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14. ABSTRACT

Enterohemorrhagic Escherichia coli (EHEC) O157:H7 is the most common infectious cause of bloody diarrhea in the United States, and a sequela of this infection, the hemolytic uremic syndrome (HUS), is the primary cause of acute renal failure in children in the U.S. The majority of U.S. cases of EHEC O157:H7 have occurred as a consequence of ingestion of undercooked, contaminated hamburger or produce and/or water contaminated with bovine manure. Intimin is the primary adhesin of EHEC O157:H7, and is required for colonization of neonatal calves. I hypothesized that an intimin-based vaccination strategy in calves might reduce colonization of cattle with EHEC O157:H7. To test this concept in a small animal model, I developed transgenic tobacco plant cells that expressed the full length or carboxy-terminal portion (Int261) of EHEC O157:H7 intimin and then immunized mice parenterally with intimin expressed from the plant cells, or fed mice the transgenic plant cells, or both. I was able to show that these mice not only generated an intimin-specific mucosal immune response when primed parenterally and boosted orally but also exhibited a reduced duration of EHEC O157:H7 fecal shedding after challenge. These results suggest that transgenic plants are attractive and feasible production and delivery systems for an intimin-based vaccine for cattle, and such a vaccine can reduce the duration of EHEC O157:H7 shedding in a small animal model. In addition, Shiga toxin type 2 (Stx2) is another important EHEC O157:H7 virulence factor that plays a critical role in the development of potentially fatal HUS in humans. I developed a toxoid of Stx2 by making site-directed changes to the nucleotide sequence of the Stx2 A subunit gene that abrogated cytotoxcity in vitro and in vivo. The Stx2 toxoid elicited toxin-neutralizing antibody when parenterally injected in mice. I also optimized both the Stx2 A toxoid subunit and the B subunit for expression in plants and, thus laid the groundwork for future expression of this Stx2 toxoid molecule in plants.

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Abstract

Title of Dissertation:

Toward Development of an Oral, Plant-based Vaccine Against *Escherichia coli* O157:H7

Nicole A. Judge, Doctor of Philosophy, 2004

Thesis directed by:

Alison D. O'Brien, Ph.D.

Professor, Department of Microbiology and Immunology

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tobacco plant cells that expressed the full length or carboxy-terminal portion (Int_{261}) of

EHEC O157:H7 intimin and then immunized mice parenterally with intimin expressed

from the plant cells, or fed mice the transgenic plant cells, or both. I was able to show

that these mice not only generated an intimin-specific mucosal immune response when

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animal model. In addition, Shiga toxin type 2 (Stx2) is another important EHEC

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HUS in humans. I developed a toxoid of Stx2 by making site-directed changes to the

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Toward Development of an Oral, Plant-based Vaccine Against

***Escherichia coli* O157:H7**

By

Nicole Ann Judge

Dissertation submitted to the Faculty of the

Department of Microbiology and Immunology Graduate Program of the

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in partial fulfillment of the

requirements for the degree of

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To my parents:

For giving me a love of and faith in growing things

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Introduction

I.   Enterohemorrhagic *E. coli* O157:H7

Before 1982, *Escherichia coli* O157:H7 was a rare serotype isolated from only

sporadic cases of bloody diarrhea. However, in February of 1982, two outbreaks of

severe bloody diarrhea in Oregon and Michigan thrust this virtually unknown serotype

into the national spotlight (Riley *et al.*, 1983). Riley *et al.* described the clinically distinct

gastrointestinal illness caused by *E. coli* O157:H7 as one that is characterized by severe

abdominal cramps, grossly bloody diarrhea, and the absence of polymorphonucleocytes

in the stool. In addition, epidemiological evidence suggested that consumption of

undercooked hamburgers from a fast food chain was the primary risk factor for

developing diarrhea. *E. coli* O157:H7 was isolated from the stools of infected patients, as

well as from a hamburger patty from the lot of meat used at the restaurants. These initial

findings led to the classification *E. coli* O157:H7 as a new subset of pathogenic *E. coli*,

called enterohemorrhagic *E. coli* (EHEC), and established an epidemiological link

between the bacterium and cattle. Subsequent studies on EHEC O157:H7 identified

critical factors that are required for the full virulence of this organism in animal and

tissue culture models (reviewed in Paton and Paton, 1998; Frankel *et al.*, 1998).

Currently, a major thrust of EHEC O157:H7 research is the development of methods to

reduce the incidence or prevent EHEC O157:H7 disease in humans. One approach to

reduce the likelihood that humans will come in contact with this low-infectious-dose

(Tilden, Jr. *et al.*, 1996; Griffin, 1998) bacterium is to decrease the prevalence of EHEC

O157:H7 carriage in cattle. Currently, the general estimate of the proportion of cattle that

harbor the microbe in their intestines (when manure and multiple sites on the animals are sampled by sensitive means) approaches 30 % (Elder *et al.*, 2000). Consequently, the major goal of this project is to provide proof of the principle that a transgenic plant vaccine can be constructed that will lead to a protective immune response in a surrogate animal model for cattle and reduce the level and/or length of shedding of the agent in animal feces.

Diseases caused by EHEC O157:H7. EHEC O157:H7 causes diarrheal disease that can range from mild, non-bloody diarrhea to hemorrhagic colitis. Indeed, this organism is the leading cause of infectious bloody diarrhea in the United States (Centers for Disease Control and Prevention, 1994). A potentially fatal sequela of infection with EHEC O157:H7 is the hemolytic uremic syndrome (HUS), a disease that is characterized by thrombocytopenia, microangiopathic hemolytic anemia, and acute renal failure. In fact, EHEC O157:H7-mediated HUS is the primary cause of acute renal failure in children in the United States and Canada (Centers for Disease Control and Prevention, 1994). In 1999, the Centers of Disease Control and Prevention estimated that over 73,000 cases of human disease and 60 deaths per year in the United States were caused by EHEC O157:H7 (Mead *et al.*, 1999).

Diagnosis. When patients present with symptoms indicative of EHEC O157:H7 disease, positive diagnosis of this serotype is fairly straightforward. EHEC O157:H7 is distinguished from other *E. coli* in culture by the inability to ferment sorbitol (Wells *et al.*, 1983). In the clinical laboratory, stool samples are plated on sorbitol-MacConkey

agar (SMAC) to identify sorbitol-negative colonies (white) that are confirmed as EHEC

O157:H7 by additional serological or biochemical tests. Antisera raised against the O157

and H7 antigens are used in slide agglutination assays. DNA probe and PCR assays,

commonly based on *stx* (Shiga toxin) genes and EHEC O157:H7-specific *eae* (intimin)

DNA sequences, are other procedures used to identify EHEC O157:H7. In addition,

culture enrichment, immunomagnetic separation, and multiplex and real-time PCR are

newly developed, highly sensitive methods for detection, and in some cases,

quantification of EHEC O157:H7 from fecal and environmental samples, raw meat, and

other tissues (Naylor *et al.*, 2003; Lekowska-Kochaniak *et al.*, 2002; Sharma and Dean-

Nystrom, 2003). Indeed, incorporation of enrichment steps and immunomagnetic beads

coated with anti-O157 sera into the newer O157:H7 isolation methods has lead us to

understand that the incidence of infection in cattle is much higher than estimated 5 years

ago (Elder *et al*., 2000; Keen and Elder, 2002; Laegreid *et al.*, 1999).

Epidemiology. Since widespread testing started for EHEC O157:H7 in the early 1980's,

this serotype has been found to be ubiquitous in both dairy and beef cattle. Cattle are

considered the primary reservoir for this bacterium, and although they generally remain

asymptomatic, they shed the bacteria into the environment in their feces.

Epidemiological evidence has implicated a variety of foods of bovine origin, such as

ground beef (Griffin and Tauxe, 1991), roast beef (Abdul-Raouf *et al.*, 1993a), and raw

milk (Keene *et al.*, 1997a) with human disease. In addition, human disease is associated

with numerous other foods, such as salad vegetables (Abdul-Raouf *et al.*, 1993b),

unpasturized apple cider (Centers for Disease Control, 1997) and juice (Centers for

Disease Control, 1996b), and alfalfa sprouts (Breuer *et al.*, 2001), as well as

contaminated drinking and recreational water (Swerdlow *et al.*, 1992; Keene *et al.*, 1994;

Centers for Disease Control, 1996a; Centers for Disease Control, 1999). Person-to-

person spread has also resulted in outbreaks reported from nursing homes and day care

centers (Samadpour *et al.*, 1993; Belongia *et al.*, 1993). Contact with farm animals, their

environment and livestock density are also major risk factors for EHEC O157:H7

infection (Centers for Disease Control, 2001; O'Brien *et al.*, 2001; Valcour *et al.*, 2002).

Cattle and EHEC O157:H7. The ecology of EHEC O157:H7 in cattle and on the farm

is complex and has thus far frustrated production-level intervention strategies focused on

precluding the entry of the organism into the human food chain. Although beef and dairy

cattle are considered the primary reservoir for this *E. coli* serotype, EHEC O157:H7 is

widespread in nature and can colonize a number of different species, including birds,

sheep, deer, dogs, and humans, as well as survive and multiply in certain environmental

niches (Wallace *et al.*, 1997; Kudva *et al.*, 1996; Keene *et al.*, 1997b; Armstrong *et al.*,

1996; LeJeune *et al.*, 2001). EHEC O157:H7 within the cattle population is endemic, and

although the serotype can be found on most farms, its prevalence can fluctuate from less

than 0.5% to approximately 28% (Hancock *et al.*, 1998; Elder *et al*., 2000). EHEC

O157:H7 is more frequently isolated from cattle during the warmer months of spring and

summer, and this pattern correlates with the increased incidence of human disease during

these months (Hancock *et al*., 1998; Griffin, 1995). Recent studies by Naylor *et al.* show

that EHEC O157:H7 colonizes the terminal 3 to 5 cm of the rectum in the bovine host

(Naylor *et al*., 2003). In addition, they observed that the majority (13 out of 15) cows in

their study persistently shed EHEC O157:H7 for more than 3 weeks, a finding that

suggests that the duration of colonization may be greater than previously suspected. This

prolonged colonization in cattle could provide a greater opportunity for EHEC O157:H7

contamination of beef products, the environment, and other cattle in the herd that are not

yet colonized. In addition to colonizing the gastrointestinal tract, EHEC O157:H7 can

persist and proliferate on cattle hide surfaces, oral cavities, and environmental water

sources, such as water troughs and contaminated lakes and drinking wells (Elder *et al*.,

2000; Keen and Elder, 2002; Conradi, 1903; Samadpour *et al.*, 2002; Centers for Disease

Control, 1999). These extra-gastrointestinal sites of EHEC O157:H7 persistence are not

only potential sources of human infection but also increase the possibility of re-

colonization of the cattle and spread of the bacterium in the cattle population. Several

studies showed that irrigation can spread EHEC O157:H7 onto crops and that the

proximity of vegetable fields or processing facilities to cattle farms can provide a

significant potential for contamination (Solomon *et al.*, 2002; Hilborn *et al.*, 1999).

Based on such reports, a number of investigators have concluded that a decrease in the

amount of EHEC O157:H7 shed and in the number of cattle that excrete the serotype

could lead to a significant reduction in the prevalence of the bacteria in cattle and the

farm environment and a decline in the incidence of human EHEC O157:H7-related

disease (Jordan *et al.*, 1999; Hancock *et al*., 1998).   In particular, the stochastic

simulation study performed by Jordan *et al*. suggested that vaccination of cattle with an

agent that would result in the reduction of colonization and shedding of the serotype held

the greatest potential impact for reducing EHEC O157:H7 contamination of cattle

carcasses (Jordan *et al*., 1999). Not only would this approach reduce carcass

contamination, it would also diminish the amount of EHEC O157:H7 entering the

environment and decrease potential pollution of wells, and of recreation and irrigation

water. A decrease in contamination of irrigation water would likely reduce secondary

contamination of non-bovine foodstuffs, such as fruits and vegetables.

Virulence Determinants of EHEC O157:H7. *E. coli* first emerged as potential

pathogens in the 1940's when two researchers hypothesized that "summer diarrhea" that

plagued infants and young children in England and North America was due to pathogenic

E. coli (Bray, 1945; Bray and Beavan, 1948). In these experiments, Bray and Beavan

isolated and raised anti-sera against homogenous strains of *Bacterium coli neapolitanum*

(*E. coli*) obtained from stools of infants with diarrhea. The anti-sera agglutinated 87.5%

of *E. coli* strains isolated from 90 children with diarrhea and only 4% of strains from 180

control patients. Since then, research involving pathogenic *E. coli*, especially EHEC

O157:H7, has grown and converged with numerous fields of laboratory-based and

epidemiology-based investigations. All pathogenic *E. coli* have underlying

commonalities with respect to virulence characteristics and pathogenesis, of which EHEC

O157:H7 is no exception. Three of these common factors are that: 1) critical virulence

components are plasmid encoded; 2) pathogenic *E. coli* have specific and characteristic

interactions with the intestinal mucosa; and, 3) enterotoxins and cytotoxins are often

produced.

EHEC O157:H7 Plasmid. Almost all EHEC O157:H7 strains have a large, 90 Kb

plasmid (pO157), but experimental findings about the contribution of pO157 to EHEC

O157:H7 virulence are contradictory (Karch *et al.*, 1987; Toth *et al.*, 1990; Tzipori *et al.*, 1987; Wadolkowski *et al.*, 1990a). These disparate conclusions about the importance of pO157 genes may reflect the absence of any animal model or culture system that completely mimics human infection. That pO157 is likely to play some role in EHEC O157:H7-evoked disease is suggested by the numerous plasmid genes that encode potential virulence factors, e.g., hemolysin (Schmidt *et al.*, 1994), catalase (Brunder *et al.*, 1996), and serine protease (Brunder *et al.*, 1997).

