production also informs the use of antibiotics to treat Stx-related disease. Since the diseases are caused by bacterial infections, the conventional paradigm dictates treating them with antibiotics. This works well in the case of infection by *S. dysenteriae* type 1, for which antibiotic treatment ablates the production of Stx concomitant with killing the infecting bacteria. However, this practice can be problematic when the source of the toxins is STEC. Treatment with antibiotics can induce the SOS response and the consequent production of Stx and intact phages (Walterspiel et al. 1992), worsening patient condition and favoring disease progression to HUS (Al-Qarawi et al. 1995). Fortunately, the realization that phages control the production of Shiga toxins led to refinement of treatment strategies and more successful outcomes (Molbak et al. 2002; Thielman and Guerrant 2004; Wong et al. 2000).

Because antibiotics kill bacteria by a variety of mechanisms, it is possible that a subset of antibiotics will prove useful in treating STEC infections. In a broad study, it was found that antibiotics that interfere with DNA replication, folate metabolism, or the cell envelope increase Stx production by as much as 140-fold (Kimmitt et al. 2000). The antibiotic geldanamycin, an inhibitor of heat shock protein 90 (Hsp90) enhanced the retrograde transport of Shiga toxins, delivering them more effectively

to the cytoplasm and thereby potentiating their toxicity (Dyve Lingelem et al. 2015). Stx-inducing antibiotics include quinolones, nitrofurans, β-lactams, monobactams, and cephalosporins. Another antibiotic is polymyxin, a non-ribosomal polypeptide antibiotic that alters the outer membrane of the bacterial cell wall. Polymyxin induces over-expression of Shiga toxins under some conditions. In contrast, the non-ribosomal polypeptide antibiotic, colistin, reduces production of Stx by STEC (Percivalle et al. 2016). It apparently disrupts the bacterial cell membrane without inducing the SOS response. Metronidazole, a nucleic acid synthesis inhibitor, and fosfomycin, an inhibitor of bacterial cell wall biosynthesis, kill STEC without inducing the overexpression of Stx. As a class, antibiotics that interfere with protein synthesis do not induce Shiga toxin production (Kimmitt et al. 2000). These translation-disrupting antibiotics include gentamicin (an aminoglycoside), tetracyclines, and erythromycin (a macrolide). In summary, some antibiotics are useful in treating STEC infections, but experimental results dictate that consideration be given to the antibiotic mechanism of action, particularly whether the antibiotic triggers the SOS response (Zhang et al. 2000).

5.6 Variations in Disease Associated with Stx Type and Subtype

The only property common to all STEC is phage-mediated production of Stx; their modes of pathogenesis vary. Each Stx-producing phage is capable of producing one toxin, but more than one phage may infect a single host. Astoundingly, the sequence of one *E. coli* O157:H7 strain revealed 24 active or inactive prophages in its genome (Hayashi et al. 2001). When infected by different phages, a single serotype can produce more than one Shiga toxin (Kruger et al. 2011). Thus, a patient may be infected with a STEC strain that expresses type 1, type 2, both type 1 and type 2, or combinations of type 2 Shiga toxins. These possibilities have clinical significance since the observed toxicity of Shiga toxins is highly varied. Although both type 1 and type 2 Shiga toxins can cause HC, some subtypes of Stx2 are more frequently associated with the development of serious HUS than are other subtypes or Stx1. Thus, recent outbreak strains of *S. sonnei* infection in California produced Stx1 exclusively. Though most patients infected with it showed symptoms of HC, none developed HUS (Lamba et al. 2016).

A further complication is that the relative toxicities of Shiga toxin types and subtypes depend upon the model used to study them (Fuller et al. 2011). For example, the archetypal type 1 Shiga toxin is more toxic to Vero cells and human kidney cells, than are Stx2a, Stx2b, Stx2c, or Stx2d (Fuller et al. 2011). Other Stx1 subtypes also have a higher toxicity in the Vero cell or human kidney cell-based assay than do Stx2 subtypes. In contrast, Stx1 is less toxic than Stx2 in mice. The LD_{50} for Stx1 is approximately 400 ng, while the LD_{50} for Stx2 is 1 ng (Tesh et al. 1993). Mouse bioassay (IP inoculation) showed that Stx2d was somewhat more toxic than Stx2a, but about 100-fold more toxic than Stx2b or Stx2c (Fuller et al. 2011). Using a Vero

cell assay Stx2f was found to be 3 to 5-fold less toxic than Stx2a (Skinner et al. 2013). Stx2g is approximately tenfold more toxic than Stx2a, using the Vero cell assay (He et al. 2012). As measured by mouse bioassay, the LD_{50} of Stx2e is between 100 and 1000 ng/mouse, which makes it more toxic than Stx2c, but less toxic that Stx2a (Ran et al. 2008). Mice infected with STEC that produce Stx2, but not Stx1, succumbed to Shiga toxin poisoning (Wadolkowski et al. 1990). Baboons were similar to humans with regard to Stx2 sensitivity (Siegler et al. 2003). When inoculated (IV) with Stx2, they developed serious disease that included HUS, but they were insensitive to Stx1. These results indicate that extrapolating cell-based and even animal-based assays to human disease is problematic, since both Stx2a and Stx2c cause HUS, but have dramatically different toxicities in the mouse.

Epidemiological and experimental evidence suggests that STEC producing type 2 Shiga toxins are more commonly associated with HUS (Boerlin et al. 1999; Melton-Celsa 2014; Ostroff et al. 1989; Soborg et al. 2013). This is surprising, since, as noted previously, *S. dysenteriae* type 1 produces Stx1 and shigellosis can lead to HUS. An analysis of STEC O157:H7 outbreaks in Denmark showed that only strains producing Stx2a or Stx2c were associated with HUS (Soborg et al. 2013). Summarizing the structure-function relationships among types and subtypes of Shiga toxins, a recent review listed Stx1a, Stx2a, Stx2c, and Stx2d as associated with HUS, while Stx1c, Stx1d, Stx2b, Stx2e, Stx2f, and Stx2g were not (Melton-Celsa 2014).

STEC serotypes associated with HUS express their *stx* genes at very different levels, yet are still able to cause HUS. Even though it seems reasonable to assume that STEC expressing higher levels of Shiga toxin are responsible for the more serious sequelae associated with a STEC infection, the relationship for STEC isolated from HUS patients is unclear (Olavesen et al. 2016). These researchers determined the *stx* messenger RNA (mRNA) expression levels of serotypes infected with different Stx-phages. They found that the expression levels among the HUS-related strains were variable. Expression level did not correlate with the infecting phage, but rather, with the serogroup. Another study compared STEC serotypes that were human pathogens to those which were not pathogenic (de Sablet et al. 2008). Both sets of serotypes expressed levels of Shiga toxins that varied in a similar fashion. These results indicate that strains can produce dramatically different amounts of toxin, but still cause serious disease (Figs. 5.1 and 5.2). The results also suggest that, under experimental conditions, an apparently non-pathogenic serotype can produce Shiga toxins at a high level, similar to levels seen in human pathogens. In short, expression levels of Shiga toxins seem related to the serotype and not the infecting phage.

5.7 Inferences from PCR Data

The complexities of phage infection and host bacterial response dictate that the mere presence of an intact Stx operon is no guarantee that it will be expressed. The host bacterial cells have the means to inactivate phages, preventing lytic replication.

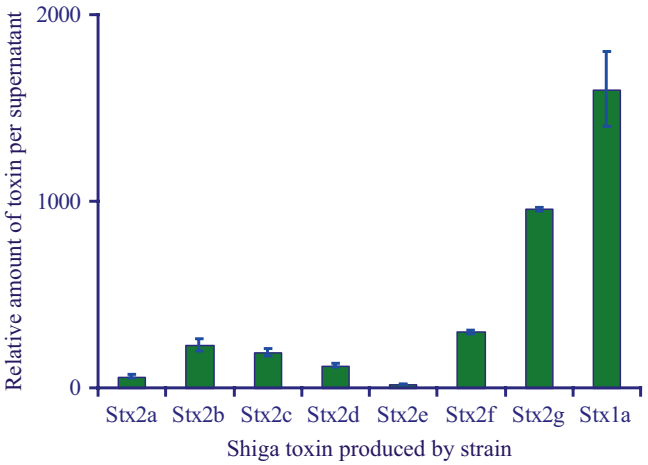

Fig. 5.1 Relative amount of Shiga toxins produced by eight different strains of STEC. The values were measured by mass spectrometry and the strains were induced under identical conditions (Silva et al. 2014)

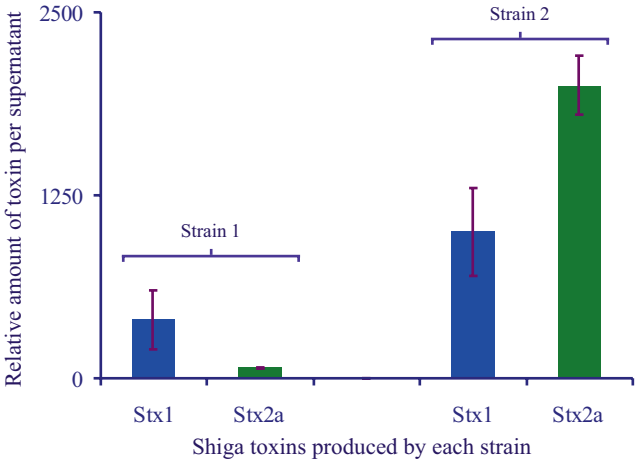

Fig. 5.2 Relative amounts of Stx1a and Stx2a produced by two different STEC strains. The values were determined by mass spectrometry and the strains were induced under identical conditions (Silva et al. 2014)

As discussed above, the expression of the Shiga toxin operon is controlled by genes expressed in the late stage of the phage lytic cycle. Therefore phages that are no longer capable of lytic reproduction are unable to express the Shiga toxins encoded in the intact operon. This assertion is supported by analysis of the STEC present in waste water from various sources (Martinez-Castillo et al. 2012). The study identified 55 strains of *E. coli* comprising 21 different serogroups that were determined to have *stx* genes and classified as STEC. Of the 55 strains, only 16 were actually able

to produce Shiga toxins under their experimental conditions, confirming that the presence of *stx* genes does not guarantee the production of Shiga toxin. On a broader level, this means that inferring Shiga toxin production from PCR data is problematic.

The pathologies associated with Shiga toxins result from the complex interactions among the toxins, the Stx-phages, and the bacterial "factories" that produce the phage-encoded toxins. *S. dysenteriae* type 1 has taken control of Shiga toxin expression and eliminated the ability of the infecting prophage to replicate. Although the stx genes of *S. dysenteriae* type 1 are no longer mobile, the bacterium may still be infected by other phages, including Stx phages. STEC produce Shiga toxin(s) under the control of the infecting phage, but the levels of that expression are influenced by the host. Unlike the situation with *S. dysenteriae* type 1, the *stx* genes remain mobile in STEC. Stx-producing phages carry the Shiga toxin virulence factor, and they can also carry other genetic factors that affect virulence. Their bacterial hosts may contain transposons (or integrons), which can move genes throughout the bacterial genome. If the phage is integrated into the bacterial chromosome, then these elements may move the transposon genes into the prophage genome. If the transposon genes insert themselves inside the Stx operon, they may disrupt the production of Shiga toxin. If the genes insert in the prophage genome without disrupting lytic replication, they can be transferred to a new bacterial host. Transposons can move a variety of genes, including those that encode antibiotic resistance and other virulence factors. In summary, a Stx-phage may acquire additional genes that encode virulence factors other than Shiga toxin and move them to new hosts.