II.   Intimin and Adherence to the Intestinal Mucosa.

History. EHEC O157:H7 has the capacity to produce attaching and effacing (A/E) lesions in the colonic epithelium of cultured human intestinal tissue and animals (Phillips *et al.*, 2000; Dean-Nystrom *et al.*, 1997; Donnenberg *et al.*, 1993). A/E lesions are characterized by the adherence of the bacteria to the apical surface of the intestinal epithelium and the subsequent destruction of the microvilli. While investigating A/E lesion formation by EHEC O157:H7 in gnotobiotic piglets, Tzipori *et al.* (Tzipori *et al.*, 1986) observed that the lesions were very similar to those produced by enteropathogenic *E. coli* (EPEC), though at different intestinal sites. In addition to extensive damage to the brush border of the intestine, intimate adherence of EPEC and EHEC O157:H7 was also characterized by the accumulation of actin under the adherent bacteria (Knutton *et al.*, 1989). Through the use of fluorescene-labeled phalloidin to bind the accumulated actin, a fluorescent actin staining (FAS) test was developed by Knutton and colleagues

(Knutton *et al*., 1989) and proved to be a powerful tool in further investigations of the factors influencing the adherence of EHEC O157:H7. FAS was used to screen EPEC for genes required to facilitate the adherence of the bacteria to HEp-2 cells, a human laryngeal cell line (Jerse *et al.*, 1990). Jerse *et al.* discovered a chromosomally-encoded gene in EPEC, *eae* (for <u>*E. coli*</u> <u>a</u>ttaching and <u>e</u>ffacing, and originally referred to as *eaeA*), that is necessary for the formation of A/E lesions, and that the *eae* gene produces a 94 kDa outer membrane protein, later appropriately termed intimin due to its capacity to facilitate intimate attachment of the bacteria to host cells. The *eae* gene is also present in EHEC O157:H7 (Yu and Kaper, 1992; Beebakhee *et al.*, 1992) and plays a role in inducing A/E lesions *in vitro* and *in vivo* (Donnenberg *et al*., 1993; McKee *et al.*, 1995). The amino-terminal region of intimin mediates outer membrane localization of the protein and facilitates the functional surface expression of the host cell binding domain of the molecule: the carboxy-terminus (Fig. 1) (Liu *et al.*, 1999). While EPEC and many EHEC strains share intimin as a common virulence factor, EHEC strains produce Shiga toxins, very potent enterotoxins, and lack bundle forming pili (BFP) which are important for the initial adherence of EPEC to the intestinal epithelium. In addition, EPEC, but not EHEC, regulates the expression of intimin using the plasmid-encoded regulatory (*per*) region present on the 60 MDa plasmid, which also encodes BFP (Gomez-Duarte and Kaper, 1995).

LEE. The EPEC and EHEC O157:H7 *eae* genes are located in a ~35 kb chromosomal pathogenicity island named LEE (<u>l</u>ocus for <u>e</u>nterocyte <u>e</u>ffacement) that also includes additional genes that comprise a type III secretion system and mediate adherence in

Figure 1. Homology of the intimin proteins from EHEC O157:H7 strain EDL933 (γ-intimin) and EPEC O127:H6 strain 2348/69 (α-intimin). The EHEC EDL933 *eae* gene is 2802 bp and is predicted to encode a protein of 934 amino acid residues. The EHEC *eae* gene is slightly smaller than the EPEC O127:H6 *eae* gene, which is 2817 bp and is predicted to encode a 939 amino acid residue protein. The overall homology of the structural genes is 83% at the amino acid level. The first 2/3 of the proteins (amino acids 1-704) share 94% identity and the remaining 25% of the proteins share 49% amino acid identity (Yu and Kaper, 1992).

EHEC O157:H7 Intimin
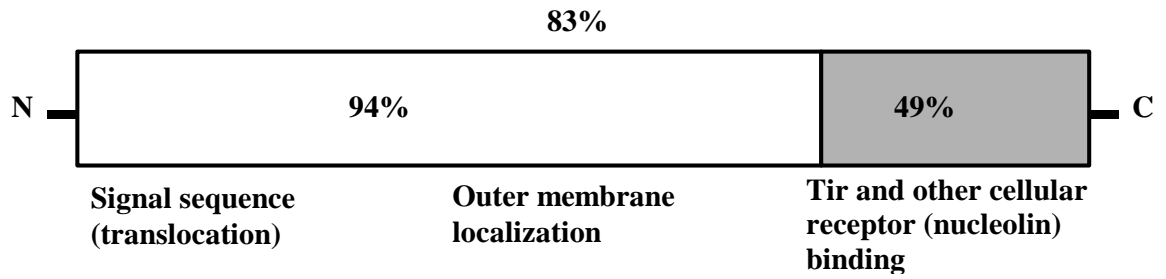
conjunction with *eae* (McDaniel *et al.*, 1995). The LEE can be divided into 3 regions. The middle region contains the genes for intimin and Tir, the translocated intimin receptor. Tir is a bacterial protein that acts as an intimin receptor after it is translocated to the host cell via the type III secretion system. The genes that comprise the type III secretion system are present in the second region, upstream of *eae* and *tir* in the LEE (Kenny *et al.*, 1997). This *E. coli* type III secretion system shares similarities to systems found in other enteric pathogens such as *Yersinia*, *Shigella*, and *Salmonella*. The third region of the LEE encodes several proteins that are secreted through the type III secretion system. One such protein, Esp A (for *E. coli* secreted protein A) forms a tube-like structure that is critical for the secretion of other LEE encoded proteins such as Tir (Kenny *et al.*, 1997). The complete sequence for EHEC O157:H7 strain 933 shows that EPEC LEE and EHEC O157:H7 LEE share 41 genes with an average nucleotide identity of 93.9% across the shared genes (Perna *et al.*, 2001). The degree of identity is not consistent over the 41 common genes. Some EPEC and EHEC O157:H7 LEE genes, like the *esc* genes that make the type III secretion apparatus show 98-100% identity and other genes, such as *eae* and *tir*, display 87% and 33% identity, respectively. These LEE-encoded effector proteins interact directly with host cells and their variability suggests different host cell tropisms for EPEC versus EHEC. Indeed, when *eae* from EPEC O127:H6 is cloned into an *eae* mutant of EHEC, the resulting EHEC clone is able to colonize piglet intestine, though in a manner more characteristic of EPEC (small intestine) than EHEC (large intestine) (Tzipori *et al.*, 1995).

Intimin Affects Colonization of EPEC in Humans and EHEC O157:H7 in Cattle.

Intimin is required for EHEC O157:H7 to colonize host mammalian cells *in vitro* and *in vivo* (Donnenberg *et al*., 1993; McKee *et al*., 1995). Moreover, antibodies specific to intimin can block adherence of the bacteria to cultured cells and intestinal mucosa (Gansheroff *et al.*, 1999; Dean-Nystrom *et al.*, 2002). Experimental infection of human volunteers with EPEC by Levine *et al*. showed that the patients made an antibody response to a 94 kDa outer membrane protein that was later identified as intimin (Levine *et al.*, 1985; Jerse and Kaper, 1991). In addition, one volunteer who had preexisting antibody to intimin did not develop diarrhea when challenge with the EPEC strain, a finding that suggests that antibody to intimin may protect from EPEC-mediated disease. Other studies confirmed the induction of intimin-specific antibody, along with other antibodies to additional *E. coli* virulence factors such as the Esps or Tir, upon natural infection with EPEC or EHEC strains (Martinez *et al.*, 1999; Li *et al.*, 2000; Karpman *et al.*, 2002).

Intimin interacts with mammalian cells through two receptors. When EHEC O157:H7 comes in contacts with target host cells, Tir is injected into the cells by the bacterium through the type III secretion system. Tir incorporates into the host cell outer membrane and acts as an intimin receptor (DeVinney *et al.*, 1999) that mediates the close adherence of the bacteria to the eukaryotic cell surface leading to A/E lesion formation. The most severe symptoms of EHEC O157:H7 infection are associated with the productions of Shiga toxins, however the ability to form A/E lesions is an essential virulence factor for EHEC and EPEC as well. The absence of Tir, or the inability to deliver Tir to host cells abrogate pedestal formation in host cells by both EHEC and

EPEC strains (Kenny *et al*., 1997; DeVinney *et al*., 1999). Both EHEC O157:H7 and

EPEC share the ability to form A/E lesions within the host cell, though the mechanisms

by which they initiate host cell cytoskeletal changes are very different. One major

difference is that EPEC Tir is tyrosine phosphorylated within the host cell whereas EHEC

O157:H7 initiates pedestal formation by an as yet unknown tyrosine phosphorylation-

independent mechanism. EPEC Tir can complement a Tir deficient EHEC strain, but

EHEC Tir can not complement an EPEC Tir mutant (Kenny, 2001; DeVinney *et al.*,

2001) because EHEC O157:H7 Tir lacks a critical tyrosine residues at position 474 and is

not phosphorylated in host cells. EPEC strains that deliver EPEC Tir lacking this

tyrosine residue are unable to form pedestals (DeVinney *et al*., 2001). These results

suggest that while EPEC Tir is dependent on tyrosine phosphorylation to induce

cytoskeletal changes, EHEC Tir does not have the same requirements. It is still unknown

how EHEC initiates A/E lesion formation in the absence of tyrosine phosphorylation,

though it is interesting to speculate that EHEC O157:H7 has evolved into a more self-

sufficient pathogen compared to EPEC. EHEC O157:H7 requires other bacterial encoded

factors, delivered by the type III secretion system, for intimate adherence and pedestal

formation via the Tir-intimin$_{O157}$ interaction (DeVinney *et al*., 2001). Thus EHEC

O157:H7 is not limited to relying on the host cell tyrosine kinases at least at this early

stage of attachment. In addition, intimin can interact with a host-cell surface-expressed

protein called nucleolin (Sinclair and O'Brien, 2002). Both Tir and nucleolin bind

intimin on the carboxy-terminal portion of the molecule (Frankel *et al.*, 1994; Frankel *et

al.*, 1995; Sinclair and O'Brien, 2002). This observation suggests that although this

portion of intimin is the most divergent among the different intimin types (Yu and Kaper,

1992) (Fig. 1; Table 1), the carboxy-terminus is very important for intimin host-cell interactions and may influence binding affinity to cells in different anatomical sites of the intestine (Tzipori *et al*., 1995).

In addition, antibodies to intimin$_{O157}$, specifically antibodies directed to the carboxy-terminal third of intimin$_{O157}$, can block adherence of wild type EHEC O157:H7 to HEp-2 cells (McKee and O'Brien, 1996; Gansheroff *et al*., 1999). Furthermore, colostrum from sows immunized intramuscularly with intimin from EHEC O157:H7 contains anti-intimin$_{O157}$ antibodies that can protect suckling piglets from colonization with wild type EHEC O157:H7 (Dean-Nystrom *et al*., 2002). These studies suggest that antibodies specific to the carboxy-terminal third of intimin$_{O157}$ play an important role in blocking adherence of the bacterium to host cells and can protect a host from EHEC O157:H7-mediated disease. EHEC O157:H7 can colonize calves and adult cattle, sheep, and a variety of other domestic and wild animals (Cray, Jr. and Moon, 1995; Kudva *et al*., 1996; Keene *et al*., 1997b; Wallace *et al*., 1997), and intimin$_{O157}$ is required for EHEC O157:H7 to colonize neonatal calves and adult sheep and cattle (Dean-Nystrom *et al.*, 1998; Cornick *et al.*, 2002). These data, along with the finding that antibodies specific to intimin$_{O157}$, especially the carboxy-terminal third of intimin$_{O157}$, can block bacterial adherence to HEp-2 cells and prevent infection of suckling piglets, strongly suggest that intimin$_{O157}$ is an attractive candidate for an EHEC O157:H7 anti-transmission vaccine for cattle.

Table 1: Intimin Types and Associated Bacterial Serotypes

Intimin Type	Bacterial strain	Reference or Accession number
Alpha	EPEC O127:H6	(Adu-Bobie *et al.*, 1998)
Alpha 2	EPEC O125:H6	(Acheson *et al.*, 1995)
Beta	EHEC O26:H11	(Adu-Bobie *et al*., 1998)
	RDEC-1	(Adu-Bobie *et al*., 1998)
	Citrobacter rodentium	(Adu-Bobie *et al*., 1998)
Gamma	EHEC O157:H7	(Adu-Bobie *et al*., 1998)
	EHEC O55:H7	(Adu-Bobie *et al*., 1998)
Gamma 2	EHEC O111:H-	(Acheson *et al*., 1995)
Delta	EPEC O86:H34	(Adu-Bobie *et al*., 1998)
Epsilon	EHEC O121:H19	(Acheson *et al*., 1995)
Iota	EHEC O145:H4	(Ramotar *et al.*, 1990)
Kappa	EHEC O118:H5	(Ramotar *et al*., 1990)
Lambda	NT strain 97-3 (China)	AAL32028
Eta	EPEC O125:H-	(Ramotar *et al*., 1990)
Zeta	EHEC O84:NM, EHEC O84:H4	(Tarr and Whittam, 2002; Ramotar *et al*., 1990)
Theta	EHEC O111:H8	(Tarr and Whittam, 2002)

III.   Shiga Toxins in *E. coli*

History. Shiga toxin (Stx), produced by *Shigella dysenteriae* serotype 1, is the prototype toxin of a large family of structurally and functionally-related toxins that was first discovered in 1903 by two independent groups (Conradi, 1903; Neisser and Shiga, 1903). O'Brien *et al.* in 1977 and 1982 reported that certain *E. coli* strains produce a cytotoxin that cross-reacts with anti-sera raised against Shiga toxin (O'Brien *et al.*, 1977; O'Brien *et al.*, 1982). EHEC O157:H7 isolates from the 1982 hamburger outbreak were also found to produce a cytotoxin that was neutralized by anti-Shiga toxin antibodies (O'Brien *et al.*, 1983). Shiga toxins of Shiga-toxin producing *E. coli* (STEC) were called Shiga-like toxins and, alternatively, Vero toxins due to their similarity to Stx from *Shigella* and their Vero cell cytotoxicity (Calderwood *et al.*, 1996; Karmali *et al.*, 1996). Since then, *E. coli* strains have been shown to produce two main forms of Stx: Stx1 and Stx2 (Strockbine *et al.*, 1986). Stx and Stx1 are antigenically identical and only differ in one amino acid (Strockbine *et al.*, 1988). Stx2 is sufficiently antigenically distinct from Stx and Stx1 such that polyclonal antisera to Stx or Stx1 does not neutralize Stx2 or any of its numerous variants that include Stx2c, Stx2d [Stx2d1, Stx2d2], Stx2e, and Stx2f (Ito *et al.*, 1990; Melton-Celsa *et al.*, 1996; Marques *et al.*, 1987; Schmidt *et al.*, 2000; Schmitt *et al.*, 1991; Teel *et al.*, 2002).