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Chapter 6
Detection Methods for Shiga Toxins and Shiga Toxin-Producing *E. coli*

Abstract Shiga toxin detection methods reflect the complicated biological processes that deliver these toxins to their target cells. Some detection methods employ techniques that indirectly detect the presence of Shiga toxins. Microbiological methods are used to identify pathogenic serotypes of *E. coli*, such as the infamous O157:H7, but the potential production of Shiga toxins is uncertain and depends on the strain. Similarly, PCR methods detect the presence of genes that code for Stx and pathogenicity factors, from which the potential presence of Stx is inferred. Bioassays detect Shiga toxins by the biological responses of cells, tissues, or animals. The ability of some antibodies to inhibit toxin activity has been exploited to make in vitro activity tests toxin-specific. Structural assays detect a 3-dimensional shape (immunoassay) or the masses of peptides that are liberated from toxin subunit chains by a protease (mass spectrometry). Many immunoassay systems have been able to detect and distinguish among Shiga toxin types, subtypes, and variants. Some antibodies have helped to elucidate toxin mechanisms and could be the basis of toxin therapeutics. Mass spectrometry has been used for direct detection of Stx and to distinguish among the Stx types and subtypes. The Multiple Reaction Monitoring (MRM) method is reviewed in depth.

Keywords Monoclonal antibody (mAb) • Polymerase chain reaction (PCR) • Enzyme-linked immunosorbent assay (ELISA) • Mass spectrometry (MS) • Multiple reaction monitoring (MRM) • Loop-mediated isothermal amplification (LAMP) • Lateral flow immunoassay (LFIA) • Quantitative polymerase chain reaction (qPCR) • *E. coli*-selective media • Vero cells

6.1 The Context and the Use of Culture Methods, Nucleic Acid Methods, and Immunoassays for STEC

As indicated in the preceding five chapters, the increase of outbreaks and illnesses linked to Shiga toxin-producing *E. coli* has necessitated the development of effective detection methods for these pathogens and their toxins in various matrices.

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Such assays are useful for routine detection of STEC contamination in food production processes, reducing the risk of STEC outbreaks. Non-O157 STEC infections have become more common and their detection is now considered critical to food safety and epidemiology of STEC disease (FSIS 2011). Shiga toxin is a primary virulence factor for STEC and is responsible for the most severe symptoms associated with STEC infection. In this chapter, methods for STEC and Stx are discussed with reference to sensitivity and specificity, cost, ease of use, and applicability to various analytical needs.

Although *E. coli* share many biochemical characteristics with other members of *Enterobactericiae*, some metabolic pathways are nearly unique to *E. coli.* These distinct metabolic traits, such as their general inability to ferment sucrose (Morooka and Ono 1953), permit the enrichment of *E. coli* and other coliform bacteria in very diverse samples. Many serotypes of STEC have additional metabolic characteristics that can be exploited for selective culture (Ojeda et al. 1995). Cultural detection of *E. coli* is exquisitely sensitive, detecting as little as one viable bacterium, operationally defined as one colony forming unit (CFU). *E. coli* detection using differential and selective culture medium is highly versatile, applicable to almost any sample suspension. Matrices include foods such as beef, milk, and produce, irrigation water, sewage, and clinical samples such as feces from infected patients. Of course, there are obvious limitations of culture-based detection as well. Culture-based methods can only detect viable bacteria, and they are incompatible with samples with non-culturable pathogen. In these samples, detection usually targets the principal virulence factor, Stx, and is the main thrust of this chapter.

Two examples of *E. coli*-distinguishing media are MacConkey (MAC) agar, which uses bile salts to inhibit the growth of Gram-positive bacteria, and Eosin Methylene Blue (EMB) agar, with dyes that are toxic for Gram-positive noncoliforms. Both use lactose as a carbon source to favor the growth of *E. coli,* which are strong fermenters of lactose. The fermentation acidifies the medium, giving *E. coli* colonies a distinctive pinkish red precipitate (MAC) or green sheen (EMB). Sorbitol-MacConkey agar (SMAC), with sorbitol replacing lactose, exploits a unique biochemical characteristic of the O157:H7 serotype. O157 STEC are generally unable to ferment sorbitol and are easily distinguishable on SMAC plates from the harmless *E. coli* found in the natural flora. However, the ability of other STEC serotypes to ferment sorbitol is inconsistent, so SMAC plates are not as effective for their detection (Karch and Bielaszewska 2001).

A number of different formulations have recently emerged from the EMB and MAC prototypes, several as proprietary commercial products. These new formulations vary in their selectivity and specificity. Tryptone bile X-glucuronide (TBX) plates tend to be more permissive and allow growth among most STEC strains. They depend on the β-glucuronidase produced by most strains of *E. coli* to convert the chromogenic X-glucuronide, resulting in blue colonies of *E. coli*. Ironically, a subset of O157:H7 does not express β-glucuronidase, making TBX media less valuable for detection of this important serotype (Yang et al. 2004). CT-SMAC is another formulation of SMAC for enhanced selectivity for STEC. It incorporates the antibiotic ceftidine, effective against most non-STEC *E. coli*, and potassium tellurite. Rainbow®

Agar O157, which contains indicators for β-glucuronidase and β-galactosidase in addition to various selective agents, can identify both β-glucuronidase-positive and -negative O157:H7 and can be supplemented with ceftidine, potassium tellurite, and novobiocin for enhanced selectivity. SMAC, TBX, and Rainbow® Agar O157 (without supplements) are very permissive, and most STEC isolates can be grown on these media (Gill et al. 2014; Verhaegen et al. 2015). However, they may present a high background of other bacteria and may not inhibit the swarming *Proteus*, which can quickly overwhelm a culture plate. When supplements (ceftidine, tellurite, and novobiocin) are added, however, some isolates of STEC will not grow. This is true for other supplemented media, such as CHROMagar™ STEC and CHROMagar™ EHEC, as well. Even with supplements, some genera of *Enterobactericiae* (*Klebsiella*, *Hafnia*, or *Citrobacter*) cannot be distinguished from STEC. It therefore behooves the analyst to use both permissive and selective media to ensure that every STEC isolate can grow and that the abundance of background flora is manageable.

Recently, new strategies to detect and serotype STEC by culture have been developed. Serotyping STEC by culture in the absence of serotype-specific antibodies was made possible by using characteristic light scattering patterns and pattern recognition software. Although the colonies for O157:H7 and each of the "Big Six" non-O157s (O26, O45, O103, O111, O121, and O145) look morphologically identical (with the exception of non-sorbitol-fermenting O157:H7) on SMAC or Rainbow® agar, the diffraction pattern for each of these serotypes was distinctive and presumably dependent upon the LPS/O antigen, shape, and other factors (Tang et al. 2014; Windham et al. 2013). This technology adds utility to culture-based detection of STEC, and permits rapid serotyping without having to resort to PCR or immunological methods.

All in all, though culture-based methods are intrinsically sensitive and can be valuable for STEC detection and isolation, confirmation by another method (PCR, immunoassay) is highly recommended. By the very nature of their components, all these culture methods suffer from either high selectively (preventing the growth of some STEC strains) or low selectivity (increasing the background flora to untenable levels). None of these culture methods rely on the one definitive characteristic of STEC, the production of Stx.

In 1983 Kary Mullis invented an extraordinary tool for research in molecular biology (Mullis 1990), the Polymerase Chain Reaction (PCR) technology that has revolutionized diagnostic detection (Casadevall and Fang 2016; Sloots et al. 2015). Once the sequence of genes encoding the primary virulence factors of STEC (*stx* and *eae*) were known, researchers rapidly developed STEC-specific PCR assays. The first *stx*-detecting PCR assay was described in 1989 (Karch and Meyer 1989), capable of both Stx1 and Stx2 detection (known as Shiga-like toxin (SLT)-I and SLT-II at the time). The first PCR for distinction of Stx1 and Stx2 utilized a primer set for each of the A and B subunit genes (Pollard et al. 1990).

STEC-specific PCR assays are some of the most sensitive assays for STEC and are favored by the US Department of Agriculture's Food Safety and Inspection Service (USDA-FSIS) as a front-line strategy to detect both O157:H7 and non-O157 STEC (Fratamico et al. 2014). This sensitivity is particularly important in

complex food matrices in which quantitative PCR (qPCR) is capable of detecting just 10 CFU of STEC per reaction or 25 g of spiked produce (Wang et al. 2014). Combined with immunomagnetic separation (IMS), large volumes of some sample matrices can be tested for contamination without enrichment (Feldsine et al. 2016). There is a considerable variety of assay designs, and the DNA target can be various fragments of the *stx* genes, the *eae* locus, and portions of various virulence plasmids. The BAX O157:H7 and STEC qPCR methods are currently (as of 2016) used by the USDA-FSIS to detect *eae* and *stx* in enriched samples. However, the BAX STEC PCR may be incapable of detecting some subtypes of Stx, including Stx1d, Stx2b, and Stx2f (Margot et al. 2013). Other qPCR assays, such as the Mericon VTEC stx1/2, have a wider specificity, but often still fail to detect Stx2f. Although unusual Stx subtypes like Stx1d, Stx2b, Stx2f, and Stx2g have not been commonly associated with severe clinical manifestations so far, they could become more problematic if and when they become associated with more host-adapted strains (Skinner et al. 2013b). The ABI Custom TaqMan VT1/VT2 qPCR assay was found to detect all known subtypes of Stx1 and Stx2, with the possible exception of Stx1e (Margot et al. 2013).

Quantitative PCR, while extremely sensitive and specific, does require sophisticated and expensive equipment to operate (a thermocycler and fluorescent reader), making it impractical for field use or by untrained personnel. Simpler nucleic acid amplification techniques have been developed, such as the *l*oop-mediated isothermal *amp*lification (LAMP). LAMP can be conducted within a water bath maintained at about 65 °C (Wang et al. 2012). Magnesium pyrophosphate is the insoluble byproduct of the amplification and can be measured with a turbidimeter or estimated visually or, in a variation, colorimetrically (Goto et al. 2009). LAMP can also be more rapid than PCR, with comparable sensitivity. LAMP assays have been developed against the major virulence factors of STEC: *stx1*, *stx2*, and *eae* (Wang et al. 2014) and could serve as cheap and simpler alternatives to qPCR.

Although STEC requires expression of *stx* gene(s) to reach its maximum virulence, even the mere presence of the *stx* gene(s) is a cause for considerable concern. QPCR, LAMP, and analogous assays can readily detect *stx* genes and other virulence factors in a wide variety of matrices. Nevertheless, nucleic acid detection methods for STEC are subject to limitations, as are culture-based detection methods. Matrices and samples can contain toxin but no pathogen DNA or, like serum, may not be compatible with nucleic acid-based detection of STEC. However, these methods do have one enormous advantage over all other detection methods: they are capable of detecting the Stx-converting phage, something that STEC detection by culture, bioassay, and immunoassay cannot currently achieve (see discussion in Chap. 4). Stx-converting phages make an as yet unknown contribution to STEC pathogenicity in humans, but they are certainly involved in the virulent transformation of STEC strains and warrant further study (Tozzoli et al. 2014). With the remarkable advances in speed, efficiency, and economy of whole genome sequencing (WGS), this technique is likely to overtake the use of multi-tiered qPCR and confirmatory assays in the near future (Chattaway et al. 2016; Lindsey et al. 2016).