Stx Structure and Function. All members of the Stx family share genetic, antigenic, and structural features. All have an A1:B5 noncovalently associated subunit structure. The Stx A subunit (~35 kDa) is cleaved to produce A_1 (~28 kDa) and A_2 (~4 kDa)

portions. The A_2 subunit remains associated with the A_1 subunit via a disulfide bond and serves to link the A_1 to the B pentamer (Fig. 2). All of the toxins have the same enzymatic function of inhibition of protein synthesis by depurination of an adenine residue from 28S ribosomal RNA of the 60S ribosome. The A_1 subunit contains the N-glycosidase activity (Endo *et al.*, 1988; Saxena *et al.*, 1989). The glutamic acid at position 167 of the A subunit is the active site and is critical for enzymatic activity (Hovde *et al.*, 1988), though other amino acid residues in the A subunit (such as the tyrosine at position 77) are also important for toxicity (Deresiewicz *et al.*, 1992). The B subunit of Stx (~7 kDa per monomer) forms a pentameric structure that typically binds to globotriaoslyceramide (Gb_3), except in the case of Stx2e that binds to Gb_4 (Jacewicz *et al.*, 1986; Lindberg *et al.*, 1987; De Grandis *et al.*, 1989).

Stx and HUS. In 1983, Karmali *et al.* established a link between infection with Vero toxin (Stx)-producing *E. coli* and hemolytic uremic syndrome (HUS) (Karmali *et al.*, 1983), and he later confirmed that Stxs play a major role in the development of HUS (Karmali *et al.*, 1985; Griffin, 1995), Virtually all isolates of *E. coli* and *Shigella* incriminated in cases of HUS produce one or more forms of Stx. In fact, EHEC O157:H7 strains produce Stx1 and Stx2, or Stx2 alone, or, less commonly, Stx1 only (Obrig, 1998). Although Stx1 and Stx2 are equally toxic for Vero cells, Stx2, either injected in a pure form or when expressed from STEC in the gastrointestinal tract of mice, appears to be more toxic than Stx1 (Melton-Celsa and O'Brien, 1998). Louise and Obrig demonstrated that endothelial cells are 1000 times more sensitive to Stx2 than to Stx1 (Louise and Obrig, 1995). In addition, epidemiological data suggests that Stx2 may

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Figure 2. The crystal structure of Shiga toxin from *Shigella dysenteriae* (Fraser *et al.*, 1994). The Stx A and B subunit structures are shown in this figure. The Shiga toxin family has an A1:B5 noncovalently associated subunit structure. The A_2 subunit remains associated with the A_1 subunit via a disulfide bond and serves to link the A_1 to the B pentamer.

A subunit
(A_1)

A subunit
(A_2)

B subunit
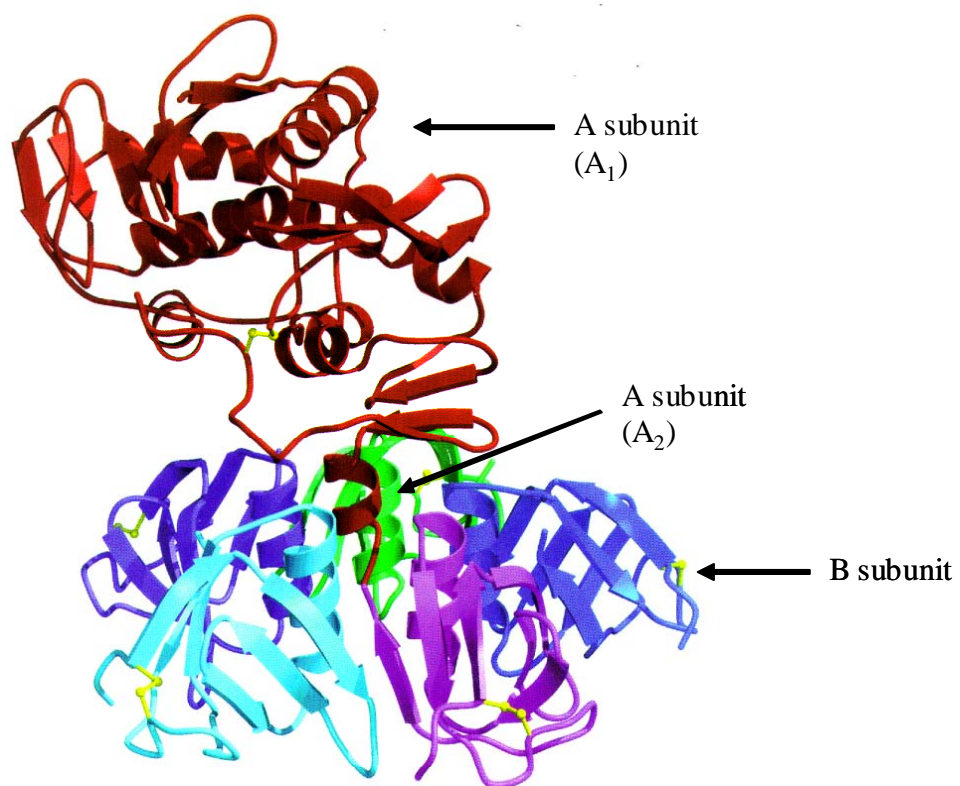
play a more critical role in the development and severity of HUS, although Stx1-only

producing strains have also been associated with cases of HUS (Scotland *et al.*, 1987;

Ostroff *et al.*, 1989).

IV.   Transgenic Plant-based Vaccines

In 1990, a consortium of philanthropic, health, and government organizations led

by the World Health Organization developed the Children's Vaccine Initiative (CVI).

The CVI's lofty goals included developing safe, orally administered, multicomponent,

heat stable vaccines that could be widely accessible to the children of underdeveloped

countries (Douglas, Jr., 1993). The CVI focused attention on technologies that would

make vaccines less expensive and more reliable, especially for the developing world. It

also drew attention to multi-component vaccines that could be delivered orally, thus

simplifying administration and eliminating the cost and risks associated with needles and

syringes. Each of the CVI's requirements for the ultimate vaccine could potentially be

met in plant-based vaccine technology.

Since the early 1990's, numerous vaccine antigens and other pharmaceuticals

(such as secretory antibodies) have been successfully expressed in plants. The vaccine

antigens include hepatitis B surface antigen (HBsAg) (Mason *et al.*, 1992; Thanavala *et

al.*, 1995; Kong *et al.*, 2001), enterotoxigenic *E. coli* heat-labile toxin B subunit (LT-B)

(Haq *et al.*, 1995; Mason *et al.*, 1998; Tacket *et al.*, 1998; Lauterslager *et al.*, 2001),

Norwalk virus capsid protein (NVCP) (Mason *et al.*, 1996; Tacket *et al.*, 2000), cholera

toxin B subunit (Arakawa *et al.*, 1997; Arakawa *et al.*, 1998), and many others. In

addition, other medically important pharmaceuticals have been successfully expressed in plants, such as secretory antibodies (Ma *et al.*, 1995; Ma *et al.*, 1998) and other human proteins, such as cytokines (Ganz *et al.*, 1996).

LT-B and NVCP expressed in transgenic potato were tested in mice and in human clinical trials. In mice, potatoes that expressed LT-B induced LT-B-specific immune responses that protected the animals from a subsequent toxin challenge (Mason *et al*., 1998). Humans fed such potatoes developed LT-B-specific serum IgG, fecal IgA, and anti-LT IgA antibody-secreting cells (Mason *et al*., 1998; Tacket *et al*., 1998). Mice immunized orally generated an antibody response to NVCP expressed in transgenic tobacco or potato (Mason *et al*., 1996). Additionally, 95% of human volunteers fed transgenic potatoes that expressed NVCP virus-like particles developed significant increases in the number of NVCP-specific IgA antibody-secreting cells and 30% and 20% developed NVCP-specific serum IgG and fecal IgA, respectively (Tacket *et al*., 2000). These results show that plants can express bacterial and viral vaccine antigens that are immunogenically similar to native proteins and that these plant-expressed antigens can induce specific immune responses upon oral immunization.

Many pathogens infect or invade via mucosal surfaces, so the capacity of plant-based vaccines to induce mucosal immunity is a great advantage. The plant cells act as a natural microencapsulation system to protect the vaccine antigens from being degraded in the upper digestive tract before they can reach the gut-associated lymphoid tissue (GALT). Recent studies on HBsAg expressed in transgenic potatoes suggests that oral delivery of this antigen can act as an immunological "prime" and can lead to the development of a strong, long-lasting secondary serum antibody response upon a

parenteral boost with a sub-immunogenic dose of yeast-derived HBsAg (Kong *et al.*,

2001). Other research suggests that plant-based oral vaccines can significantly boost

mucosal immune responses primed by parenteral injection (Van der Heijden *et al.*, 1989;

Lauterslager *et al.*, 2001). Parenteral priming of the immune system may allow the

GALT to react successfully to the small amounts of antigen delivered during oral

immunization and thus decrease the possibility of inducing oral immunotolerance to

plant-based and other orally delivered vaccine antigens.

V.   Specific Aims of this Dissertation

The underlying hypothesis of this project is as follows. When $intimin_{O157}$ or a

portion of that molecule is expressed by plant cells and delivered to an animal alone or as

a boost for parenterally injected $intimin_{O157}$, the $intimin_{O157}$-specific immune response

that is elicited leads to a reduction in the degree and/or duration of colonization of that

animal with wild-type EHEC O157:H7. Three specific aims were designed to test this

multi-tiered hypothesis. The first aim was to successfully express $intimin_{O157}$, or a plant-

optimized carboxy-terminal portion of the protein (Int_{261}), in NT-1 cells, a tobacco plant

cell system. The second aim was to determine whether the plant-expressed $intimin_{O157}$

proteins were immunogenic in mice when partially purified and parenterally injected or

when fed directly to mice in plant cell material. The final aim of this project was to

determine if the $intimin_{O157}$-specific immune response in mice could: i.) block adherence

of wild type EHEC O157:H7 strain 86-24 in a HEp-2 cell adherence assay and ii.) block

or reduce the duration of colonization with the same strain in an intimin$_{O157}$-dependent mouse model of colonization.

The final section of the dissertation describes my efforts to create a site-directed mutant Stx2 holotoxoid that retains the native structure of the toxin, is totally non-toxic, and is capable of eliciting toxin-neutralizing antibody. For this purpose, both the Stx2 A and B subunits were plant-optimized and cloned into plant expression vectors. The ultimate goal of the Stx2 toxoid studies initiated here is to express the toxoid in plants and to use these transgenic cells as an oral vaccine against Stx2-mediated disease.

Materials and Methods

Intimin

Bacterial Strains, Plasmids, and Media. The bacterial strains used in this study are summarized in Table 2. The plasmids used in this study are summarized in Table 3. EHEC O157:H7 strain 86-24 (Griffin *et al.*, 1988) was isolated in 1986 from a patient in Seattle, WA and was kindly provided by Dr. Phil Tarr. The full-length (pEB310) and histidine-tagged intimin$_{O157}$ expression plasmids, pEB313 (encodes all but the first 34 amino acids of the *eae* gene) and pMW103 (encodes the carboxy-terminal third of intimin$_{O157}$) have been described previously (McKee *et al.*, 1995; McKee and O'Brien, 1996; Gansheroff *et al.*, 1999). EHEC O157:H7 strain 86-24 expressing the GFP (green fluorescent protein) arabinose-inducible plasmid p166 was used to visualize bacteria in the adherence assay (see "adherence assay" below for details) (Sinclair and O'Brien, 2002). Streptomycin-resistant derivatives of strain 86-24 [called 86-24 Str^r (Melton-Celsa *et al.*, 1998)] and its isogenic, intimin$_{O157}$-negative mutant strain 86-24 *eae*Δ10 [called 86-24 Str^r *eae*Δ10 (McKee *et al.*, 1995)] were used to distinguish EHEC O157:H7 from other fecal bacteria during the colonization assay by plating on sorbitol MacConkey agar with 100 μg/ml streptomycin. The *Agrobacterium tumefaciens* strain EHA105, plasmids with plant-specific expression components (pBTI210.3, pBTI210.4, pIBT210), and the binary vector pGPTV-Kan were generously provided by Dr. Hugh Mason (Arizona State University, Tempe AZ) from the Boyce Thompson Institute for Plant Research (Ithaca, NY). All *E. coli* strains and clones were grown in Luria Bertani (LB) broth or on LB agar.

Table 2. Bacterial strains used in this study

Strain	Relevant characteristics	Reference
86-24	*E. coli* O157:H7, intimin positive	(Griffin *et al*., 1988)
86-24-GFP	*E. coli* O157:H7 with p166	(Sinclair and O'Brien, 2002)
86-24 Str^R	*E. coli* O157:H7, Str^R	(Melton-Celsa *et al*., 1998)
86-24 Str^r *eae*Δ10	*E. coli* O157:H7, intimin negative, Str^R	(McKee *et al*., 1995)
DH5α	*E. coli* cloning host, *recA*	(Hanahan, 1983)
XL-1 Blue	*E. coli* cloning host, *recA*, *lacI*^{q,}	(Bullock *et al.*, 1987) from Stratagene (La Jolla, CA)
Agobacterium tumefaciens EHA105	Plant transformation strain	(Hood *et al.*, 1993)

Table 3. Plasmids used in this study

Plasmid	Relevant characteristics	Reference or source
Intimin:		
pEB310	Wild-type *eae* ORF, from 86-24, in pBRKS-	(McKee *et al*., 1995)
pEB313	Truncated *eae* (bp 109-2804), in pQE32 (Qiagen)	(McKee and O'Brien, 1996)
pMW103	Truncated *eae*, (bp 1959-2804), in pQE31 (Qiagen)	(Gansheroff *et al*., 1999)
pNR37	*eae* from pMW103, plant optimized	This study
Plant Expression Vectors:		
pIBT210	CaMV 35S promoter, TEV 5' UTR, Vsp 3' UTR	(Haq *et al*., 1995)
pBTI210.3	CaMV 35S promoter, TMVΩ 5' UTR, Vsp 3' UTR	Dr. Hugh Mason
pBTI210.4	With plant-specific signal peptide sequence	Dr. Hugh Mason
pGPTV-Kan	Binary vector; Kan^R	(Becker *et al*., 1992)

Plant-specific Intimin

Expression Vectors:

pNR12	Full-length *eae* from pEB310	This study
pNR14	Truncated *eae* from pEB313	This study
pNR49	*eae* from pNR37 with signal peptide seqence from pBTI210.4	This study
pNR50	*eae* from pNR37	This study

Stx2:

pMJ100	*stx2* from *E. coli* O157:H7 86-24 in pBluescript; Amp^R	(Weinstein *et al.*, 1989)
pNR100	*stx2* Y77S, E167Q (toxoid)	This study
pNAJ58	*stx2* Y77S, E167Q; plant optimized	This study
pNAJ65	*stx2* Y77S, E167Q A subunit only from pNAJ58 in pIBT210	This study
pNAJ73	*stx2* B subunit; plant optimized (synthetic) in pBTI210.3	This study

Prior to plant cell transformation, *Agrobacterium* strains were grown in YM broth (0.4 g yeast extract, 10 g mannitol, 0.1 g NaCl, 0.2 g Mg SO_4-$7H_2O$, 0.5 g K_2HPO_4 per liter). Antibiotics were added at the following concentrations as needed for selection: ampicillin 100 μg/ml, kanamycin 50 μg/ml, carbenicillin 100 μg/ml. Plasmid DNA was isolated by the Miniprep procedure (Qiagen, Valencia, CA). Restriction enzymes were purchased from New England Biolabs (Beverly, MA) and restriction enzyme digested DNA fragments were purified by agarose gel electrophoresis and eluted from the gel using the GENECLEAN SPIN columns and reagents (Bio 101, Carlsbad, CA). T4 DNA ligase was purchased from U.S. Biochemicals (Cleveland, OH) and Pfu ligase was obtained from Stratagene (La Jolla, CA). The polymerase chain reaction (PCR) was done with AmpliTaq (Roche, Branchburg, NJ) and Pfu Turbo (Stratagene) polymerases in a MJ Research Minicycler (Watertown, MA). Sequencing was preformed with the ABI Big Dye Sequencing kit (Applied Biosystems, Inc., Foster City, CA). Products were separated and analyzed by the Biomedical Instrumentation Center at Uniformed Services University with the Applied Biosystems model 377 or 3100. Sequence results were compared to published sequences using the Wisconsin Sequence Analysis package from the Genetics Computer Group, Inc. (Madison, WI).