Culture, bioassay, and nucleic acid techniques all have utility in the detection of STEC, but one of the most critical features of these pathogens is their expression of Stx. Antibody-based detection assays, or immunoassays, detect the Stx protein directly and specifically. They are often rapid and sensitive and require modest equipment to operate. Kits based on enzyme-linked immunosorbent assay (ELISA) and lateral flow devices (LFDs, also based on immunosorbent technology), are entirely self-contained, making it exceptionally convenient and simple to operate. This class of assays tends to be rapid, requiring less than 4 h to operate, and some can be conducted in a few minutes. Although immunosorbent methods are generally less sensitive than qPCR, they are highly effective on stool samples and enriched samples. Immunoassays are often combined with qPCR for mutual confirmation, ensuring that suspected STEC samples are properly evaluated. Details of Stx immunoassay are in Sects. 6.3–6.6.

6.2 Detection of Shiga Toxins by Bioassay: Animals, Cells, and Receptors

One of the earliest assessments of Shiga toxin lethality was done by live animal bioassay (Van Heyningen and Gladstone 1953). For ethical reasons, contemporary animal use protocols, when possible, define an endpoint other than death per se. When animals are moribund, animals are euthanized to mitigate suffering. Typical bioassays are conducted using groups of five to ten mice for each of four or five dose levels per experiment. Randomly grouped mice are inoculated by the route of toxicity under investigation (for example, IP or IG by gavage). Animals are monitored for 7 days for signs of intoxication, a moribund state, or death. LD_{50} values are calculated from several individual experiments, using accepted statistical methods, such as that of Reed and Muench (1938) and/or Weil (1952). An alternative measure of toxicity useful for highly potent toxins is the minimum lethal dose (MLD). This is typically determined with two mice per dose level, with MLD defined as the minimum dose that kills both mice (Schantz and Kautter 1978). Statistical significance is determined by unpaired t-tests.

Reviews of animal studies of STEC-caused pathogenesis have been published using data from several species, from rodents to primates (Melton-Celsa and O'Brien 2003). The development of renal injury and HUS in mouse models was discussed extensively above and reviewed by Mohawk & O'Brien (2011). From a food safety point of view, study of oral toxicity is especially important, and several animal studies have elucidated the pathogenesis resulting from enteric exposure to Stx. In the absence of STEC and other pathogenicity factors in a mouse model, Rasooly et al. (2010) estimated that about 1% of toxin administered IG is absorbed in an active state into the circulation and that Stx is pathogenic in the absence of STEC.

Animal bioassay is also used to assess potential clinical interventions, including the provision of active or passive protective immunity. Several studies have established the ability of a monoclonal antibody (mAb) or a mixture of mAbs to protect

mice from Stx-induced pathogenesis (Cheng et al. 2013; Melton-Celsa et al. 2015; Smith et al. 2009). Russo et al. reported that the LD_{50} for Stx2a IG in BALB/c mice is 2.9 μg, about 1000-fold higher than the IP LD_{50} (Russo et al. 2014). Mice were protected from oral Stx2a intoxication by passive immunity using a mAb that binds the Stx2a A-subunit. In studies of antibody protection, the statistical significance of antibody effects can be quantified by using log-rank tests of the survival curves (Bland and Altman 2004).

Vero cells were found to be highly sensitive to filtered culture media from multiple clinical *E. coli* isolates, in a way that was distinct from the effects of both *E. coli* exotoxins that had already been well characterized (Fig. 6.1) (Konowalchuk et al. 1977). After a 48- to 96-h incubation with Stx-containing media, most of the affected Vero cells detached from the plate and were found dead in the culture medium. Some spindle-shaped debris persisted on the culture surface (Fig. 6.1). Stx cytotoxicity was readily observable by microscopy, but difficult to quantify. Still, these early experiments paved the way for the development of more sophisticated, quantitative bioassays.

HeLa cells were used in a bioassay for Stx from *Shigella* quantitatively for the first time in 1980 (Gentry and Dalrymple 1980). In this prototype assay, the dye, crystal violet, was applied to fixed, Stx-treated cells, and absorbance was measured at 595 nm. Although this method was quantitative, the sensitivity of HeLa cells to Stx is considerably lower than that of Vero cells (He et al. 2012). The median cytotoxic dose (CD_{50}) for this bioassay was a 10^7-fold dilution of culture medium. Standardization of detection assays became practical when purified Stx became available. Using Stx purified from *Shigella*, the Takeda laboratory performed mouse, rabbit ileal loop, and Vero cell bioassays (Yutsudo et al. 1986). They determined the LD_{50} for the mouse (28 ng), the

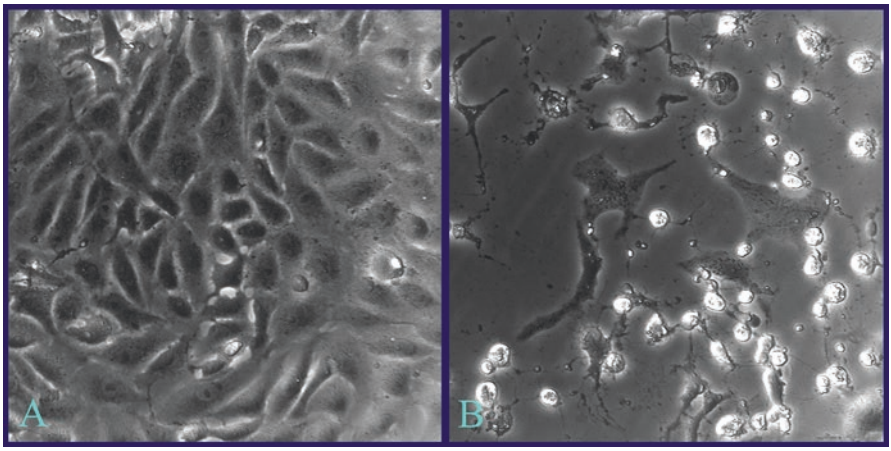

Fig. 6.1 Images of untreated Vero cells (**A**) or Vero cells treated with Stx2a (**B**). Vero cells were plated, exposed to either phosphate-buffered saline (PBS) or 1 ng/mL of Stx2a for 1 h, and then grown overnight. Panel (**A**) shows the confluent growth of untreated cells. Panel (**B**) shows the remnants of dead cells after exposure to Shiga toxin

minimal detectable dose for the rabbit ileal loop assay (1.25 μg for significant fluid accumulation), and the CD_{50} for Vero cells, a remarkable 1 pg/100 μL. Even at this early stage of Stx bioassay development, the Vero cell assay was very sensitive, though time-consuming and nonspecific. Assays lasted 48–72 h, and Vero cells were sensitive to a number of other cytotoxins (Gupta and Siber 1996; Mahony et al. 1989). Due to the high homology of Stx from *Shigella* and the Stx1 of STEC, these results allow comparison to later data on Stx1 from STEC.

Engineered cell lines have made quantitative Stx bioassays faster and more sensitive. Luciferase-expressing Vero cells (Zhao and Haslam 2005) and cells that express d2EGFP, a destabilized, enhanced green fluorescent protein (Quinones et al. 2009) were designed to estimate Stx concentration based on the inhibition of reporter protein expression by Stx. Using a microscope or a plate reader to evaluate the cultures, the d2EGFP-Vero cells enabled detection of Stx in 16–18 h, with an LOD of 1 pg/mL.

Cellular Stx-detecting bioassays have great utility in the laboratory due to their sensitivity, versatility, and ability to detect bioactive toxin. Because they require cell culture they are currently unsuitable for use in the field or by untrained individuals. However, portable GFP readout is becoming easier and cheaper. For example, a charge-coupled device (CCD) for excitation and imaging of the fluorophore has been described (Rasooly et al. 2015). Cell-free translation (CFT) assays for Stx enable analysis of RIPs such as Stx by determining translated luciferase activity using luminescence (Hale 2001; He et al. 2009). Because the toxin does not have to enter a cell in this assay, the activity of isolated A chains, as well as intact toxin can be measured. If needed, specificity for an individual toxin or toxin subtype is conferred on the assay by measuring activity with and without an antibody that specifically inhibits the activity of the target toxin. Because they sense the primary toxic effect of Shiga toxins, bioassays based on inhibition of ribosomal activity remain the "gold standard" for Stx detection.

Receptor binding assays for Stx generally measure binding to Gb3 or Gb4 receptor mimics, with an antibody detection step. This "receptor/ELISA" or RELISA was found to be 100% specific for Stx1 (Basta et al. 1989), with sensitivity comparable to the Vero cell assay (5 pg/100 μL sample). A similar approach was used by Togashi et al. (2015) to develop an assay for Stx2e based on its binding to Gb4 receptors, with linear detection only to 20 ng/mL. The glycolipid binding preferences of Stx variants were analyzed by Karve and Weiss (2014), and receptor binding has been shown to correlate with lethality for at least one unusual subtype, Stx2d (Bunger et al. 2013). It may be anticipated that receptor-binding assays have a future for measurements of biologically active Stx variants.

6.3 Structure-Based Assays for Stx: General Considerations and ELISAs Using Polyclonal Antibodies

Both polyclonal antibodies (pAbs) and mAbs (Fig. 6.2) have been produced against Stx and used in cross-neutralization studies, epitope-mapping experiments, and the development of rapid diagnostic tests for the presence of Stx or STEC in clinical

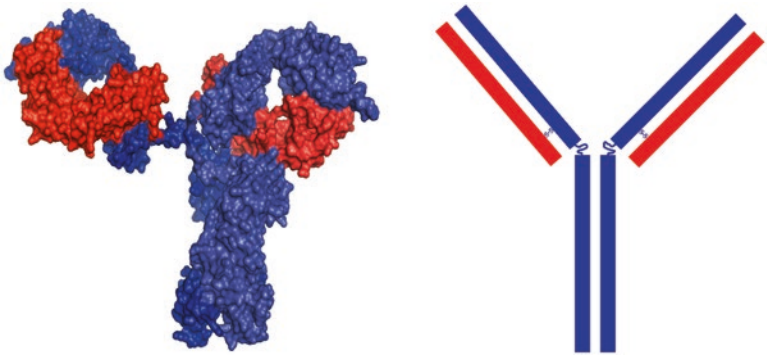

Fig. 6.2 Crystal structure surface image (*left*) and schematic representation (*right*) of an IgG molecule. The two heavy chains are colored *blue*; the two light chains, *red*. The tetrameric IgG has two binding sites for antigens such as the A or B subunit of Shiga toxin. The structure is a PyMOL rendering of the 1HZH structure from the PDB database (Saphire et al. 2001)

Table 6.1 Representative immunoassay systems for Stx

Method	Format	Typical readout
ELISA	Multiwell	Colorimetric/luminescent
LFA	Disposable cartridge	Colorimetric/visual
Immuno-PCR	Multiwell	Cycle threshold (C_T)
Bead array	Instrumental system	Fluorescent
Magnetic bead array	Instrumental system	Fluorescent
Solid array	"Printing" on glass slide	Colorimetric

and food samples. In view of the importance of toxin subtype to pathogenicity (Orth et al. 2007), robust assays with exquisite specificity are needed to provide optimal protection of the food supply. Table 6.1 lists representative immunoassay systems that will be discussed. Mass spectrometry provides structural determination of molecules based on molecular mass and also provides methodology for analysis of Shiga toxins in complex samples.

Detection of expressed toxin is critical for both clinical and environmental diagnostics. Antibody-based Stx detection assays, or immunoassays, detect the Stx protein directly and specifically. Some formats are both rapid and sensitive and require modest equipment to operate. Although immunosorbent methods are generally less sensitive than qPCR because of lower amplification factors, they are highly effective on stool samples and enriched samples. Immunoassays are often combined with qPCR for mutual confirmation, ensuring that suspected STEC samples are properly evaluated. Array-based methods generally require sophisticated instrumentation, but often deliver a much higher degree of multiplexing.

Matrix effects must be considered in all analytical schemes. The evaluation of STEC in environmental samples, including foods, is difficult because of the high number of non-target bacteria and the low number of pathogens needed to cause the illness. Routine pasteurization of milk is sufficient to kill STEC, but not sufficient

to inactivate Stx that may have accumulated or may have been intentionally added to raw milk prior to processing (Rasooly and Do 2010). ELISAs for food safety applications are compatible with many food matrices, including beef, milk, and produce (Brandon and Carter 2012). They are also useful for quantitating Stx in clinical samples such as serum and filtrate that lack STEC bacterial DNA. The ability to detect Stx in serum could be valuable for clinical diagnosis, as well as for elucidating how Stx toxification leads to HUS and neurological damage. Stx2 detection by immunoassay in mouse serum samples is routine and sensitive, but Stx2 detection in human serum is complicated by the human serum amyloid P protein (HuSAP), which binds Stx2 with high affinity (Marcato et al. 2003) and could limit the access of antibodies to Stx2 epitopes.

ELISA (Fig. 6.3) has been an increasingly widely used detection method for proteins and other biomolecules over the past 50 years. Its ease of use and versatility, requiring only one specific antibody in the simplest format, makes it ideal for commercial assay development as well. ELISAs can be specific or general (sometimes called generic), and exceptionally sensitive, depending on the format and antibody or antibodies used. ELISAs can be multiplexed to target a number of different antigens, for example multiple toxins and additional pathogenicity factors associated with STEC.

The first ELISA designed for Stx detection was developed in 1987 using a pAb prepared in rabbits to "capture" the toxin and the same pAb, conjugated to alkaline

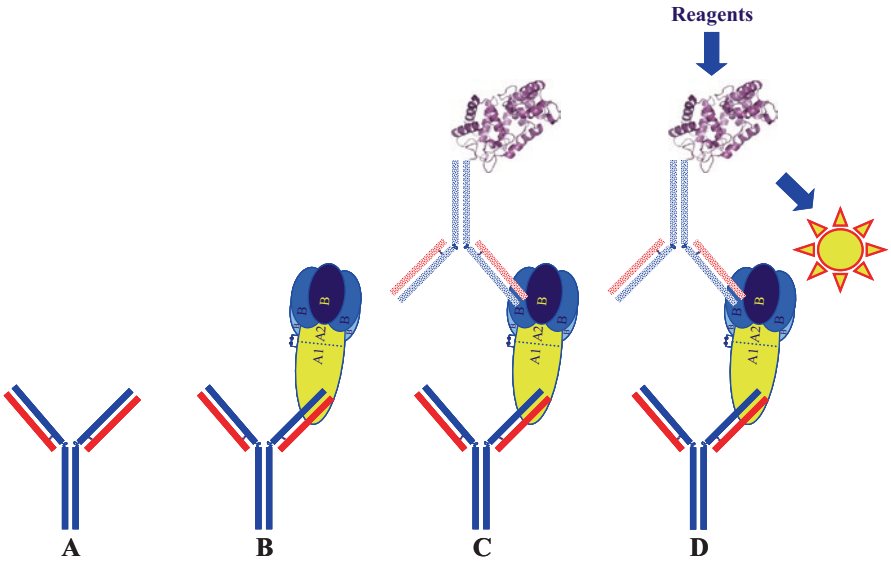

Fig. 6.3 Principal steps in sandwich ELISA. (**A**) "Capture antibody" (e.g., monoclonal IgG anti-A subunit of Stx) is coated on the surface of wells of a microplate. (**B**) Coated Ab captures Stx present in the applied sample. (**C**) "Detection antibody" (e.g., monoclonal IgG anti-B subunit of Stx) conjugated to a reporter enzyme such as horseradish peroxidase binds to another antigenic site of the Stx molecule. (**D**) Substrate is added to generate a signal, usually color, fluorescence, or luminescence

phosphatase, to detect it (Kongmuang et al. 1987). The immunogen was purified Stx1 that had been formalinized for 1 week. The ELISA boasted a detection limit of a few ng/mL and a dynamic range of 10–1000 ng/mL. Although much less sensitive than the Vero cell assay (1 pg/mL), the assay offered considerable benefits. Like with countless ELISAs developed subsequently, there was no need to grow cells in an incubator or maintain sterility, and the reagents were stable for months. Although these polyclonal antibodies were not characterized for their fine specificity, they neutralized the cytotoxic activity (presumably that of Stx2) of the three strains of *E. coli* O157:H7 tested. This result is surprising given that pAbs generated with a Stx1 immunogen generally do not cross-react with Stx2, and vice versa (Strockbine et al. 1986).

Another milestone in the evolution of pAb-based ELISA for Stx is demonstrated by a method developed using a recombinant Stx toxoid, Stx2 (E167Q), as the immunogen (He et al. 2013b). Genetic toxoids had been used as a vaccine (Liu et al. 2009), but the use of this recombinant molecule as an immunogen led to a major advance in analytical technology for Stx. The toxoid replaces glutamic acid with glutamine at position 167 (He et al. 2013a), a conserved active-site residue among Shiga and ricin toxin families (Hovde et al. 1988), thereby eliminating Stx2 activity. The polyclonal antibody obtained binds specifically to the A subunit of Stx2 and is capable of neutralizing Stx2 toxicity in a cellular assay. The strategy proved successful for detecting Stx2 in all 36 STEC strains tested that were confirmed to be *stx2*-positive by qPCR. The pAb-based ELISA was the first reported immunoassay able to detect all seven subtypes of Stx2 in bacterial cultural supernatants. The assay used a chemiluminescent substrate and offered sensitivity of about 100 pg/mL, determined with Stx2a in enrichment broth used to detect STEC in samples of soil or feces.

Other pAb-based methods for Stx2 have used IgY antibodies prepared from immunized hens. IgY antibodies were demonstrated to neutralize Stx2 (Arimitsu et al. 2014; Feng et al. 2013; Parma et al. 2011) and to provide detection in ELISA (Brandon and Korn 2016). Single-chain camelid antibodies and single-domain engineered polypeptides derived from the camelid antibody repertoire have been prepared and tested for protective effects in model systems in vitro or in vivo (Lo et al. 2014; Mejias et al. 2016; Tremblay et al. 2013). The utility and potential advantageous use of non-traditional antibodies and binders for detection remains to be fully developed.

6.4 Monoclonal Antibodies for Stx Detection, Inactivation, and Protection

ELISAs based on mAbs have often been more sensitive and specific than pAb-based methods (Perera et al. 1988). Moreover, antibodies recognizing the B subunit can neutralize Stx, providing Vero cell assays with a valuable confirmatory tool (Arimitsu et al. 2015). The development of a wide variety of mAbs and pAbs to Stx facilitated

the commercialization of Stx detection ELISAs. The Premier EHEC (Meridian Bioscience Inc., Cincinnati, OH, USA), Ridascreen Verotoxin (r-Biopharm AG, Damrstadt, Germany), ProSpecT STEC Microplate Assay (Remel Inc., Lenexa, KS, USA), and VTEC Screen Seiken RPLA (Denka Seiken Co., Tokyo, Japan) are among the most common ELISA-based commercial Stx detection kits available. The Premier EHEC is offered in a 96-well plate format and is a highly sensitive assay, with a limit of detection of 7 pg/mL for Stx1 and 15 pg/mL for Stx2. The ProspecT STEC assay has been estimated to have a comparable sensitivity to that of the Premier EHEC (Willford et al. 2009), but the Ridascreen Verotoxin assay is approximately tenfold less sensitive (Willford et al. 2009). These three assays are all colorimetric and can be read visually or using a spectrophotometer. They have similar development times (around 90 min), and are approved for use with stool or enriched broth cultures (Willford et al. 2009). The VTEC Screen Seiken RPLA assay requires a much longer incubation time with samples (overnight) and its limit of detection is about 25 pg/mL for Stx1 or Stx2 (Beutin et al. 2002). As a latex agglutination assay, it is typically read visually, so Stx concentration may be difficult to measure accurately. Although not well documented as yet, these immunoassays are probably compatible with more diverse samples such as ground beef or fresh produce. Both ELISA and qPCR (despite extraordinary sensitivity) generally require enrichment, but ELISA typically has a lower false-positive rate than qPCR.

Monoclonal Abs have been produced against Shiga toxins and used in neutralization studies, epitope-mapping experiments, and the development of rapid diagnostic tests for the presence of Stx or STEC in clinical and food samples (Gupta et al. 2010; He et al. 2009, 2011, 2013a, 2016; Jeong et al. 2010; Smith et al. 2006, 2009). An example of sandwich ELISA using commercial mAbs, with the enhanced amplification of PCR, immuno-PCR (iPCR), was effectively used to detect Stx2 in environmental samples (He et al. 2011). The identification of STECs based on Stx in culture supernatants gave 100% agreement with the results of qPCR and culture methods. He et al. 2013a used the genetic toxoid mentioned above to develop mAbs for Stx2 and a second genetic toxoid (Stx1E167Q) to develop mAbs that bind Stx1 B-subunit (Skinner et al. 2014). All three mAbs had toxin-neutralizing activity in the Vero cell assay and two were used to develop a sandwich ELISA with a LOD of 8.7 pg/mL. Hybrid Stx1/2 molecules are produced by some STEC and were detected with these mAbs.

6.5 Monoclonal Antibodies for Differentiating Stx Subtypes

With so many subtypes of both Stx1 and Stx2, obtaining broad specificity by ELISA can be a problem, especially for monoclonal antibodies. Although all commercial Stx ELISA kits can detect Stx1a and Stx2a, no kits currently available are capable of detecting all Stx subtypes. However, other Stx subtypes are increasingly being reported, making detection of diverse subtypes important (Delannoy et al. 2015; Prager et al. 2009). Moreover, Stx subtype appears to be important in the

pathogenicity and host range of STEC. Therefore, robust assays with well defined specificity are needed to provide optimal diagnostics for environmental and clinical applications. For example, Stx2f is a subtype with newly recognized relevance to foodborne infection. This subtype is heat- and acid-stable and a strong binder to model receptors. Stx2f has been increasingly identified in clinical samples from patients infected with STEC. A new purification scheme for Stx2f permitted its structure and toxicity to be characterized (Skinner et al. 2013a). Four new mAbs and a sandwich ELISA with a LOD of 120 pg/mL were developed (Skinner et al. 2013b). A general scheme for purifying and characterizing Stx2 variants was based on initial purification of toxins by immunoaffinity chromatography (He et al. 2012). This work demonstrated the importance of using purified, well characterized Stx preparations and detection reagents. In addition, the results showed that cytotoxicity did not correlate with enzymatic activity when different variants were compared. For example, Stx2g was less active than three other variants in inhibiting protein synthesis in rabbit reticulocyte lysates, but more active in the HeLa cell cytotoxicity assay. How these subtype characteristics relate to human clinical toxicity and pathogenicity remains to be elucidated. New mAbs for Stx2e were developed (Skinner et al. 2015) and the resulting ELISA developed with these antibodies produced the lowest LOD yet reported for this subtype (12 pg/mL) (Fig. 6.4). The new Stx2 subtype-specific mAbs were recently commercialized and the Stx2 ELISA kit was evaluated (Kong et al. 2016).