Plant Optimization – *eae* (2165-3010). Thirteen independent changes were made to the nucleotide sequence of the intimin gene from pMW103 with the QuikChange™ Site-directed Mutagenesis Kit (Stratagene). The seven sets of oligonucleotides listed in Table 4 were designed according to the protocol listed in the QuikChange™ manual. The initial mutagenesis reaction was done with 50 ng of

Table 4: Oligonucleotides sets for *eae* (1959-2804) plant expression optimization by

QuikChange™ site-directed mutagenesis

Oligo set	Oligo
1-forward	GGGTCAGCCAGTTAA**C**AA**C**CAATCCGTTACATTCTCAACAAACTTTGG
1-reverse	CCAAAGTTTGTTGAGAATGTAACGGATTG**G**TT**G**TTAACTGGCTGACCC
2-forward	GGCTGCAATATGGTCA**A**TTTAAACTGAAAGCAAGCGG
2-reverse	CCGCTTGCTTTCAGTTTAAA**T**TGACCATATTGCAGCC
3-forward	GGGAAAGTCACTTTGAATGGTAAAGGC**TC**TGTCGTAATTAAAGCC
3-reverse	GGCTTTAATTACGACA**GA**GCCTTTACCATTCAAAGTGACTTTCCC
4-forward	GGTGATAAGCAAACAGTAAGTTACACTAT**C**AA**G**GCACCGTCG
4-reverse	CGACGGTGC**C**TT**G**ATAGTGTAACTTACTGTTTGCTTATCACC
5-forward	GCTATGTCCATTTGCAAAAA**C**TT**G**TTACCATCCACACAGACGG
5-reverse	CCGTCTGTGTGGATGGTAA**C**AA**G**TTTTTGCAAATGGACATAGC
6-forward	GGTATTGTCAGATAT**C**TATGACTCATGGGGGGCTGCAAA**C**AA**G**TATAGCC
6-reverse	GGCTATA**C**TT**G**TTTGCAGCCCCCCATGAGTCATA**G**ATATCTGACAATACC
7-forward	GGATTAAACAGACATCTAGTGAGCA**A**CGTTCTGGAGTATCAAGC
7-reverse	GCTTGATACTCCAGAACG**T**TGCTCACTAGATGTCTGTTTAATCC

pMW103 and 125 ng of each primer from primer set #1 in a total volume of 50 µl. In addition, 1 µl of Pfu Turbo polymerase was added to each reaction tube immediately prior to placing the sample tubes into the Minicycler. The samples were subjected to a 30 second hot start at 95°C, then 12 cycles of 95°C for 30 seconds, 55°C for 60 seconds, and 68°C for 12 minutes. After the cycles were completed, each reaction mixture was digested with *Dpn*I, and 1-5 µl of the digested reaction were transformed into the supercompetent cells provided with the kit as outlined in the QuikChange™ manual. The transformed cells were plated on LB agar with ampicillin. Several ampicillin-resistant colonies were selected, and 3 ml overnight LB broth cultures prepared from each colony. Bacteria were then harvested by centrifugation, and plasmid DNA purified from the pelleted organisms. The *eae* sequences were amplified by PCR from the plasmids, and the PCR products were then eluted from an agarose gel and sequenced. Clones in which the sequence of the mutated, PCR-amplified *eae* gene had been confirmed were used in the next round of mutagenesis with the next set of mutagenesis primers. The final plant-optimized intimin$_{O157}$ clone from pMW103 (Gansheroff *et al*., 1999) that contained all 13 nucleotide changes was called pNR37. The intimin$_{O157}$ protein encoded by this gene on this plasmid has an amino-terminal histidine tag and consists of the carboxy-terminal 261 amino acids of intimin$_{O157}$ from EHEC O157:H7 (Int$_{261}$).

Plant-expression Plasmid Construction. The nucleotide sequence for the VspA signal peptide was amplified by PCR from pHB306 (Richter *et al.*, 2000). A fragment that contained the nucleotide sequences for tobacco etch virus (TEV) 5'-UTR and the VspA signal peptide (encodes M A M K V L V F F V A T I L V A W G A) was ligated

into the *Xho*I-*Sac*I sites of pIBT210.1 (Haq *et al.*, 1995) to make pBTI210.4. Plasmid

pBTI210.4 codes for a plant-specific signal peptide 5' to a *Sac*I site, a cauliflower mosaic

virus (CaMV) 35S promoter, TEV 5'-UTR, and a VspB 3' flank. Plasmid pBTI210.3

was created by digestion of pHB211.1 (Richter *et al.*, 2000) with *Nco*I . This restriction

enzyme digested DNA was 3' end-filled with Klenow polymerase and then further

digested with *Xho*I and mung bean nuclease. The resultant blunt-ended vector fragment

was ligated to generate pHB211. The HindIII-NcoI fragment of pHB211 that contained

the sequences for the CaMV 35S promoter fused to the tobacco mosaic virus (TMV) 5'-

UTR was ligated into pIBT210.1 to create pBTI210.3.

Plasmids that encoded full-length or histidine-tagged fragments of intimin$_{O157}$ for

expression in plants were constructed by standard procedures. PCR was used to amplify

full-length intimin$_{O157}$ from pEB310 (McKee *et al.*, 1995). The primers used,

TGGTGGATCCATAACATGATTAC (forward) and

CTAGGAGCTCTTATTCTACACAAACCGCATAG (reverse), contained *BamHI* and

SacI sites respectively. The amplified product was cut with *BamHI* and *SacI* and ligated

into pBTI210.3 to make pNR9. Plasmid pNR9 was cut with *NcoI*, subjected to mung

bean digestion, and the resultant blunt-ended vector was religated to create pNR23. The

EcoRI-HindIII fragment of pNR23 was cloned into pGPTV-Kan to create pNR12, a

plant-expression construct that was designed to express full-length intimin$_{O157}$. Plasmid

pNR12 was then transformed by electroporation into *A. tumefaciens* EHA105.

PCR was used to amplify the DNA that contained the gene for histidine-tagged

intimin$_{O157}$ from pEB313 (McKee and O'Brien, 1996). The primers used,

TTAACCATGGGAGGATCTCAC (forward) and

CAGATTTTACCCGGGGTGGTTATGG (reverse), contain *NcoI* and *SmaI/XmaI* sites

respectively. The amplified product was cloned into a T-tailed bluescript vector from the

TA cloning® kit (Invitrogen, Carlsbad, CA). The resulting plasmid was cut with *NcoI*

and *SmaI* and ligated into pBTI210.3 to generate pNR27. The *EcoRI-HindIII* fragment of

pNR27 was cloned into pGPTV-Kan to make pNR14, a plant-expression construct that

was designed to express histidine-tagged, slightly truncated (minus 35 amino acids from

the amino-terminus) intimin$_{O157}$. Plasmid pNR14 was then transformed by

electroporation into *A. tumefaciens* EHA105.

The DNA that contained the gene for the histidine-tagged carboxy-terminal third

of intimin$_{O157}$ (the carboxy terminal 261 amino acids; Int$_{261}$) was amplified by PCR from

pNR37. The primers used, GAGAGGAGCTCCACCATCACC (forward) and

ACTGGAGCTCGACCCGGGGTACC (reverse), each contain a *SacI* restriction site.

The amplified product was cut with *SacI* and cloned into pBTI210.4 to make pNR45.

The *EcoRI-HindIII* fragment of pNR45 was cloned into pGPTV-Kan to create pNR49, a

plant-expression construct that was designed to express the histidine-tagged carboxy

terminal third of intimin$_{O157}$ with a plant-specific signal peptide on the amino-terminus of

the protein. Plasmid pNR49 was transformed by electroporation into *A. tumefaciens*

EHA105.

The DNA that contained the gene for the histidine-tagged carboxy-terminal third

of intimin$_{O157}$ was amplified from pNR37 by PCR. The QuikChange™ Site-directed

Mutagenesis Kit (Stratagene) was used to introduce an NcoI site just 5' of the histidine-

tagged gene. The primers used to create the NcoI site were,

CAGAATTCATTAAAGAGGAGAAATTAACCATGGGAGGATCTCACC (forward)

and GGTGAGATCCTCCCATGGTTAATTTCTCCTCTTTAATGAATTCTG (reverse).

The plasmid was cut with *NcoI* and *KpnI*, and a fragment that contained the Int_{261} gene

was cloned into pBTI210.3 to make pNR44. The *EcoRI-HindIII* fragment of pNR44 was

cloned into pGPTV-Kan to make pNR50, a plant-expression construct that was designed

to express the carboxy-terminal third of $intimin_{O157}$ without a signal peptide. Plasmid

pNR50 was transformed by electroporation into *A. tumefaciens* EHA105.

Adherence Assay. HEp-2, human laryngeal epithelial cells (ATCC# CCL23)

were maintained by serial passage in complete MEM (Eagles minimal essential medium

[BioWhittaker, Walkersville, MD], 10% fetal bovine serum, 20 mM L-glutamine, 100

μg/ml gentamicin and 100 U penicillin G). Assays to assess adherence of EHEC to HEp-

2 cells were done as previously described (Gansheroff *et al*., 1999) with slight

modifications. HEp-2 cells were seeded into eight well plastic Lab-Tek® chamber slides

(Nunc, Inc., Naperville, IL) at $2x10^5$ cells per well and grown for 24 hours to form

subconfluent monolayers. Freshly grown bacterial cultures were inoculated into 0.5 ml

Luria broth and grown overnight statically. Preimmune sera and sera from immunized

animals were diluted directly into 300 μl of adherence medium (Eagles minimal essential

medium, 1% mannose, 0.4% sodium bicarbonate). After preliminary testing, sera

dilutions of 1:60 and 1:100 were chosen to assess serum antibody adherence-blocking

activity. Similar dilutions of PBS (phosphate buffered saline pH 7.2) also served as

additional controls. Bacteria and diluted sera were preincubated at 37°C for 35 minutes.

The tubes were gently mixed 3 times during this period. The HEp-2 cell monolayers

were washed 2 times with Hank's balanced salt solution (with Mg^{2+} and Ca^{2+}), the

preincubated solutions of sera and bacteria were added to the cells, and the HEp-2 cells

and bacteria were then incubated for 2 1/2 hours at 37°C. The cell layers were washed

once with Dulbecco's PBS (with Mg^{2+} and Ca^{2+}), and fresh arabinose adherence medium

(Eagles minimal essential medium, 2% arabinose, 0.4% sodium bicarbonate) that

contained sera (or PBS) was added for a second 2 1/2 hours incubation period. The

monolayers were washed 6 times with PBS and fixed with 300 μl 3% formaldehyde for

20 minutes at room temperature. The fix was removed and the cell layers washed an

additional 2 times with PBS. The slides were then mounted by means of the SlowFade™

Antifade kit (Molecular Probes, Eugene, OR). The cell layers were examined with an

Olympus BX60 system microscope with a BX-FLA reflected light fluorescence

attachment. All images were obtained with a SPOT RT CCD digital camera (Diagnostic

Instruments, Inc., Sterling Heights, MI). Reduced adherence in the HEp-2 cells

adherence assay was determined by visual, qualitative analysis of the digital images.

In addition to the bacterial adherence assay, the serum samples were also tested

for the capacity to kill EHEC O157:H7 strain 86-24 in a bactericidal assay. Bacteria and

sera, or PBS control, were added to 300 μl adherence medium as described above. After

3 hours at 37°C, the samples were diluted in PBS and plated on MacConkey agar with

sorbitol to determine the CFU per ml.

Plant Materials, Growth, and Transformation. Untransformed *Nicotiana*

tabacum cv Bright Yellow 2 (NT-1) cells (An, 1985) were obtained from The Boyce

Thompson Institute for Plant Research (Ithaca, NY). The NT-1 cells were grown at 25°C

on a rotary shaker (150 rpm) in 40 ml NT medium (Murashige minimal organics medium

[LifeTechnologies, Gaithersburg, MD], 30 g/L sucrose, 3 μM thiamine, 0.58 mM myoinositol, 1.3 mM KH_2PO_4, 1 μM 2,4-Dichlorophenoxyacetic acid (2,4-D), 2.5 mM 2[N-morpholino]ethanesulfonic acid, pH 5.7). These cells were subcultured (1 part cells:19 parts NT medium) every 7 days. To facilitate transformation, binary vectors of interest were introduced into *A. tumefaciens* EHA105 by electroporation (200 Ω, 2.5 kV, 25 μF). *Agrobacterium* strains that contained the construct of interest were used to transform NT-1 cells as previously described (An, 1985) with minor modifications. Three days after subculture, acetosyringone was added to the NT-1 cells to a final concentration of 20 μM, and the cells were abraded by repeated pipeting (~20 times with a 10 ml pipet). Four milliliters of cells were cocultivated with 10-15 μl of *Agrobacterium* [*Agrobacterium* strains were grown at 30°C overnight in YM medium (0.4g/L yeast extract, 10g/L mannitol, 0.1g/L NaCl, 0.2 g/L $MgSO_4$-$7H_2O$, and 0.5 g/L K_2HPO_4) to an OD_{600} of approximately 0.5] for 3 days. The cells were washed 3 times with 50 ml NTC (NT-1 medium that contained 500 μg/ml carbenicillin) followed by sedimentation at 1000 rpm for 4 minutes to remove the *Agrobacterium*. The cells were then suspended in 4 ml NTC and plated on 2 NTCK plates [NT-1 medium that contained 500 μg/ml carbenicillin and 200 μg/ml kanamycin with 0.7% Agar type A (Sigma, St. Louis, MO)]. After 3-4 weeks, individual transformed calli were transferred to fresh NTCK plates and grown at 25 °C (Figure 3).