Stx1 detection has become a higher priority in recent years, as *S. dysenteriae,* which produces this toxin, is a re-emerging pathogen in the developed world. Stx1 is generally less important in human foodborne infections than are STEC, but it causes infections that can be lethal. New anti-Stx1 mAbs (Skinner et al. 2014) were used to develop a sandwich ELISA with a LOD of 8.7 pg/mL. As cited above (Skinner et al. 2014) identified Stx1/Stx2 hybrid toxins in STEC culture media using new anti-Stx1 mAbs. This observation opens new aspects of STEC disease to study, and we anticipate that new, highly specific antibodies will help elucidate the role of hybrid toxins in pathogenesis. Recently, a new Stx1 subtype, Stx1e, was identified from a clinical sample containing an atypical host, *Enterobacter cloacae.* Stx1e is not detectable by most Stx assays. Using a new mAb developed for Stx1e and an anti-Stx1 pAb, an ELISA was reported with an LOD of 4.8 pg/mL (Skinner et al. 2016). The mAbs reported by Skinner et al. (2014, 2016) were incorporated into a commercial ELISA kit and evaluated (Kong et al. 2016). The LOD was reported as 25 pg/mL.

6.6 Special-Purpose Immunoassay Methods: Amplification, Portability, and Arrays

Amplified and ultra-sensitive immunoassay formats are powerful adjuncts to conventional ELISA. A “traditional” approach includes the use of tertiary antibodies in addition to standard secondary antibodies such as labeled rabbit anti-mouse IgG. A significant problem is that the background signal is often increased as well, and the

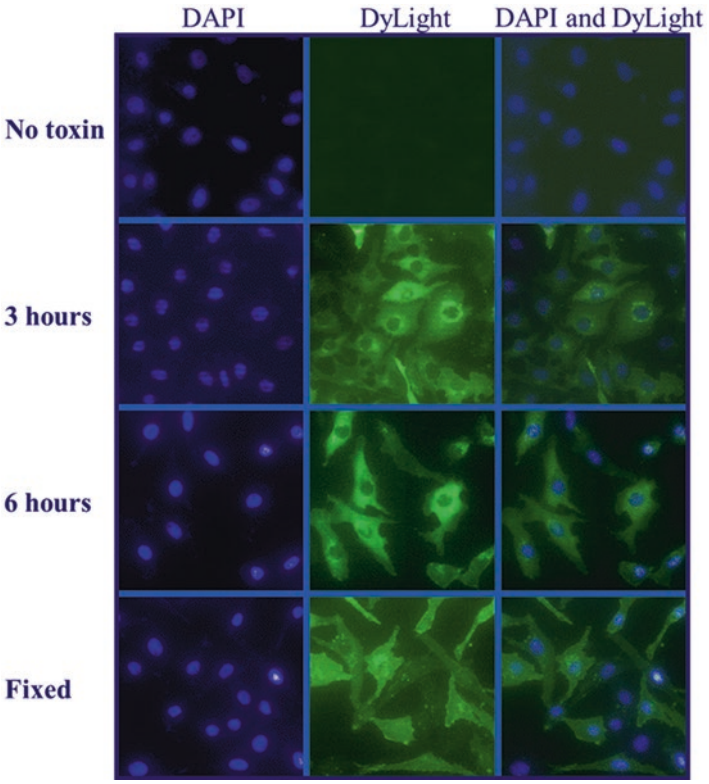

Fig. 6.4 Visualizing internalized Shiga toxin in Vero cells. 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) was used to visualize cell nuclei and intact DNA. MAb to Shiga toxin A subunit (Stx2e-3; anti-A subunit) was used to identify Stx with a DyLight reporter. Conditions are no toxin, toxin after 3 h, and toxin after 6 h without fixation or after fixation (Skinner et al. 2015). The digital combination of the DAPI and DyLight images are shown in the third column

overall signal-to-noise ratio may not be improved. It should be noted that conventional ELISAs as well as amplified formats often take advantage of the high sensitivity and low background achievable with state-of-the-art chemiluminescent substrates available from commercial suppliers. One amplified format, immuno-PCR, already cited above, exploits the huge amplification factor available via PCR (He and Patfield 2015). Highly sensitive ELISAs and related iPCR formats were developed with the new anti-Stx2 mAbs, with LODs of 1 pg/mL in milk for ELISA with chemiluminescent detection and 10 fg/mL for the iPCR format, an increase of 100-fold over ELISA (He et al. 2013a).

Useful portable assays fulfill one or both of the following objectives: (1) Requirement for only minimal end-user training; and (2) Suitability for use in the field in non-laboratory settings. Portable assay technologies will enable sophisticated analyses of important biomolecules such as Shiga toxins throughout the food production stream and in mobile health applications. For example, in food safety applications, portable tests could identify potential sites of STEC contamination and

augment current testing procedures for adulteration by STEC, currently performed in regional laboratories.

Lateral flow assays (LFAs) are the prototypical portable immunoassay systems, sometimes implemented as a dipstick, but more commonly as a cartridge referred to as a lateral flow device (LFD). Methodology for this format has recently been summarized (Ching 2015) and simple, inexpensive, disposable LFDs for Stx detection in contaminated foods have been reported (Ching et al. 2015). Using two mAbs that bind epitopes common to Stx1 and six Stx2 subtypes (He et al. 2013a), the lateral flow assay produces a visible red line on the test strip in under 10 min for samples containing 100 pg/mL Stx2a. LFDs for both Stx1 and 2 and a second device for STEC detection were reported by Wang et al. (2016). The sensitivity of the LFDs is commonly 10- to 100-fold lower than comparable ELISA, but speed is greater, with assay development from sample application consuming about 30 min vs. 1–3 h. Reduced sensitivity can be accepted for some screening purposes. Stx concentrations in contaminated foods can exceed 300 ng per mL or g in model systems under ideal bacterial growth conditions (Weeratna and Doyle 1991).

An example of detection of Stx using "cell phone technology" or mobile health (mHealth) technology was reported (Rasooly et al. 2015). The prototype system is essentially a Vero cell assay, providing data based on inhibition of translation of green fluorescent protein (GFP) in a transduced Vero cell. It is important to note that this is a measurement of active toxin, the parameter most generally relevant to food and clinical diagnostics. The fluorescence is read using an inexpensive CCD camera, rather than an analytical fluorometer. The system showed an excellent negative linear relationship between Stx2 concentration and fluorescence intensity ($R^2 = 0.85$). It remains to be determined how to make the cell culture system sufficiently robust for use outside the laboratory, but sensitivity in the 0.1 pg/mL range in buffer was achievable. Portable detectors have an enormous potential for providing onsite foodborne toxin diagnostics and point-of-care tests, and low-cost systems have special relevance for the underdeveloped world.

Gehring et al. (2014) published results from a high-throughput planar array system based on a precipitating colorimetric sandwich ELISA for Stx. This system detected Stx1 and/or Stx2 in cultures of STEC following treatment with antibiotic and/or a cell-disrupting, protein extraction reagent. The prototype system utilized an inexpensive flatbed scanner to provide quantitative measurements. As expected for ELISA, test results from artificially contaminated foods could be obtained within 2 h via the formation of a colored product in positive assays. A second-generation implementation of this system used array printing of antibody and fluorescence detection to detect both STEC and Stx in about 75 min (Gehring et al. 2015).

A microbead-based immunoassay for simultaneous detection of Stx1 and Stx2 and isolation of *E. coli* O157 in foods was developed (Clotilde et al. 2011). This magnetic fluorescent microbead format (Luminex® 100, Luminex Corp., Austin, TX) was 1000-fold more sensitive than sandwich ELISA using the same commercial antibodies. The method is capable of simultaneously detecting O157 serotypes directly and other STEC via detection of Stx in foods such as ground beef, lettuce, and milk spiked at levels as low as 2 CFU/g. The test provides results in less than 24 h and

could be used in food processing plants to allow in-house testing of products prior to sale. A related study reported proof-of-principle for the use of an automated ELISA workstation (Dynex Technologies, Chantilly, VA) to analyze toxin and STEC (Clotilde et al. 2012). In this format, assay cost and sample size are reduced by performing all tests simultaneously on a plastic microbead, on which antibodies are passively absorbed. Up to ten bead-based ELISA-like assays could be performed in each well of 96-well plates, with chemiluminescent detection. Assay specificity is determined by the antibody coated onto the bead. For example, like traditional ELISA, this system could be used for 10-plex assays of Stx subtypes.

In addition to their use in ELISA-like applications, bead arrays are applicable to the analysis of PCR products. Specific probe sequences can be conjugated to uniquely colored fluorescent beads and analyzed using Luminex xMAP® technology. Multiple analytes can be analyzed in a single (multiplex) reaction. Lin et al. (2011) used this system to detect genes encoding the ten most important STEC O-serotypes. Importantly, this system has the capability of high-throughput, ideal for analysis of multiple environmental or clinical samples. The same technology was used for a 7-plex microbead-based immunoassay for seven STEC O-serotypes (Clotilde et al. 2013). Both microbead techniques were applied to the analysis of 161 STEC isolates from cattle feces (Clotilde et al. 2015). With this powerful, multiplexed technology, the researchers determined the serotypes of 11 isolates that had not been typeable using conventional antisera.

6.7 Mass Spectrometric Methods for Detecting Shiga Toxins

Mass spectrometry has been used in various ways to analyze Shiga toxins: for detection of Stx and other toxins in aerosols (Alam et al. 2012); to study the stability of the holotoxin (Conrady et al. 2010; Kitova et al. 2005, 2009); to analyze the binding of the sugars to toxin (Kitova et al. 2001, 2007); and to detect and discriminate among Shiga toxins (Fagerquist and Sultan 2010, 2011; Fagerquist and Zaragoza 2015; Kondo et al. 1997, 2003; Meisen et al. 2005). These methods employ a "top-down" approach, where the entire protein is analyzed. A more sensitive and specific approach we describe in depth uses the multiple reaction monitoring method (MRM) (Fig. 6.5).

MRM is a well established method of detecting and quantitating proteins that does not require intact protein for instrumental analysis. Instead, MRM relies on the detection of characteristic peptides derived from enzymatic digestion (Domon and Aebersold 2006; Pan et al. 2009; Picotti and Aebersold 2012). A sample of the target protein is first reduced to cleave its disulfide bonds, and the free thiols are then alkylated to prevent the reformation of the disulfide bonds. A protease, commonly trypsin, is used to digest the reduced and alkylated protein to produce a characteristic set of peptides that are unique for the analyte protein. These peptides are analyzed by MS to select ones with chromatographic properties (e.g. peak shape and retention time) and signal intensities appropriate for a MRM analysis. Instrument parameters are

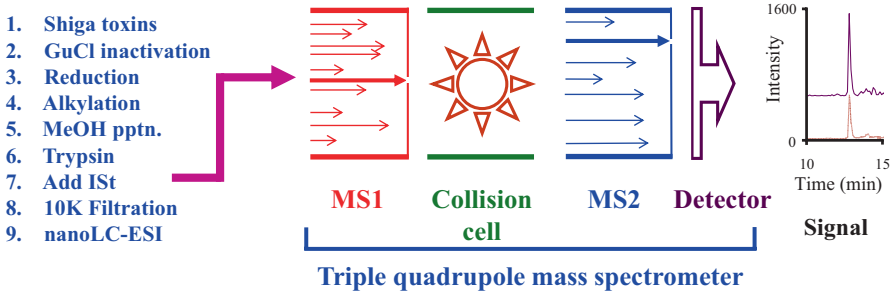

Fig. 6.5 Schematic diagram of the Multiple Reaction Monitoring method. Shiga toxin-containing samples are prepared for analysis (steps 2–8) to yield a complex mixture of peptides that is chromatographed using a nanoLC-electrospray ionization (ESI) system. As peptides elute from the column, they are analyzed by the triple quadrupole mass spectrometer. Peptides of a preprogrammed mass pass through the first quadrupole (MS1) and are fragmented in the collision cell (second quadrupole). The fragments next enter the third quadrupole (MS2), where only those with a preprogrammed mass pass through and register a signal. An internal standard (ISt, ^{15}N-labeled analyte peptide, *pink trace*) is used to confirm that the identified peak (*purple trace*) has the previously specified physico-chemical properties (chromatographic retention time, mass, fragmentation, etc.). Since a known, fixed amount of ISt is added, the absolute amount of analyte peptide can be calculated (Silva et al. 2014, 2015)

then optimized separately for each peptide. The analyte peptide producing the most intense signal is generally selected as the target peptide, and other peptides can be used for confirmation. The presence and identity of the analyte protein is confirmed by the presence of multiple peptides from the set of characteristic peptides. This is accomplished without introducing intact toxin into the spectrometer and typically allows the detection and quantification of peptides in the attomole (10^{-18} mole) range. Since the MRM method is based on the analysis of peptides and not intact toxins, the scientists and technicians analyzing the samples are not exposed to the toxins. This is a safe and effective means of detecting Shiga toxins (Fig. 6.6).

Shiga toxin subunit stoichiometry makes MRM an especially appealing approach for detecting Stx (Silva et al. 2014). Briefly overviewed, the B subunits bind the target cell gangliosides, but the toxic enzyme activity of the holotoxin resides in the A subunit. Because there are five B subunits per holotoxin, five peptides are derived from B subunits for each one from the A subunit. An analysis based on the B subunits thus affords an inherent fivefold increase in sensitivity relative to an analysis based on the sole A subunit. Furthermore, the analyte peptides (*vide supra*) are located in conserved regions of the B subunits (Fig. 6.7).

Like many other MS methodologies, MRM can employ an internal standard (ISt) to identify and quantitate the analyte peptides. A suitable ^{15}N-labeled ISt is prepared by cloning and overexpressing a B subunit or a synthetic gene containing the relevant peptides in minimal medium supplemented with $^{15}NH_4Cl$ (Silva et al. 2015). Furthermore, the MRM method can be easily adapted to detect new Shiga toxin variants, as they are identified. This methodology has been used to safely detect and discriminate among the Shiga toxin types in the low attomole range in complex media without having to purify the toxin (Silva et al. 2014).

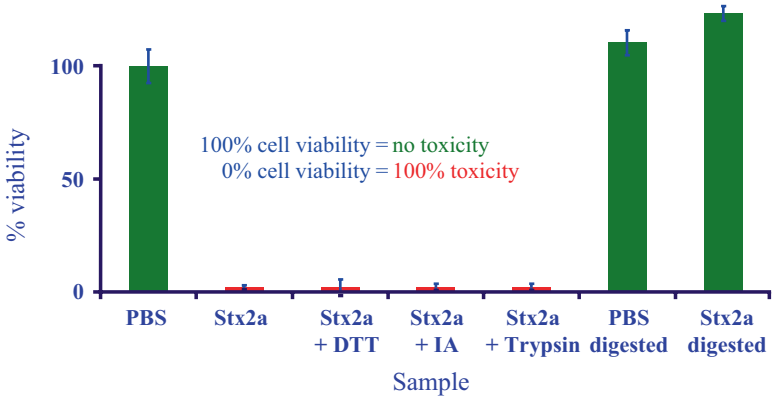

Fig. 6.6 Graphical representation of a Vero cell assay of samples prepared for a mass spectrometry-based analysis. 100% cell viability corresponds to no toxicity; 0% cell viability, to 100% toxicity. Samples consist of PBS alone; Stx2a reduced with dithiothreitol (DTT) alone; Stx2a treated with iodoacetamide (IA) alone; Stx2a digested with trypsin alone; PBS with added trypsin; or Stx2a sequentially treated with DTT and IA, and then digested with trypsin

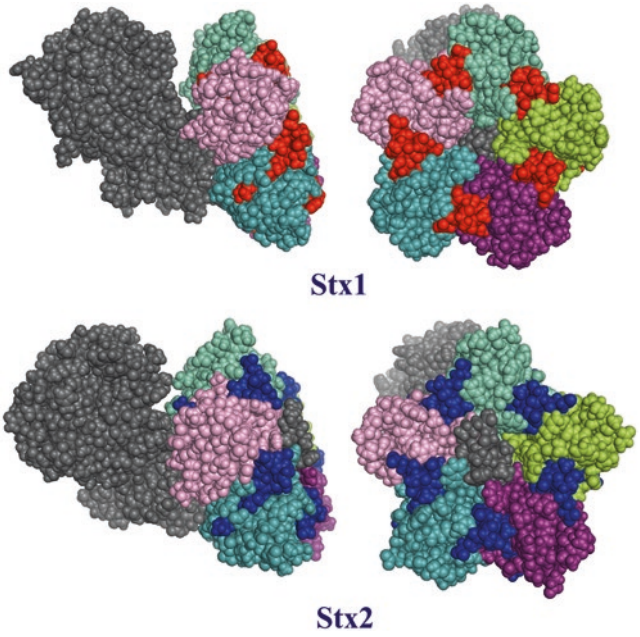

Fig. 6.7 Location of analyte peptides (Fig. 3.4) in Stx1 and Stx2. Analyte peptide for Stx1 is indicated in *red*; for Stx2, *blue*. Figures are derived from a PyMOL script based on the RCSB PDB structures 1DM0 and 4M1U, respectively (Fraser et al. 1994; Jacobson et al. 2014)

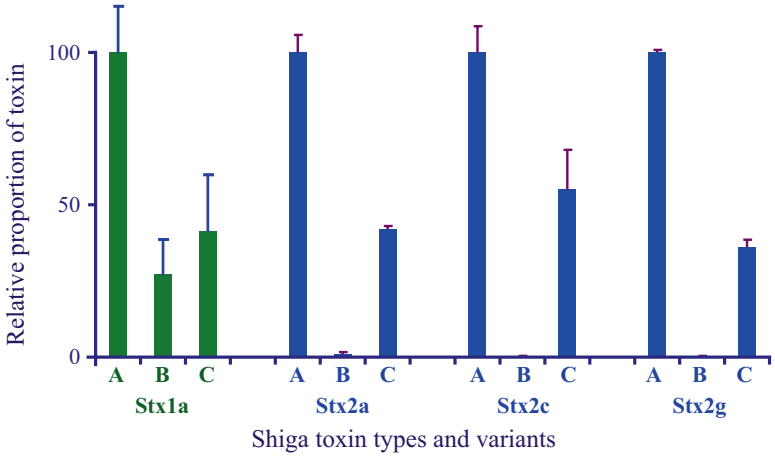

Fig. 6.8 Relative amounts of Stx1a, Stx2a, Stx2c, or Stx2g detected in bacterial supernatant (*A*), bacterial supernatant spiked into human serum (*B*), or bacterial supernatant spiked into human serum and then processed with guanidinium chloride, (GuCl) (*C*). Each set of three samples for each toxin is normalized to the amount of toxin in the bacterial supernatant spike (*A*). The values were determined by mass spectrometry (Silva et al. 2015)

As noted above, HuSAP, a common component of normal human serum, interferes with the binding of Stx2 to the gangliosides on the target cell surface. Therefore, Stx2 may be present in a human serum sample, but unable to bind detection antibodies or receptors in a bioassay system. The binding of HuSAP also interferes with MS analysis. Unlike Stx2, Stx1 is readily detectable in human serum because it does not bind to HuSAP (Fig. 6.8). However, Stx2 is associated with more serious human disease than Stx1, and the inability to detect it can be a significant problem.

Fortunately, the MRM procedures for detecting Stx in complex media can be adapted to detecting Shiga toxins in human serum, by using GuCl, a strong chaotrope that denatures proteins. The denatured proteins are then susceptible to the reduction/alkylation/trypsin cleavage used in the MRM method. Precipitation of the protein by methanol removes the GuCl, and trypsin digestion can then be conducted to produce the analyte peptides (Fig. 6.8). This approach also allows a sample to be concentrated prior to analysis. With a larger sample volume, there is an increase in sensitivity. In addition, ultrafiltration, with retention of the Stx2/HuSAP complex for subsequent treatment with GuCl and analysis, can be used to further increase the sensitivity of this method. The modified MRM method has been used for safe and rapid detection of all types of Stx in human serum in the low attomole range (Silva et al. 2015).

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Chapter 7
Conclusions and a Glimpse into the Future

Abstract Shiga toxins are a significant, continuing threat to human health due to their intrinsic toxicity, prevalence in the environment, and continuing evolution. Their structures and binding preferences are mutable by recombination in the lambdoid phages that produce them. The toxins are delivered by several bacterial species, comprising many strains and serotypes. Advances in understanding the basic biology of Stx production has led researchers to new methods of inactivating or thwarting these toxins. Animal vaccines to reduce STEC populations in food animals have been developed and approved, and similar strategies for the human population are actively being pursued.

A variety of approaches have been taken to interfere with the binding of Shiga toxins to their ganglioside receptors on target cell surfaces, including antibody-based approaches. Lead compounds have been developed that interfere with the RNA-N-glycosidase activity of Shiga toxins or disrupt the intracellular trafficking of the toxins. Antibiotics are not generally recommended for the treatment of Stx-related infections, but new antibiotics are actively being sought to kill the pathogenic bacteria without inducing the production of Stx. Understanding the basic science of Stx intoxication suggests many opportunities for more effective control of Shiga toxin-producing bacteria and treatment of infections.

Keywords RNA-N-glycosidase inhibitors • Anti-tumor drugs • Shiga toxin vaccines • Anti-*Escherichia coli* O157:H7 vaccines • Bacteriophage therapy • Probiotics • Toxin-blocking drugs • Eculizumab • Biocides • Fosfomycin

7.1 Exploiting Shiga Toxins for Beneficial Uses

Shiga toxins are effective at killing cells that express Gb3 on their cell surfaces. A variety of cancer cells overexpress Gb3, including astrocytoma tumors and centrofollicular cells (Devenica et al. 2011; Engedal et al. 2011). Because of their specific binding to Gb3 and Gb4 gangliosides, Shiga toxins may be used for specific targeting and killing of cancer cells that express these gangliosides.

Stx B subunits bind to Gb3 and/or Gb4 gangliosides, setting off a series of membrane events that transport the toxin into the cell. Appropriate modification of the

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Shiga toxin structure may allow a hybrid molecule to deliver therapeutic molecules to the kidney or to other Shiga toxin-binding cells. Researchers have used this approach to bring immunogens to the immune system for developing an anti-tumor immune response (Vingert et al. 2006) and to selectively label tumor cells (Viel et al. 2008). When the hybrid molecule, a toxin-chromophore conjugate, was injected into mice, the chromophore moiety was slowly secreted by kidney cells but the toxin remained in the tumor cells. In this way the binding properties of the Shiga toxin B subunit could possibly be exploited to deliver molecules that heal damaged cells. The long and complex journey of Stx in its human victims provides opportunities for therapeutic "hijacking" of the toxic mechanism.