Arabidopsis thaliana transformation by *Agrobacterium tumefaciens* was done at Boyce Thompson Institute for Plant Research in Ithaca, NY by means of the floral-dip transformation protocol outlined by Clough and Bent (Clough and Bent, 1998). *Arabidopsis* plants were transformed with *Agrobacterium* strains that contained pNR12,

pNR14, pNR49, or pNR50. Seeds from transformed plants were grown and leaf material was sent to Uniformed Services University. The leaf material was processed in either NT-1 cell extraction buffer or directly into sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer before being subject to SDS-Page and subsequent Western blot analysis.

Detection of Intimin Expressed in Plant Cells. A small amount of plant cell material (~0.03 g), resuspended in an extraction buffer (25 mM sodium phosphate, pH 6.6, 100 mM NaCl, 10 mM EDTA, 0.1% Triton X-100, 10 µg/ml leupeptin, and 50 mM sodium ascorbate), was disrupted by sonication (25 second pulse; 5 sec. on, 10 sec. off). The lysed material was clarified by centrifugation (12,000 x g, 3 minutes, 4°C), and proteins in the extract were subjected to SDS-PAGE (4% stacking gel, 10% separating gel) using a Mini Protean II slab cell apparatus according to the manufacturer's instructions (BioRad, Hercules, CA). The separated proteins on the Tris Glycine SDS-PAGE gels were transferred onto nitrocellulose and probed with anti-intimin monoclonal or polyclonal antibodies (Levine, 1987; Gansheroff *et al*., 1999).

Immunoblotting. The proteins separated by SDS-PAGE were electroblotted onto nitrocellulose. The membranes were blocked with 5% nonfat dried milk in Tris-buffered saline pH 8, with 0.1% (vol/vol) Tween 20 (TBS+T) overnight and incubated 1 hour with primary antibodies diluted 1:5000 in TBS+T plus 5% milk. Membranes were washed in TBS+T and incubated for 1 hour in TBS+T plus 5% milk with the appropriate horseradish peroxidase-conjugated secondary antibody (BioRad). The signal was

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Figure 3. Examples of NT-1 cells transformants. NT-1 cells newly transformed with *A. tumefaciens* strains that carried plant expression constructs were plated as a lawn on NT agar plates supplemented with carbenicillin and kanamycin (NTCK). After approximately three weeks, kanamycin resistant NT-1 cell clones were distinguished as actively growing cell clusters on a lawn of susceptible, not actively growing cells (panel A). At 6 weeks, the kanamycin resistant cell clusters were sufficiently large to be subcultured onto to fresh NTCK plates (panel B). After an additional month of growth on the fresh NTCK plate, the transformed NT-1 cell clone clusters were of the appropriate size to start NTCK broth cultures or to harvest samples of the cluster for testing of antigen protein expression (panel C).
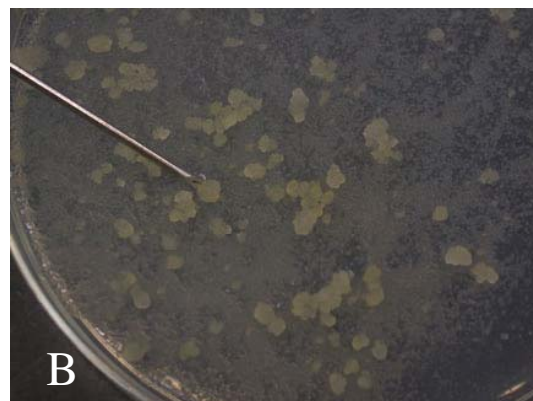
visualized by chemiluminescence with the ECL™ (Enhanced Chemiluminescence)-Plus detection kit from Amersham (Piscataway, NJ). Antigen amounts were quantified by comparing the Western band intensity to that of a control of known concentration with NIH Image 1.61 (Sinclair and O'Brien, 2002).

Mouse Feeding Assay

   NT-1 cell feeding assay. NT-1 cells or transgenic NT-1 cell clones that expressed full length or his-tagged $intimin_{O157}$, or Int_{261} were grown in 40 ml suspension culture to confluency (about 7 days). The cells were pelleted by gentle centrifugation, and culture medium was removed. Five grams of NT-1 cell material were aliquoted into individual small, plastic weigh dishes, and 0.5 grams of sucrose was added to each sample. The amount of intimin protein delivered in the 5 g dose was determined semi-quantitatively as described above. A 7.5 μg dose of purified cholera toxin (Sigma) was also added to appropriate samples to serve as an oral adjuvant. The addition of this dose of CT did not have any detrimental effects on the mice (i.e., loose stool or diarrhea). Female BALB/c mice, 16-18g, (Charles River Laboratories, Inc.) were fasted overnight before they were allowed to eat the plant material *ad libitum*. The 5 g dose of the plant material was usually consumed within 8 hours. The mice were bled by tail vein cut, and fecal pellets were collected both prior to and after feeding. Blood samples were allowed to clot at room temperature for 15 minutes and centrifuged at 10,000 x g for 10 minutes at 4°C to pellet red blood cells. A second, 2 minute centrifugation step was used to further clarify each serum sample. These clarified serum samples were stored at -20°C until assayed. Fecal samples were frozen, lyophilized overnight, and then homogenized in

PBS, pH 7.2 (for every 15 fecal pellets, 0.8 ml PBS was used). The resuspended fecal

material was centrifuged for 5 minutes, and each clarified supernatant was transferred to

a clean tube and stored at -20°C until assayed. Mice immunized intraperitoneally (i.p.)

with purified his-tagged $intimin_{O157}$ [from pEB313 (McKee and O'Brien, 1996)], or Int_{261}

[from pMW103 (Gansheroff *et al*., 1999)] + TiterMax®, 20 µg on day 0 and 10 µg on

days 7 and 14, served as the positive control.

   ELISA. To measure anti-$intimin_{O157}$ antibodies, an ELISA was developed. For

that purpose, U-bottomed 96-well microtiter plates were coated with 50 ng/well purified

histidine-tagged Int_{261} from pMW10 (Gansheroff *et al*., 1999). Serum samples were

serially diluted from a titer of 1:50 to 1:781250 and fecal pellet extracts were diluted

from 1:50 to 1:3200 and incubated overnight at 4°C. The plates were washed, and goat

anti-mouse IgG (1:3000) or goat anti-mouse IgA (1:3000) conjugated to alkaline

phosphatase was added to the appropriate plates. The plates were then incubated at room

temperature for 1 hour. The antigen-antibody reactions were detected colorimetrically by

incubation with tetramethylbenzidine (TMB) peroxidase substrate (BioRad) for 15

minutes followed immediately by measurement of the absorbance at 600 nm with an

ELx800 microtiter plate reader (Bio-Tek Instruments Inc., Winooski, VT). The titer was

defined as the reciprocal of the highest dilution of serum or fecal extract that gave an

absorbance reading above both background and pre-immune levels. Mice were

considered responders if they had detectable serum or fecal titers at the last collection

point. Mice with no titer or that lost titer by the last collection point were called non-

responders. The geometric mean of the responders' titers was used to determine the mean response per group.

Nickel Affinity Chromatography of His-tagged Intimin from Plant Cells.

Approximately 20 ml of packed NT-1 cells from a 7 day shaking culture was resuspended in 25 ml modified extraction buffer (25 mM sodium phosphate, pH 6.6, 100 mM NaCl, 0.1% Triton X-100, 10 μg/ml leupeptin, and 50 mM sodium ascorbate) and subjected to sonic disruption (5 minutes; 30 sec. on/off). The sonic lysate was centrifuged to remove any cellular debris and was mixed with 4 ml of equilibrated Ni-NTA slurry (Qiagen Inc.) at 4°C for 1 hour. Intimin was purified by a non-denaturing protocol as outlined in the Qiagen manual. The protein was eluted with 10 ml of 250 mM imidazole, pH 7.8 and collected in 5, 2 ml fractions. Samples from the clarified sonic lysate, wash, and each elution fraction were subject to SDS-PAGE, electroblotted to nitrocellulose, and were visualized with an anti-intimin$_{O157}$ polyclonal antibody (Gansheroff *et al*., 1999). Elution fractions that contained the intimin$_{O157}$ protein of interest were pooled and dialyzed against 1 liter of PBS pH 7.2 overnight at 4°C. The pool of samples was concentrated, and the amount of protein was determined by BioRad protein assay. The purity of the sample pool was estimated by Coomasie stain (Bio Rad) of the material after separation on SDS-PAGE gels. Western blot analysis was done to ascertain that the bands seen were indeed intimin$_{O157}$.

Mouse Model of Intimin$_{O157}$ -dependent Colonization. Female BALB/c mice, 16-18 g, (Charles River Laboratories, Inc.) were fasted over night and water was removed

from cages at least 4 hours prior to the inoculation. The bacterial inoculum was prepared

by seeding a 25 ml LB culture that contained 100 μg/ml streptomycin with 2-3 colonies

of streptomycin-resistant EHEC O157:H7 strain 86-24 Str^R or 86-24 Str^R *eae*Δ10 each

from a fresh overnight plate culture. The broth culture was shaken at 37°C for 3 hours.

The entire culture was centrifuged and resuspended in 2.5 ml freshly-prepared, filter-

sterilized 20% sucrose. The mice were scruffed and allowed to drink 50 μl of the inocula

from a micropipet. Animals received a total inoculum of 10^8-10^9 colony forming units

(CFU) in each of two doses administered 4 hours apart. Fecal pellets were collected

from individual mice daily, weighed, serial dilutions were made in PBS, and plated on

sorbitol-MacConkey agar (Wells *et al*., 1983) with 100 μg/ml streptomycin to determine

CFU/g feces and duration of colonization. In addition, several sorbitol-negative colonies,

from different mice at each collection time point were tested by slide agglutination with

O157 antiserum (Difco Laboratories, Sparks, MD) to further confirm they were colonized

with the challenge strain. All mice were free of Str^R, O157 agglutination-positive

colonies prior to challenge.

Statistics. The SPSS 11.0 statistical package was used to calculate the mean

duration of colonization and the duration of colonization among mouse groups. The Log-

rank test was used to determine the statistical significance of the difference in duration of

colonization among the mouse groups. The two-sided t-test for independent samples was

used to determine the statistical significance between the groups' serum and fecal

antibody titers. The Fisher exact test was used to compare the significance of the

difference in the number of mice per group making an intimin$_{O157}$-specific immune response.

Stx2 Toxoid

Vero Cell Cytotoxicity Assays. Ninety-six well microtiter plates were seeded with $1x10^4$ Vero cells (Green monkey kidney cells) per well, 24 hours before the start of the assay. Bacterial cell lysates were prepared by sonically disrupting cells from broth cultures. The lysates were then centrifuged to remove cellular debris. The clarified supernatants were serially diluted in tissue culture media and added to the wells of the Vero cell microtiter plates (Gentry and Dalrymple, 1980). The plates were incubated for 48 hours then the adherent cells in each well were fixed with 10% formalin and stained with crystal violet. The absorbance at 600 nm was measured in each well with the ELx800 microtiter plate reader (Bio-Tek Instruments, Inc.). The cytotoxic titer per milliliter of culture lysate was expressed as the reciprocal of the highest dilution required to kill 50% of the cells in a well (CD$_{50}$/ml).

Stx2 Toxoid Construction and Purification. Two changes were made to the nucleotide sequence of the Stx2 A subunit gene from pMJ100 (Weinstein *et al*., 1989) with the Stratagene QuikChange™ Site-directed Mutagenesis Kit (Stratagene). The first change was the substitution of a serine for a tyrosine at amino acid position 77 (Y77S) by means of the primers,

GATTATTGAGCAAAATAATTTAT<u>C</u>TGTGGCCGGGTTCGTTAATACGG (forward)

and CCGTATTAACGAACCCGGCCACA<u>G</u>ATAAATTATTTTGCTCAATAATC

(reverse). The second change was the substitution of a glutamine for a glutamate at

amino acid position 167 (E167Q) by means of the primers,

GTTTTGTCACTGTCACAGCA<u>C</u>AAGCCTTACGCTTCAGGCAG (forward) and

CTGCCTGAAGCGTAAGGCTT<u>G</u>TGCTGTGACAGTGACAAAAC (reverse). The

resulting plasmid that contained both changes was called pNR100. Bacterial cell lysates

from this clone were tested for cytotoxicity by the Vero cell cytotoxoicity assay (as

described above), and the toxoid protein was visualized by Western blot analysis with

rabbit anti- Stx2 antibodies (prepared by Ms. Edda Twiddy).

Stx2 toxoid protein was expressed by growing 500 ml pNR100 cultures (OD_{600} ~

0.5) with 1mM IPTG for 5 hours shaking at 37°C. The bacterial cultures were harvested

and centrifuged to remove the culture supernatants, and the bacterial pellets were frozen

overnight. The pellets were thawed and then resupended in 20 ml TEAN (50mM Tris pH

7.5, 1mM EDTA, 0.2M NaCl, 0.02% sodium azide) for every 500 ml of starting culture.

The resuspended cells were sonically disrupted (10 min; 30 sec on/off) and then pelleted

by centrifugation to remove cellular debris. The clarified sonic lysates were stored at -

20°C until used.

A 3 ml antibody affinity column with 3 mg/ml of the anti-Stx2 A subunit

monoclonal antibody (MAb) 11E10 was kindly prepared by Ms. Edda Twiddy. (Perera *et

al.*, 1988). The 11E10 MAb was linked to AminoLink plus resin (Pierce, Rockford, IL)

by the pH 10 coupling method according to the Pierce manual. The column was stored in

TEAN at 4°C.

The pNR100 Stx2 toxoid clarified sonic lysate was thawed, and the 11E10 antibody affinity column was brought to room temperature for 15 minutes prior to addition of the lysate. A 5 ml sample of sonic lysate was added to the column and allowed to bind at 4°C overnight. The column was washed with 10 bed volumes (~30 ml) TEAN until the OD_{280} was ~0.05, then the toxoid was eluted from the column with 0.1M glycine, pH 3 in 1 ml fractions. The OD_{280} values of these fractions were recorded, and the 3-4 fractions that showed a spike in OD_{280} (~0.06 or greater) were pooled, dialyzed against 1 liter PBS pH 7.2 overnight at 4°C, and concentrated. Small samples (~10 μl) of sonic lysate, column wash, pre-dialysis pooled elution fractions, and post-dialysis concentrated toxoid were subject to SDS-PAGE. The gels were Coomassie (Bio Rad) stained or transferred to nitrocellulose and visualized by immunoblot with Stx2 polyclonal antibody to ascertain the purity and approximate concentration of the toxoid (Stx2 polyclonal antibody prepared in rabbits by Ms Edda Twiddy). The isolated toxoid was then tested in a Vero cell cytotoxicity assay before evaluation as an immunogen in mice.