Shiga toxin's travel from the cell surface to its cytosol affords scientists a unique probe to establish the steps involved in this journey. Shiga toxin can be rendered non-toxic by replacing a single specific glutamic acid with a glutamine (Gordon et al. 1992). This means that researchers can work with a protein that is identical to Shiga toxin in all ways except toxicity. Shiga toxins provide scientists with a valuable tool to elucidate the binding of protein toxins to glycolipid receptors at the atomic level. The toxins can be used to study the lipid-protein interactions needed for endosome formation, sorting, and movement. Shiga toxins are xenoproteomic, i.e., not derived from the host genome, and possess distinct amino acid sequences and enzymatic activities that provide researchers with unique tools to study intracellular protein transport. Shiga toxin may prove to be a helpful tool for deciphering complicated cellular trafficking, with applications in toxinology (the biology of toxins) and molecular medicine.

7.2 Future Strategies to Treat and Prevent Shiga Toxin-Related Disease

Currently there is no antidote for Shiga toxin poisoning, but researchers are trying a variety of approaches to prevent or treat Stx intoxication. Various strategies have been developed to vaccinate domestic cattle against *E. coli* O157:H7 and prevent its transmission to food. Parallel research seeks to develop human vaccines against STEC and Shiga toxins. The following biologicals and chemotherapeutics hold promise for future treatment and/or prevention of Stx-associated disease: (1) Vaccines against the host bacteria; (2) Lytic (non-temperate) phages to kill the host bacteria; (3) Antibiotics that can kill STEC without inducing an SOS response; (4) Vaccines against the Shiga toxin; (5) Stx-binding small molecules or antibodies that will prevent the toxin from binding to target cell surfaces; (6) Small molecules that interfere with trafficking of Shiga toxin once it has entered the cell; (7) Small molecules to inhibit the enzymatic activity of the A1 domain, preventing ribosomal inhibition.

Effective vaccines have been developed for use against *E. coli* in domestic cattle. The vaccine against O157:H7 targets two surface components of the

bacterium, the siderophore receptor and the porin protein, and was effective in reducing the shedding of O157:H7, but not other *E. coli* serotypes (Cernicchiaro et al. 2014; Paddock et al. 2014). Other potential vaccine targets are the Stx B subunits. They are efficiently "presented" by antigen-presenting cells of the immune system via the major histocompatibility complex and elicit strong humoral responses (Haicheur et al. 2000; Lee et al. 1998; Noakes et al. 1999; Vingert et al. 2006). The O, H, and other surface antigens of STEC are potentially good targets for vaccine development. Both experimental and epidemiological data indicate that *E. coli* surface antigens meet the prerequisites for vaccine development. For example, about 14% of children living on farms have antibodies to O157:H7 and this is associated with less severe bouts of diarrhea (Belongia et al. 2003). Mouse and other antibodies to a number of toxins, including Shiga toxin, have been developed, with an eye towards therapeutic use (Chow and Casadevall 2012). For example, not only is the Stx 1 and 2 B subunit fusion protein immunogenic in mice, but it elicits protective antibodies against O157:H7 infection (Gao et al. 2011; Gao et al. 2009).

Non-temperate bacteriophages can be exploited to kill various pathogens, including O157:H7 (Hudson et al. 2010; Mahony et al. 2011; Sulakvelidze 2013). Found in cow manure, these phages have been naturally selected for their ability to kill O157:H7 and other strains that infect domestic cattle (Viazis et al. 2011). The phages lack the ability to produce Shiga toxins and lytically replicate in the bacterial host, reducing the bacterial population. This approach is appealing because bacteriophages have a narrow, very specific host range. In experimental model systems, some lytic phages have been shown to reduce the population of *E. coli* O157:H7 on meat and produce (Hong et al. 2014; Hudson et al. 2013), while other phages are effective on experimentally infected cantaloupes and lettuce (Sharma et al. 2009) and surface-treated tomatoes and spinach (Abuladze et al. 2008). Relevant to contamination of ground beef, phages added to the wash water used on cattle prior to slaughter successfully reduced the amount of O157:H7 on the animals. Among the hurdles that the phage approach must overcome are both technical and regulatory concerns. Unfortunately the O157:H7 strain, and presumably others, can develop resistance to phage infection and limit this approach (O'Flynn et al. 2004). Experimental work has been conducted under a temporary exemption from EPA to allow field testing outside the food production channel (EPA 2011). Additives, especially biologics, that may find their way into the food supply are a perennial safety concern and often a "hot-button" political issue. Nonetheless, the power of lytic phage reproduction seems likely to provide a successful, practical strategy to control STEC in the food animal population and at other interfaces between humans and pathogenic bacteria.

As discussed in Sect. 5.5, antibiotics have been used to treat Stx-related diseases, particularly severe cases of *Shigella dysenteriae* infection (Christopher et al. 2010), but are not generally recommended for treatment of STEC infection. The main challenge is to avoid induction of the bacterial SOS response (*vide supra*) (Freedman

et al. 2016) that may even lead to the development of HUS. Aside from the clinical use of antibiotics, growth-promoting or subtherapeutic levels of antibiotics in food animal husbandry introduce the additional indirect risk of inducing phage reproduction. This, in turn, increases the chance that Stx-phages will infect other *E. coli* and create new STEC strains (Kim et al. 2016). On the positive side, there is a continuing research effort to develop antibiotics such as the polyketide azithromycin, that seem not to induce phage reproduction as they kill the host bacterium. Also of concern is the development and spread of antibiotic resistance. In the context of Stx-related disease, integrons are able to transmit antibiotic resistance to phages and could derail any antibiotic-based approach to STEC reduction. However, another positive to note is that some antibiotics provide alternative mechanisms for treating STEC infection. As recently reported, polymyxin impairs interaction between human neutrophils and Shiga toxins (Carnicelli et al. 2016). Finally, novel combinations of treatment regimes, such as immuno- and antibiotic therapy (Skinner et al. 2015) may enable clinicians to avoid triggering an SOS from STEC. In summary, antibiotics may be used to treat some Stx-related bacterial infections in food animals and humans, but their use depends on the bacterium, the ability of the antibiotic to induce the SOS response in that bacterium, and other risk factors.

Researchers have explored a number of approaches to interfere with the binding of Stx to target gangliosides (Nishikawa 2011). Bacteria engineered to express Gb3-like molecules on their surfaces are potential toxin-blocking agents (Paton et al. 2000). When these bacteria are formalin-fixed and fed to mice, the mice are protected from the effects of Stx intoxication (Paton et al. 2001). *Lactobacillus casei* has been shown to reduce the colonization by O157:H7 in a rabbit model, resulting in lower Stx levels (Ogawa et al. 2001). Single-domain antibodies (nanobodies) derived from llamas have been used to prevent Stx2e from binding to target cells (Fig. 7.1) (Lo et al. 2014). Phage display libraries have been used to identify peptides that inhibit the binding to Shiga toxins to their ganglioside receptors (Bernedo-Navarro and Yano 2016). The synthetic ligands “Starfish“ and “Daisy“ were developed based on Gb3 sugars and have been shown to inhibit the binding of Stx to target cells (Figs. 7.1 and 7.2) (Kitov et al. 2000; Mulvey et al. 2003). Other synthetic ligands, a series of dendrimers bearing globotriaose ends (“SUPER TWIG”), were effective in neutralizing Shiga toxin in animal models (Matsuoka et al. 2006). A tetravalent peptide ligand has proven to be successful in treating STEC-infected mice (Nishikawa et al. 2006). The peptide binds the B subunit of Shiga toxin and then diverts its retrograde transport to an acidic compartment for degradation. Ingestion of chitosan conjugates of Gb3 and Gb4 sugars rescued mice infected with a strain of *E. coli* O157:H7 (Li et al. 2012). These intriguing approaches have yet to yield an approved treatment for Shiga toxin poisoning. The transition from in vitro to in vivo use is the first hurdle. For example, analogs of Gb3 sugars attached to resins were able to bind Shiga toxins in vitro (Armstrong et al. 1991), but were ineffective in clinical trials to treat diarrhea-associated HUS in children (Trachtman et al. 2003).

Researchers have developed high throughput cell-based assays to screen for small molecules that can rescue cells from Stx poisoning (Saenz et al. 2007). Rescue could involve interference with one or more steps in the development of intoxication. One

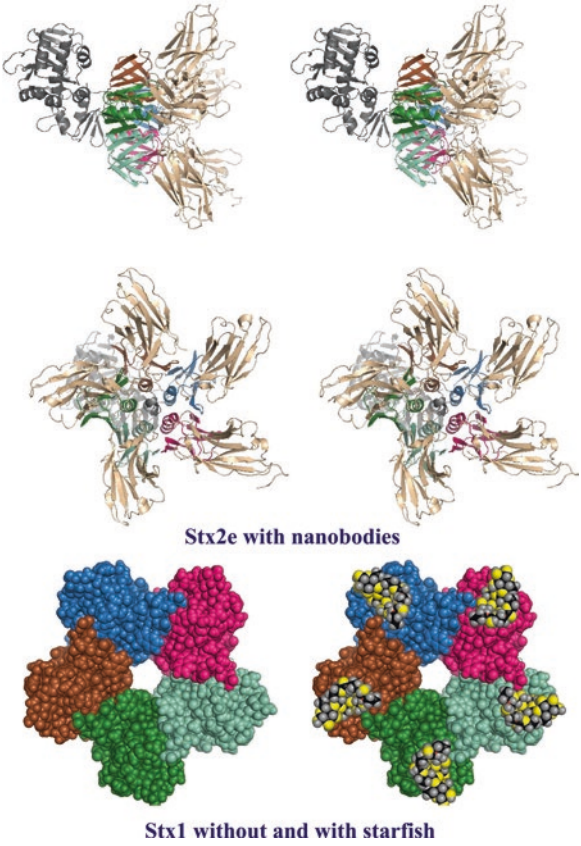

Fig. 7.1 Interactions between the Stx2e B subunits and nanobody inactivators or Starfish sugars. *Upper panel*, interaction between single-domain antibodies (nanobodies; beige) and Stx2e. The 3D image is rendered in PyMOL from RCSB PDB structure 4P2C (Lo et al. 2014). *Lower panel*, spherical image, based on the crystal structure of the B subunits of Stx1 or the B subunits of Stx1 bound to the sugar residues (*black*, *yellow*, and *grey spheres*) of the Starfish ligand. The images are derived from the RCSB PDB structure 1QNU and rendered in PyMOL (Kitov et al. 2000)

example is inhibition of enzymatic activity essential for toxicity. Synthetic small molecule inhibitors of the RNA-N-glycosidase activity common to both Shiga toxin and ricin have been identified (Wahome et al. 2010; Wahome et al. 2011). An earlier step in Stx toxicity is intracellular trafficking. Manganese has been shown to inhibit trafficking of Shiga toxins and has been used to rescue STEC-infected mice (Mukhopadhyay and Linstedt 2012). The small-molecule drugs, Retro-1, Retro-2, and Golgicide A (Fig. 7.3), interfere with retrograde transport (Noel et al. 2013; Park et al. 2012; Saenz et al. 2009; Stechmann et al. 2010). Effective in thwarting ricin toxicity in mice, molecules similar to Retro-2 may be useful in treating Stx poisoning after the toxin has entered its target cell (Secher et al. 2015). High-throughput screening of large libraries of small molecule drug candidates enables identification of the structural features responsible for desired pharmacological activities. This process can then be iterated to guide medicinal chemists as they prepare libraries of analogous compounds for evaluation as potential therapeutic agents.

Most cases of HUS are referred to as “typical” and are caused by Shiga toxin poisoning. Activation of complement, a set of blood plasma proteins involved in innate immunity, occurs in typical HUS (Keir and Langman 2016). However, a

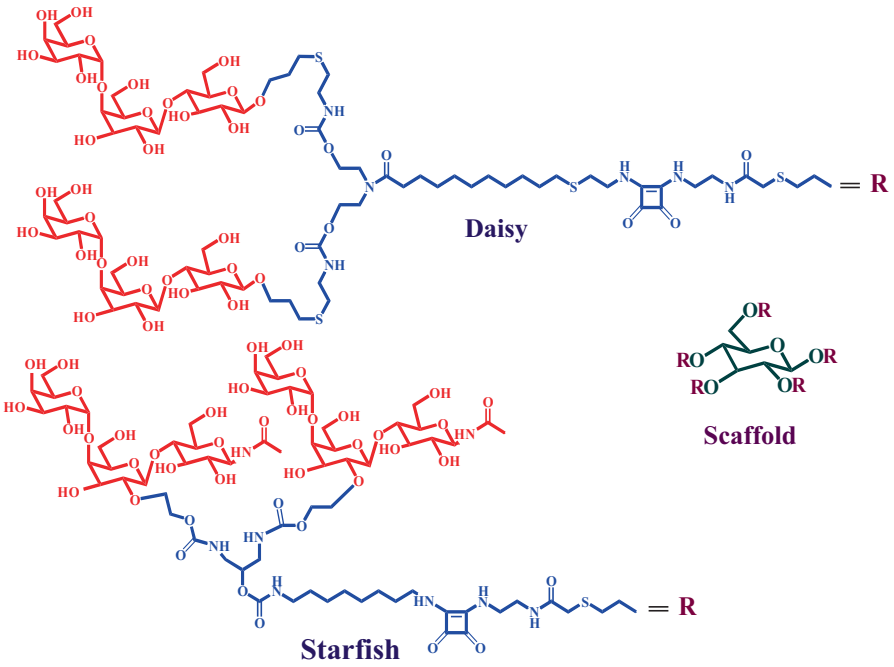

Fig. 7.2 Structures of "Daisy" and "Starfish," two Stx- binding small molecules. Sugar residues are shown in *red*. Each of the R groups is bound to the scaffold

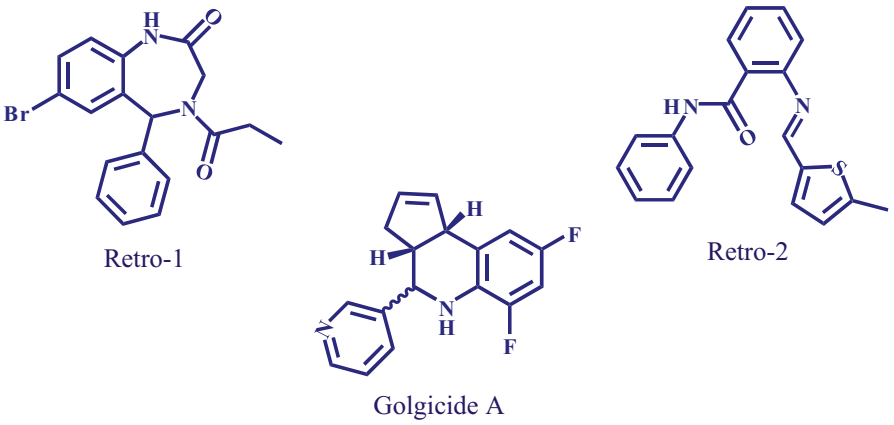

Fig. 7.3 Structures of Retro-1, Retro-2, and Golgicide A

heritable defect in an innate immune response results in "atypical HUS" (Kavanagh and Goodship 2011), in which red blood cells are lysed by defective complement. A therapeutic humanized antibody (Eculizumab) impedes complement and has been successfully used to treat patients suffering from atypical HUS. The same antibody has been used to treat typical HUS caused by Shiga toxin poisoning, with

mixed success. Although ineffective in adults (Kielstein et al. 2012), Eculizumab produced more promising results when children were treated soon after onset of neurological symptoms (Lapeyraque et al. 2011; Pape et al. 2015). However, another small study of children yielded equivocal results (Gitiaux et al. 2013). These studies reveal the potential utility of compounds that interfere with the complement cascade for treating HUS.

There are reasons to be optimistic about the future for prevention and treatment of Shiga toxin-caused disease. Basic research has provided us with a clearer understanding of how the toxins exert their effects on cells. There are a number of methods under development to minimize the entry of STEC into the food supply. These methods include vaccination of food animals and use of biocides, including bacteriophages, to kill STEC. Intense concern by the public, regulatory oversight by government, and action by industry and producer groups have resulted in heightened awareness by those who handle and process our foods and development of action plans based on HACCP principles (Hazard Analysis and Critical Control Points, acronym pronounced "Hah-sip") to minimize Stx-related problems. Anti-phage compounds may be developed to prevent phage induction in host animals and human patients. In spite of the likelihood of future STEC outbreaks, one expects that more effective treatments will soon become available.

7.3 Conclusions

Unlike other toxins, Shiga toxins are the end product of two interacting conveyances (host bacteria and toxin-expressing phages) that together deliver a toxin to an animal's intestine. Basic research has shown that the *E. coli* bacterial host associated with a particular Stx is an efficient means of delivering a Stx-producing phage to a vulnerable location. The properties of the *E. coli* strain influence its efficiency in both infecting a host and protecting the phage that it carries. Shiga toxins are unstable in the acidic environment of the human stomach, but when encoded in a "pop-up toxin factory" (Stx phage) they readily pass through the human stomach. The phage largely controls its own induction and the levels of Stx produced. Once in the intestine, Shiga toxin is protected by its very structure from the action of trypsin. As formidable as it appears, this secure conveyance system affords researchers multiple points of intervention to deny the delivery of active Shiga toxins to their ribosomal site of action.

As can be seen in the diversity of emerging STEC serotypes and strains, the expanding host range of Stx-phages is changing the nature of STEC infections. After the deadly outbreak of *E. coli* O157:H7 in Seattle in 1993, the primary regulatory focus was on the O157:H7 serotype. Indeed, up to 2008 most STEC outbreaks were associated with the O157:H7 serotype. Between 2008 and 2014, the estimated STEC outbreak serotypes were mostly non-O157 serotypes. The transition in serotypes may be due, in part, to more practiced surveillance regimes, but it also reflects the mobility of the phages that control the production of Shiga toxins. The future will undoubtedly see more outbreaks occurring as the Shiga toxin-producing

phages adapt to new *E. coli* serotypes and new species of bacteria. Shiga toxin production is not restricted to *E. coli* and can be found in *Enterobacter cloacae* (Paton and Paton 1996; Probert et al. 2014). These strains can even produce novel variants of Shiga toxin (Probert et al. 2014). The moniker "Shiga toxin-producing *E. coli*" has become overly restrictive and should be recast to reflect this new reality. "Shiga Toxin-producing Enteric Colonizers," would reflect the broadening host range of these phages, but retain the familiar and pronounceable acronym.

The genetics of the lambdoid Stx-phages will undoubtedly influence future outbreaks. The genetic sequence of phages isolated from STEC already show evidence of significant recombination, as well as integron-based modification. The consequences of this recombination are complicated and poorly understood. However, it is reasonable to assume that these genetic changes will result in phages that have additional selective advantages, such as antimicrobial resistance and enhanced host range. In any event, recombination provides phages with greater genetic diversity and, as a consequence, a greater ability to respond to natural selection. Most of these changes can be passed on to their progeny.

The structure of Stx is paramount in its toxicity and is subject to modification. The A1 domain of Stx1 is much more catalytically active than the corresponding domain of Stx2, and Stx1 holotoxin is more stable than Stx2. In spite of these apparent "molecular advantages," Stx1 is generally considered to be much less toxic than Stx2. It is the structure of the B subunits that determines the cells to which the toxin binds, as well as the pathological consequences. Shiga toxins bind in vivo primarily to Gb3 glycolipids found on variety of human cell types (*vide supra*), including kidney epithelial cells and neurons, making those cells vulnerable to the toxic effects of Stx. However, Gb4 glycolipids must also be considered. The B subunits of Stx2e and Stx2f bind to both Gb3 and Gb4, conferring the potential for these subtypes to intoxicate cells that display Gb4. In this way, it is likely that future recombination events will further modify the binding characteristics of Stx B subunits, possibly producing more potent toxins that can damage a wider variety of cells.

In a textbook example of "translational medicine," the elucidation of phage control of Shiga toxin production has had a direct influence on the treatment of STEC infections. As discussed above, many but not all, antibiotics can induce the SOS response in *E. coli*, resulting in production of intact phages and Shiga toxins (Kimmitt et al. 2000). For example, both the quinolone antibiotics and fosfomycin will kill STEC, but quinolone antibiotics induce the SOS response, while fosfomycin does not (Zhang et al. 2000). Physicians were advised not to use antibiotics to treat patients with STEC infections (Wong et al. 2000). Effective clinical antibiotics must not induce Shiga toxin production. However, it is still practical to use antibiotics that do not induce lytic replications, as discussed in Sect. 5.5. This problem is an example of the interface between basic and clinical sciences, and the expected solution will likely include new and safer antibiotics for effective treatment of STEC infections.

Another example at the interface of basic and clinical science is manipulation of the toxin assembly and cell entry processes. New data indicate that much of the Stx

produced by STEC circulates as uncombined A and B subunits, with some Shiga toxin assembly occurring on the surface of host eukaryotic cells (Pellino et al. 2016). Therefore, another treatment approach could be administration of a therapeutic protein based on a catalytically inactive A subunit (A'). Such a molecule could compete with the active A subunit for assembly into a $A'B_5$ hybrid holotoxin. This process would deplete the pool of B subunits necessary for active toxin entry to cells, while any hybrid holotoxin that may enter the cell would be non-toxic. This novel approach to treating Shiga toxin infections, effective in mice, awaits clinical testing.

Looking to the future, it is appropriate to consider the broadening role of government in reducing STEC threats. In 1998, the US FDA and USDA jointly prepared the "Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables " and developed a system of voluntary audits: Good Agricultural Practices (GAP) and Good Handling Practices (GHP) (USDA 2016). These programs verify the safe production, packing, handling, and storage of produce and aim to minimize microbial food safety risks, including those attributed to STEC. In 2015, 90 commodities were audited by the Agricultural Marketing Service (AMS) of USDA throughout the US and Canada on a voluntary basis. In 2011, the US Food Safety Modernization Act (FSMA) instituted a broader program with many mandates and new enforcement authorities, particularly for the US FDA. FSMA has gradually been implemented in the US since that time, and the implementation of the Produce Safety Rule was funded in late 2016 (FDA 2016).

A similar heightened vigilance to minimize microbial risks in food can be seen worldwide, for example by the European Food Safety Authority (EFSA) and World Health Organization (WHO). With other international agencies, WHO developed its One Health initiative in 2008, to address the control of infectious diseases at the animal-human-ecosystem interface (WHO 2010). Some risk factors at this interface were cited as (1) a growing population expanding into new geographical areas; (2) climate change and intensive farming practice; and (3) ever-increasing travel and trade. STEC exemplifies an oft re-emerging infectious disease threat that warrants continuing international research, education, and outreach efforts. Global initiatives and science-based governmental guidelines can be expected to exploit these efforts and implement new approaches to prevent and treat the health threats associated with Shiga toxins.

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