Stx2 Toxoid Immunizations in Mice. Female BALB/c mice, 16-18g, (Charles River Laboratories, Inc.) were injected i.p. with ~10 μg Stx2 toxoid + TiterMax® on day 0 and ~5 μg toxoid on days 10 and 25. One week after the final immunization, the mice were bled by tail vein cut, and the serum was assayed for the capacity to neutralize Stx2 toxin as described below.

Toxin Neutralization Assay. Vero cells were prepared as in the cytotoxicity

assay. Both pre and post-immune sera from mice immunized with the Stx2 toxoid were

serially diluted and added to the Vero cell plates. Purified Stx2 toxin lysate was diluted

to ~ 2.0 CD_{50} and ~20 CD_{50}. Two-fold dilutions of the serum samples were tested in

duplicate for capacity to neutralize both the low and high cytotoxic dose by incubating

the serum samples with the toxin for 3 hours at 37°C.   Samples of the mixtures (100 μl)

were added to the Vero cells, and the plates were incubated for 48 hours at 37°C then

fixed, stained, and read at 600 nm with a microtiter plate reader.

Stx2 Toxoid Plant Optimization. Three additional changes were made to the

nucleotide sequence of the Stx2 toxoid A subunit with the QuikChange™ Site-directed

Mutagenesis Kit to optimize the gene for expression in plants. The first and second

changes disrupted RNA destabilization motifs located from base 253 to 257 and 528 to

532. The primers used to alter base 253 were,

GTGTATATT<u>G</u>TTTAAATGGGTACTGTGCCTGTTACTGGG (forward) and

CCCAGTAACAGGCACAGTACCCATTTAAA<u>C</u>AATATACAC (reverse). The

primers used to alter base 532 were,

CTGATTATTGAGCAAAATAATTT<u>G</u>TCTGTGGCCGGGTTCG (forward) and

CGAACCCGGCCACAGA<u>C</u>AAATTATTTTGCTCAATAATCAG (reverse). The third

change disrupted a polyadenylation motif from base 1115 to 1120. The primers used to

alter base 1119 were,

GGCGACAGGCCTGTTATAAAAATAA<u>G</u>CAATACATTATGGG (forward) and

CCCATAATGTATTG<u>C</u>TTATTTTTATAACAGGCCTGTCGCC (reverse). All changes

were confirmed by sequencing. The optimized Stx2 toxoid A subunit was amplified by

PCR with primers designed to introduce 5' *XbaI* (GGAACACCTCTAGATGAAGTG)

and 3' *SacI* (GCTGAGCTCCTTTATTTACCCG) sites flanking the Stx2 A subunit. The

XbaI-SacI fragment was cloned into pIBT210 (Haq *et al*., 1995) to create pNAJ65.

Stx2 B: Plant Optimization and Synthesis. To optimize the Stx2 B subunit for

expression in plants, extensive changes to the nucleotide sequence of the gene were

required. It was not feasible to make the 37 nucleotide changes by site-directed

mutagenesis, so the entire Stx2 B subunit gene was re-synthesized by a ligase chain

reaction (LCR) protocol. The nucleotide sequence of the plant optimized Stx2B subunit

is listed in Figure 4. The primers used in the Stx2 B subunit synthesis are summarized in

Table 5.   The LCR method of Chalmers and Curnow, with minor modifications, was

used for the LCR (Chalmers and Curnow, 2001). Briefly, all of the oligonucleotides

were resuspended in water to 250 μM, and 15 μl of each was premixed and

phosphorylated with T4 polynucleotide kinase for 2 hours at 37°C. The reaction was

stopped by heating the samples to 80°C for 10 minutes. A 40 μl sample of the heat-

inactivated phosphorylation reaction was aliquoted to a fresh tube, and 4 μl 10X Pfu

ligase buffer and 2μl Pfu ligase were added. The LCR reaction consisted of a 1 minute

hot-start at 95°C, then 40 cycles of 1 min 30 s at 55°C, 1 min 30 s at 70°C, and 30 s at

95°C, and final incubations at 55°C for 2 min and 70°C for 2 min. The resulting LCR

product (~360 base pairs) was amplified three times using the oligos 2B1 and 2B10

(Table 5) at 10 μM each. The LCR product was gel purified, digested with *BspHI* and

SmaI, and ligated into the *NcoI-SmaI* site of pBTI210.3 to create pNAJ73. The sequence

of the synthetic B subunit gene in pNAJ73 that was made by LCR was confirmed and contained no additional PCR-induced mutations despite amplification of the LCR product 3 times to produce sufficient product to cut and clone.

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Figure 4. The nucleotide sequence of the plant-optimized Stx2 B subunit gene. Thirty seven individual nucleotide base changes (in bold) were made to the original Stx2B subunit gene to optimize the gene for expression in plants. Flanking sequence that incorporated specific restriction enzyme sites (*BspHI* and *SmaI*, underlined) was added for cloning purposes. The ATG start and TAA stop codons are designated in italics.

5' - GCAT GCAT CGAT CGAT GCTA GCTA TAGC TAGC ATCG ATCG TCAG

AA<u>TC</u> *<u>ATG</u>* <u>A</u>AG AAG ATG TT**C** ATG GC**T** GT**G** **C**T**C** TTT GCA TT**G** GCT TCT

GT**G** AAT GCA **T**TG GC**A** GC**T** GAT TGT GCT AA**G** GGT AA**G** ATT GAG TT**C**

TCC AAG TA**C** AAT GAG GAT GA**T** ACA TT**C** ACA GTG AAG GT**G** GA**T** GGG

AA**G** GA**G** TAC TGG ACC AGT **A**G**G** TGG AA**C** CT**C** CAA CC**A** **C**T**C** CT**C** CAA

AGT GCT CA**A** TTG ACA GGA ATG ACT GTC ACA ATC AA**G** TCC AGT ACC

TGT GA**G** TCA GGC TC**T** GGA TTT GCT GA**G** GTG CA**A** TT**C** AA**C** AAT GAC

TAA <u>CCCGGG</u> ATGC GCAT AGTA TCAG GATC CGAT CATG ACTG CGTA – 3'

Table 5. Stx2 B Subunit Ligase Chain Reaction Oligonucleotides

2B1	GCAT GCAT CGAT CGAT GCTA GCTA TAGC TAGC ATCG ATCG
2B2	TCAG AATC ATG AAG AAG ATG TTC ATG GCT GTG CTC TTT GC
2B3	A TTG GCT TCT GTG AAT GCA TTG GCA GCT GAT TGT GCT AAG
2B4	GGT AAG ATT GAG TTC TCC AAG TACAAT GAG GAT GAT ACA T
2B5	TC ACA GTG AAG GTG GAT GGG AAG GAG TAC TGG ACC AGT AG
2B6	G TGG AAC CTC CAA CCA CTC CTC CAA AGT GCT CAA TTG ACA
2B7	GGA ATG ACT GTC ACA ATC AAG TCC AGT ACC TGT GAG TCA G
2B8	GC TCT GGA TTT GCT GAG GTG CAA TTC AAC AAT GAC TAA CC
2B9	CGGG ATGC GCAT AGTA TCAG GATC CGAT CATG ACTG CGTA
2B10	TACG CAGT CATG ATCG GATC
2B11	CTGA TACT ATGC GCAT CCCGGG TTA GTC ATT GTT GAA TTG
2B12	CAC CTC AGC AAA TCC AGA GCC TGA CTC ACA GGT ACT GGA C
2B13	TT GAT TGT GAC AGT CAT TCC TGT CAA TTG AGC ACT TTG GA
2B14	G GAG TGG TTG GAG GTT CCA CCT ACT GGT CCA GTA CTC CTT
2B15	CCC ATC CAC CTT CAC TGT GAA TGT ATC ATC CTC ATT GTA C
2B16	TT GGA GAA CTC AAT CTT ACC CTT AGC ACA ATC AGC TGC CA
2B17	A TGC ATT CAC AGA AGC CAA TGC AAA GAG CAC AGC CAT GAA
2B18	CAT CTT CTT CAT GATT CTGA CGAT CGAT GCTA GCTA TAGC
2B19	TAGC ATCG ATCG ATGC ATGC

Results

I.   **Expression and antibody response to transgenic plant-expressed intimin$_{O157}$.**

NT-1 plant cells that express full-length and his-tagged intimin$_{O157}$. We began our studies by constructing two expression vectors, pNR12, that contained the full-length intimin$_{O157}$ gene from pEB310 (McKee *et al*., 1995) and pNR14, that contained the gene for histidine-tagged intimin$_{O157}$ from pEB313 lacking the signal peptide (McKee and O'Brien, 1996) (Fig. 5). The expression of both proteins was driven by a cauliflower mosaic virus 35S promoter (CaMV 35S). The expression constructs also included a tobacco mosaic virus Ω 5' untranslated region (TMV Ω 5'-UTR) and a 3' soybean vegetative storage protein polyadenylation sequence (3' VSP) to facilitate translational initiation and 3' end processing, respectively. A neomycin phosphotransferase gene (*ntp2*) that conferred kanamycin resistence was included in the constructs to facilitate detection of successfully transformed plant cell clones. When these plant expression constructs were transformed into NT-1 cells by *Agrobacterium*-mediated transfer, 6 clones (3 full-length intimin$_{O157}$, 3 his-tagged intimin$_{O157}$) were obtained out of approximately 100 tested (~50 clones from each transformation) that expressed appreciable levels of intimin$_{O157}$ protein. Furthermore, the expressed protein appeared to be the same size (~94 kD) as purified intimin$_{O157}$ expressed from pEB313 (McKee and O'Brien, 1996) (Fig. 6).

Figure 5. Full-length and his-tagged intimin$_{O157}$ plant-expression constructs. The full-length intimin$_{O157}$ plant-expression construct that contained the intimin$_{O157}$ gene from pEB310 (McKee *et al*., 1995) was called pNR12. The his-tagged intimin plant-expression construct that contained the intimin$_{O157}$ gene from pEB313 (McKee and O'Brien, 1996) was named pNR14. The his-tagged intimin$_{O157}$ gene was missing 35 amino acids (signal peptide) from the amino-termius and was his-tagged on the N-terminus as well. These expression vectors shared common plant expression elements such as a cauliflower mosaic virus 35S promoter (CaMV 35S), and a tobacco mosaic virus Ω 5' untranslated region (TMV Ω 5'-UTR) and a 3' soybean vegetative storage protein polyadenylation sequence (3' VSP) to facilitate translational initiation and 3' end processing, respectively. A neomycin phosphotransferase gene (*ntp2*) was incorporated into the constructs to confer kanamycin resistence to successfully transformed plant cell clones.

Full-length Intimin Expression
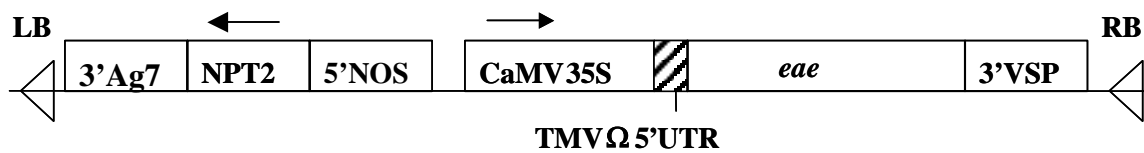
His-tagged Intimin Expression

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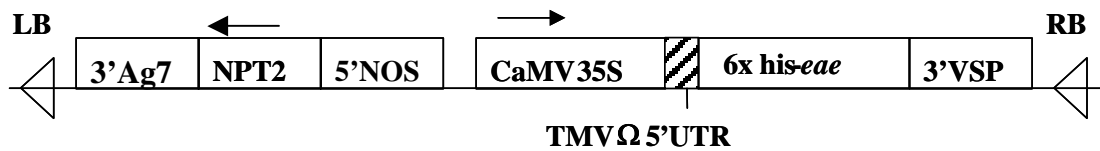
Figure 6. Western blot analysis of NT-1 cells that expressed his-tagged (H) and full-

length (F) inimtin$_{O157}$. Intimin$_{O157}$ (100 ng) purified from pEB313 (McKee and O'Brien,

1996) was used as a positive control. Extracts (~ 100 μg total protein) of NT-1 cells only

(NT-1 only) and NT-1 cells transformed with the pGPTV-Kan vector only (NT-1 +

vector) served as negative controls. Extracts of NT-1 cells transformed with pNR14 that

expressed his-tagged intimin$_{O157}$ (~94 kD) were named H11, H17, and H42. Extracts of

NT-1 cells transformed with pNR12 that expressed full-length intimin$_{O157}$ (~94 kD) were

named FL7, FL11, and FL25. The intimin$_{O157}$-specific proteins on this blot were detected

with an intimin$_{O157}$ monoclonal antibody (Gansheroff *et al*., 1999).
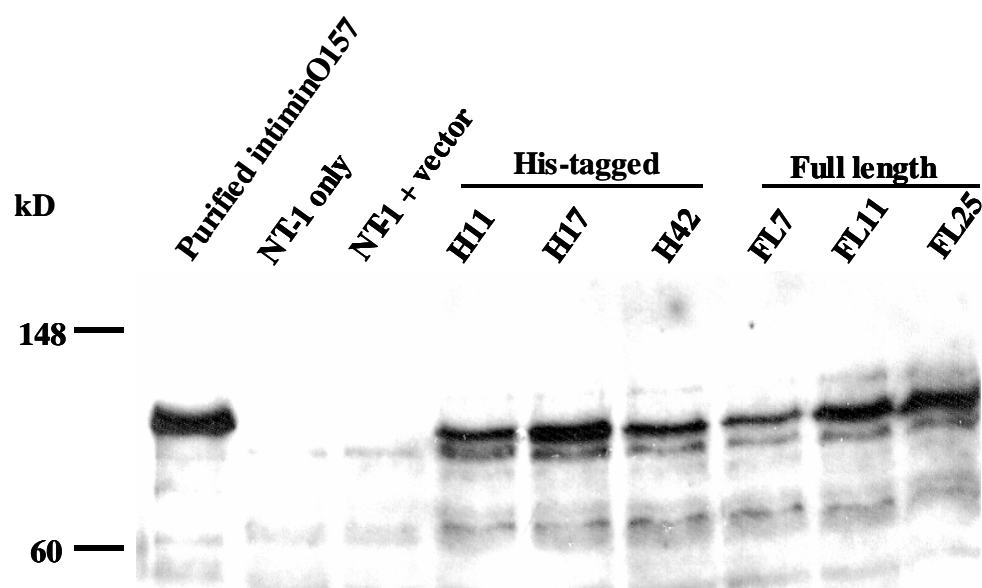
These $intimin_{O157}$-expressing transgenic NT-1 cell clones produced 8-10 μg of $intimin_{O157}$ per gram of total plant material. $Intimin_{O157}$ amounts were determined semi-quantitatively as described in the Materials and Methods (p. 39) The $intimin_{O157}$ expressed from plant cells reacted on Western blot analysis with an $intimin_{O157}$ polyclonal antibody, as well as an $intimin_{O157}$ monoclonal antibody (Levine, 1987; Gansheroff *et al.*, 1999), but not with an isotypic monoclonal antibody BC5 against another antigen, Stx 2B (Downes *et al.*, 1988) (Fig. 7). These latter Western blot findings demonstrate the specificity of the binding of the anti-$intimin_{O157}$ monoclonal antibody to the $intimin_{O157}$ produced in the plant cells. The majority of the $intimin_{O157}$ protein produced in the transgenic plant cells was soluble and present in the clarified sonic lysate; only a small amount of $intimin_{O157}$ was detected in the pellet of cellular debris after sonication and processing of the transgenic plant cells (Fig. 8).

Antibody response to full-length $intimin_{O157}$. Once we had established that $intimin_{O157}$ could be expressed in transgenic NT-1 cells at levels comparable to other proteins produced by plant cells {Dr. Hugh Mason, personal communication}, we chose one of our highest $intimin_{O157}$- expressing clones (FL25) to proceed with mouse i.p. and oral immunization assays. The first group of 5 mice was immunized i.p., three times over a period of six weeks, with FL25 plant cell extract that had been dialyzed overnight against PBS pH 7.4 and emulsified one to one with TiterMax®. The mice received about 300 ng $intimin_{O157}$ protein per injection of FL25 material containing approximately 500 μg total protein. The mice immunized with the FL25 material made a modest serum IgG

Figure 7. An isotypic monoclonal antibody to Stx2B did not recognize plant-expressed intimin$_{O157}$ from NT-1 cells. BC5 recognized Stx2 B subunit (~7 kD) in the Stx2 toxin lane, but did not react with any proteins from the plant cell extracts nor the purified intimin$_{O157}$ control (100 ng).

kD	Purified intiminO157	NT-1 only	NT-1 + vector	H17	FL25	Stx2 toxin		Purified intiminO157	NT-1 only	NT-1 + vector	H17	FL25	Stx2 toxin
148													
60													
42													
30													
22													
17													
6-4													

1° Antibody: Anti-intimin MAb   BC5 - anti-Stx2 B MAb

2° Antibody: Goat anti-mouse HRP   Goat anti-mouse HRP
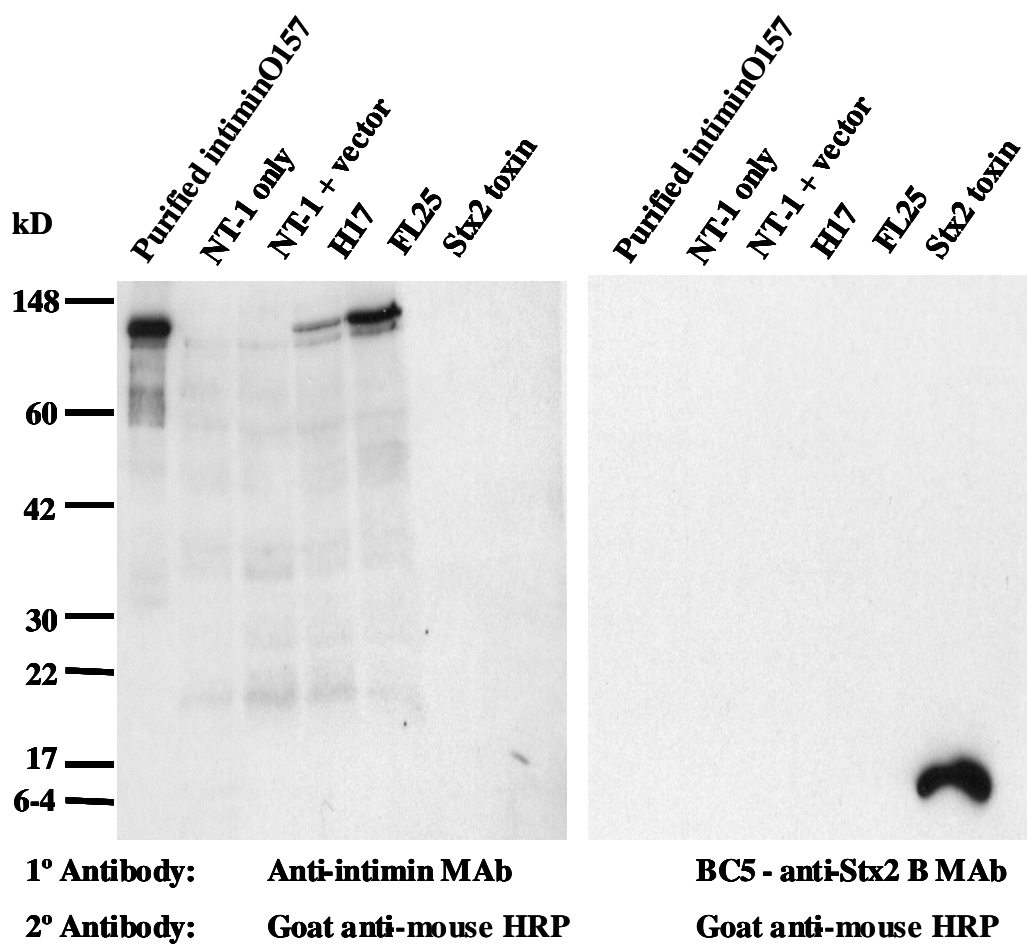
Figure 8. Western blot analysis of soluble intimin$_{O157}$ expressed in NT-1 cells. The

majority of the intimin$_{O157}$ protein produced in the transgenic plant cells was soluble and

present in the clarified sonic lysate (H17 and FL25 clarified lysate). Only a small amount

of intimin$_{O157}$ was detected in the pellet of cellular debris after sonication and processing

of the transgenic plant cells (H17 and FL25 pellet). 100 ng of purified intimin$_{O157}$ was

used as the control.
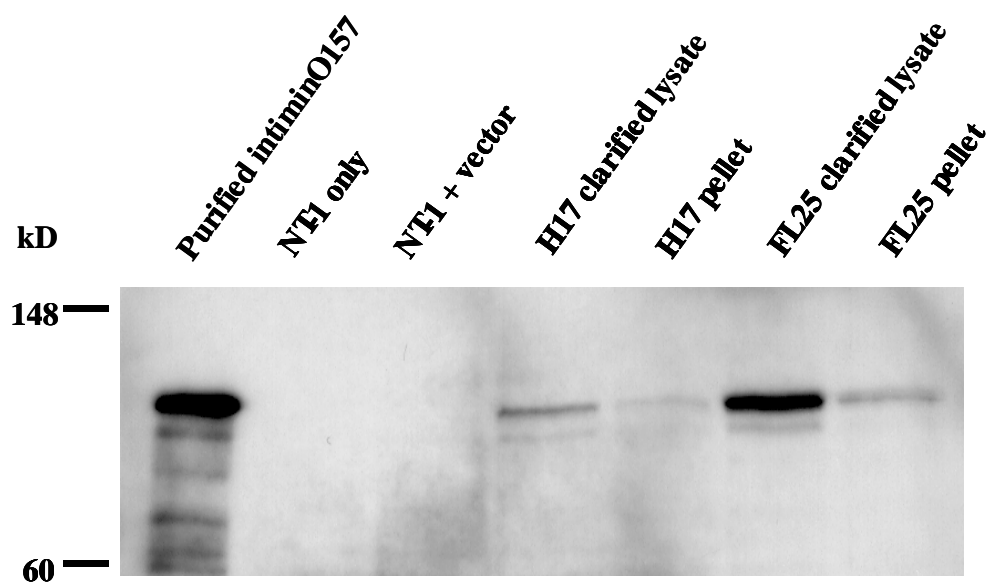
response to intimin$_{O157}$ (based on Western blot analysis), even though they received a

much smaller dose of intimin$_{O157}$ than the positive control mice (group n=5) that received

25 µg intimin$_{O157}$ per injection (Table 6). These results indicate that mice can make an

antibody response to the plant-expressed, full-length intimin$_{O157}$ when delivered i.p and

that these antibodies recognize intimin$_{O157}$ expressed from bacterial cells. Another 2

groups of mice (group n=5) were fed FL25 plant material that contained approximately

40 µg total of intimin$_{O157}$ in 4 grams of plant material, either with or without cholera

toxin (CT) as an oral adjuvant. The CT-B subunit has been shown to have strong adjuvant

properties for stimulating both serum IgG and mucosal IgA responses to unrelated, non-

coupled antigens after intranasal as well as oral co-administration (Tochikubo *et al.*,

1998). Mice that were fed FL25 plant material with CT as an oral adjuvant made both a

serum and fecal intimin$_{O157}$-specific IgG response based on Western blot analysis results

shown in Table 6. Even mice that were fed FL25 material without cholera toxin made a

recognizable intimin$_{O157}$-specific fecal IgG response. These promising initial results

suggested that plants could express intimin$_{O157}$ and that mice injected with or fed

transgenic plant material were able to mount an intimin$_{O157}$-specific immune response.

Optimization of NT-1 cell expression of the carboxy-terminal portion of intimin$_{O157}$.
Based on our results with the transgenic plant clone FL25, we decided to make changes

in the nucleotide sequence of a small portion of intimin$_{O157}$ in an attempt to optimize its

expression in NT-1 cells. We speculated that should such an increase in antigen

Footnotes for Table 6.

[a] Serum IgG results based on reactivity of immunized mouse sera on Western blot to purified $intimin_{O157}$ from pEB313 (McKee and O'Brien, 1996) and detection with a goat anti-mouse-horseradishperoxidase secondary antibody.

[b] Fecal IgG results based on Western blot reactivity of fecal extracts on to purified $intimin_{O157}$ from pEB313 (McKee and O'Brien, 1996).

[c] Qualitative immune response references as determined by Western blot analysis: ++++ = strong $intimin_{O157}$-specific positive response, ++ = modest positive response, + = weak positive response, - = no or undetecable $intimin_{O157}$-specific response

[d] ND – not determined

[e] Cholera toxin, 7.5 µg (Sigma)

Table 6. Immune response of BALB/c mice fed NT-1 cell that express intimin$_{O157}$

Group/Immunization	Vaccination Method	Serum IgG[a]	Fecal IgG[b]
1: Intimin$_{O157}$ from pEB313 (McKee and O'Brien, 1996) given i.p.	25 µg intimin$_{O157}$+TiterMax® i.p. days 0,14,21	++++[c]	++++
2: NT-1 cell lysate given i.p.	100 µl NT-1 cell lysate+ TiterMax® i.p. days 0,14,21	-	-
3: Intimin$_{O157}$ from FL25 given i.p.	300 ng intimin$_{O157}$ i.p. on days 0,14,21	+	ND[d]
4: Intimin$_{O157}$ from FL25 given i.p.	300 ng intimin$_{O157}$ + TiterMax® i.p. on days 0,14,21	++	ND
5: Fed NT-1 cells	4 g NT-1 cells fed on days 0,7,14	-	-
6: Fed FL25 cells	4 g FL25 (40 µg intimin$_{O157}$) cells fed on days 0,7,14	-	+
7: Fed FL25 cells + CT[e]	4 g FL25 (40 µg intimin$_{O157}$) cells + 10 µg CT fed on days 0,7,14	+	++

concentration result, we would likely elicit a more vigorous immune response in our mouse model and possibly eliminate the need for an adjuvant. We elected to work with the carboxy-terminal third of the intimin$_{O157}$ protein that is comprised of the C-terminal 261 amino acids (Int$_{261}$). The carboxy-terminus of intimin is the cell-binding domain of intimin$_{O157}$ (Frankel *et al*., 1994; Frankel *et al*., 1995), and we previously found that antibodies to the carboxy-terminal third of the molecule block adherence of wild type EHEC O157:H7 to HEp-2 cells (McKee and O'Brien, 1996; Gansheroff *et al*., 1999). The gene for Int$_{261}$ (*eae* 1959-2804) was obtained from pMW103 (Gansheroff *et al*., 1999) and thirteen independent changes were made to the nucleotide sequence of this gene with the QuikChange™ Site-directed Mutagenesis Kit (Stratagene) to create pNR73 (described in detail in the Materials and Methods). The Int$_{261}$ gene from pNR73 was cloned into two different plant cell expression vectors: pNR49 and pNR50 (Fig. 9). One of these clones (pNR49) was constructed so as to contain the sequence for a plant-specific signal peptide 5' to the start of the Int$_{261}$ gene. This signal peptide was added to increase expression of Int$_{261}$ in the plant cells by directing the translation of the optimized Int$_{261}$ into the endoplasmic reticulum of the plant cell where it would also be protected from cytosolic proteases and where it would be more accessible to any chaperones required for proper protein folding. In fact, 4 out of 5 of the first Int$_{261}$-expressing NT-1 cells clones with the signal peptide (CSP) produced appreciable levels of Int$_{261}$. One of these clones, CSP5, produced about 10 µg Int$_{261}$ (~35 kD) per gram of total NT-1 cell material as determined by Western blot analysis (as described in Materials and Methods, p.39). Mice fed this clone (total of 50 µg Int$_{261}$ each on days 0,7,and 14) with (n=5) or without (n=3) CT had Int$_{261}$-specific serum IgG and fecal IgA responses as determined

Figure 9. Carboxy-terminal third intimin$_{O157}$ (Int$_{261}$) plant cell expression constructs. Plasmids pNR50 and pNR49 were constructed to contain the plant-optimized gene for Int$_{261}$ from pNR73. In addition to the shared plant-expression elements (nptII, CaMV 35S, and vsp 3'), pNR49 was also designed to contain a plant-specific signal peptide. The signal peptide sequence gene in pNR49 was located so that when expressed it would be at the N-terminus of Int$_{261}$ and target expression of that protein directly into the plant cell endoplasmic reticulum. Plasmid pNR49 was also designed to include a tobacco etch virus 5' untranslated region (TEV 5'), which has the same function as TMV 5', to facilitate translational initiation.

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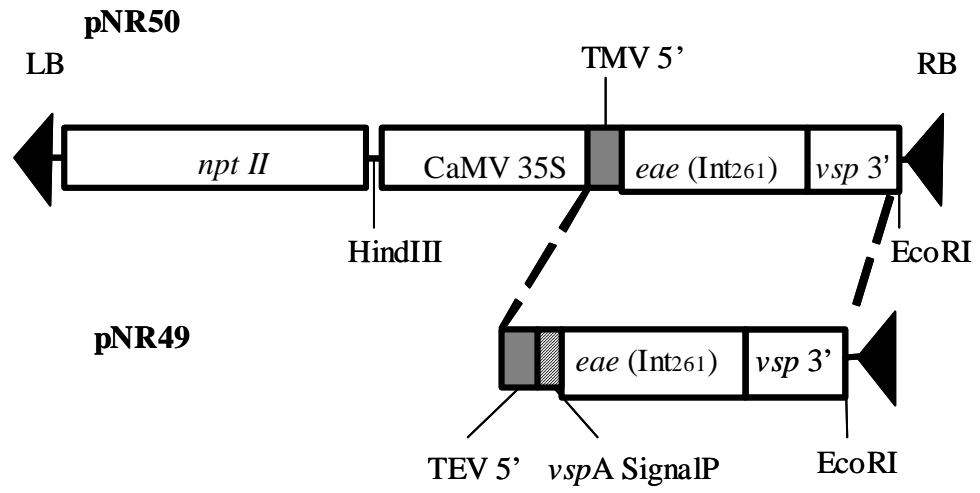
by an ELISA with purified Int_{261} from pMW103 (Gansheroff *et al.*, 1999) (Table 7) as

the antigen in the assay. The serum from mice that had an Int_{261}-specific IgG response

also blocked adherence of EHEC O157:H7 strain 86-24-GFP in a qualitative HEp-2 cell

bacterial adherence assay (Fig. 10). A bactericidal assay (described in Materials and

Methods, p. 34) done concurrently with the adherence assay showed that samples of

EHEC O157:H7 strain 86-24-GFP grown in adherence medium with immune sera grew

to the same density (avg., 1.81×10^9 CFU/ml) as bacteria grown in adherence medium

with PBS (1.66×10^9 CFU/ml). These results indicated that the reduced number of

bacteria observed on the adherence assays with immune serum was not due to bacterial

cell death. From these data with the sera from mice immunized with transgenic plant cell

material, we concluded that mice not only make Int_{261}-specific immune responses when

they are fed Int_{261}-expressing plant cells, but that this immune response is also functional

in blocking bacterial adherence of EHEC O157:H7 strain 86-24 in an *in vitro* assay.

Unfortunately, before additional mouse feeding assays could be done with CSP5, the

stock of this culture became contaminated with mold, Int_{261} expression was lost, and the

clone was unsalvageable (Fig. 11). Please note that untransformed and transgenic plant

cell stocks and clones must be kept in continuous culture because the plant cells do not

remain viable when frozen in glycerol stocks as do bacterial cells. Thus, these

continuous NT-1 cell cultures are at risk for contamination and can exhibit alterations in

protein expression (such as reduction or loss of protein expression) as the culture ages.

Further testing of NT-1 cell clones transformed with pNR49 revealed another

clone, CSP20, that expressed Int_{261} protein (~10-13 μg Int_{261} per gram of NT-1 cell

material), and this clone was used for further testing (Fig. 11). The initial mouse feeding

Footnotes for Table 7.

[a]Serum IgG and fecal IgA titers defined as the geometric mean of the reciprocal of the highest dilution of serum or fecal extract that gave an absorbance reading (A_{600}) above both background and pre-immune levels. Mice were considered responders if they had detectable serum or fecal titers at the last collection point. Mice with no titer or that lost titer by the last collection point were called non-responders. The titers reflect the geometric mean of responders only.

[b]Int_{261} affinity purified from pMW103 (Gansheroff *et al*., 1999)

*Serum of responders [in parenthesis (number of responders/total group number)] that blocked adherence of EHEC O157:H7 to HEp-2 cells *in vitro*. Serum from mice fed CSP20 was also tested but did not block adherence.

[c]Significantly higher serum IgG titer compared to other groups ($P<0.0001$; two-sided t-test). Number of serum IgG responders not significantly higher compared to other groups ($P>0.143$; Fisher's exact test).

[d]Fecal IgA titer not significantly higher compared to other groups ($P>0.1$; two-sided t-test). Number of fecal IgA responders not significantly higher compared to other groups ($P>0.487$; Fisher's exact test).

[e]Only three mice in group for sample collection after death of one mouse during blood draw.

[f]Cholera toxin, 7.5 μg (Sigma)

Table 7. Immune response of mice fed NT-1 cells expressing Int_{261}

NT-1 clone	Immunization Method	Serum IgG^a	Fecal IgA^a
CSP5	Control, i.p. 25 µg Int_{261}^b + TiterMax™, on day 0,14,21	56250 (4/4)* [c]	270 (3/4)[d]
	Fed 5g CSP5 (~50 µg Int_{261}) on days 0,7,14	50 (1/3)* [e]	156 (2/3)
	Fed 5g CSP5 (~50 µg Int_{261}) + CT^f on days 0,7,14	112 (4/5)*	156 (2/5)
CSP20	Control, i.p. 25 µg Int_{261}^b + TiterMax™, on day 0, 10 µg on days 14,21	781250 (5/5)* [c]	217 (5/5)[d]
	Fed 5g CSP20 (~65 µg Int_{261}) on days 0,7,14	137 (8/8)	75 (6/8)
	Fed 5g CSP20 (~65 µg Int_{261}) + CT^f on days 0,7,14	85.5 (6/8)	108 (6/8)

Figure 10. Serum from mice fed Int_{261}-expressing NT-1 cells block bacterial adherence to HEp-2 cells. Adherence of EHEC O157:H7 strain 86-24-GFP (Sinclair and O'Brien, 2002) to a HEp-2 cell monolayer was seen in the absence of serum (panel A). Panel B shows a similar pattern of adherence of EHEC O157:H7 strain 86-24-GFP in the presence of non-immune serum (1:60 dilution) from a mouse fed NT-1 cells only. Panel C shows reduced EHEC O157:H7 strain 86-24-GFP adherence in the presence of serum (1:60 dilution) from a mouse immunized orally with NT-1 cells expressing Int_{261} (CSP5). Reduced adherence determined by visual, qualitative analysis of the digital images.
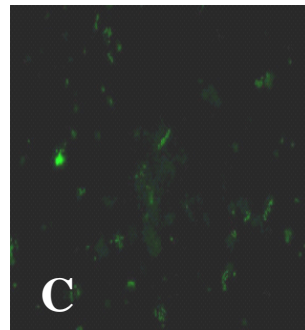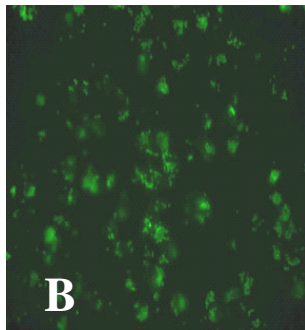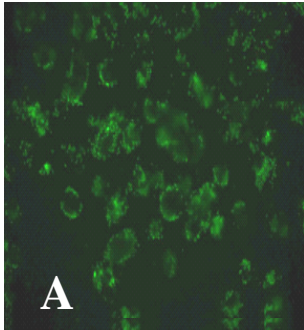
Figure 11. Western blot analysis of CSP5 extracts. CSP5 lost expression of Int_{261} and showed dramatically reduced overall protein expression levels after mold contamination. Two new NT-1 cell clones expressing Int_{261}, CSP17 and CSP20 are also shown. Int_{261} from pMW103 (75 ng) was used as a positive control (Gansheroff *et al*., 1999).
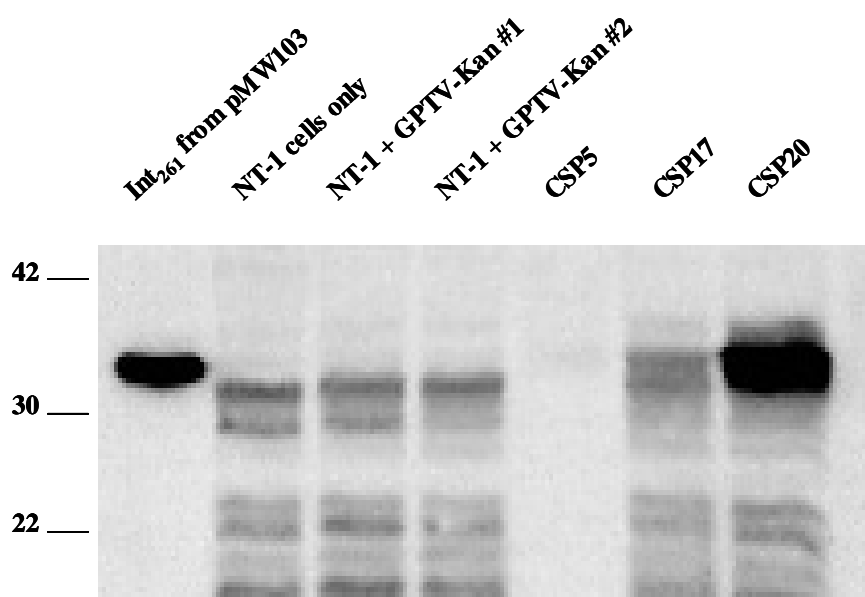
assays with CSP20 showed that mice fed CSP20 with or without CT made Int_{261}-specific serum and fecal antibody responses (Table 7, p. 70). However, mice immunized orally with CSP20 in a subsequent experiment did not elicit a detectable (titer detection limit >50) Int_{261}-specific immune response by ELISA (Table 8), and serum of immunized mice from the initial and subsequent experiments did not block adherence of EHEC O157:H7 strain 86-24 from HEp-2 cells *in vitro*. We hypothesized that the Int_{261} protein expressed from CSP20 was becoming altered over time in culture. CSP20 was in continuous culture for many months whereas CSP5 was cultured for less than a month before being used in initial feeding experiments (Table 7, p. 70). I decided to proceed by determining if and how Int_{261} in CSP20 was being modified.

Glycosylation of Int_{261} in CSP20. Prompted by the unexpected negative results detailed directly above, the Int_{261} expression from CSP20 was re-assessed. On Western blot analysis, Int_{261} appeared to express one or two slightly higher molecular weight Int_{261}-specific bands (Fig. 12). I then re-evaluated the Int_{261} deduced amino acid and noted that it contains two potential asparagine-linked glycosylation sites (NQS and NTS) that could have been modified by glycosylation in this endoplasmic reticulum-targeted form. The two higher molecular weight bands observed in Int_{261} from CSP20 (Fig. 12) could result from glycosylation of either one or both of these N-linked sites. We also noticed that sera from mice immunized with CSP20 that did not recognize Int_{261} from pMW103 (Gansheroff *et al.*, 1999) did recognize nickel affinity chromatography purified Int_{261} from CSP20 (Table 8). These results suggested that Int_{261} from CSP20 had been altered, potentially by glycosylation, and that this change in the protein had adversely affecting the immune response to the antigen. We hypothesized that the higher molecular weight

Table 8. Serum IgG titers from mice fed CSP20 does not recognize Int_{261} from pMW103 on ELISA

	ELISA Plate Bound Antigen	Int_{261} from pMW103 (Gansheroff *et al.*, 1999)	Int_{261} from CSP20 (plate culture)	Int_{261} from CSP20 (broth culture)
Immunization Method and Antigen				
Injected i.p., Int_{261} from pMW103 (Gansheroff *et al.*, 1999)		871250	6250	31250
Fed CSP20		<50[a]	250	250
Fed CSP20 with CT[b]		50	250	250

[a] 50 -titer limit of detection

[b] CT – purified cholera toxin, 7.5 μg (Sigma)

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Figure 12. Western blot analysis of higher molecular weight bands expressed by older

cultures of CSP20. After 6 months of continuous culture on NT media plates and broth,

CSP20 expressed one or two higher molecular weight intimin-specific species (CSP20)

not present in the purified intimin control (purified Int_{261} from pMW103, 75 ng). These

higher molecular weight intimin bands became more evident as the culture aged, i.e. they

were not as prominent in earlier cultures (Fig. 11).
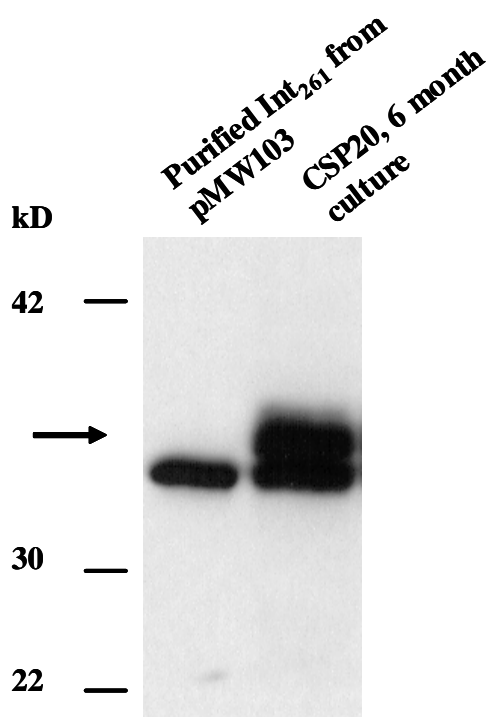
bands represent a glycosylated form of Int_{261} that was recognized by the murine host differently than the protein made by bacteria.

To determine whether Int_{261} from CSP20 was in fact glycosylated, Int_{261} from CSP20 was purified by nickel affinity chromatography and then subjected to a deglycosylation protocol with a Glyko® GlycoFree™ Chemical Deglycosylation Kit from Prozyme (San Leandro, CA). This kit contains trifluoromethanesulfonic acid (TFMS) as a reagent to non-selectively remove both N- and O-linked glycans from glycoproteins without altering the primary protein structure. The appearance of a band after treatment of Int_{261} from CSP20 with TFMS that was consistent with the size of control Int_{261} [~35 kDa when purified from pMW103 (Gansheroff *et al.*, 1999) by nickel affinity chromatography], strongly suggested that Int_{261} from CSP20 was glycosylated (Fig. 13).

The location of the two potential N-linked gylcosylation sites in Int_{261}, as illustrated on the EPEC carboxy-terminal intimin ribbon structure (Fig. 14), do not appear to be situated in either the Tir or nucleolin binding domains (Luo *et al.*, 2000; Sinclair and O'Brien, 2002). To test this prediction, we asked whether gylcosylated Int_{261} could bind to nucleolin and Tir. Based on qualitiative analysis by Far Western blot analysis (Fig. 15), we concluded that Int_{261} from CSP20 does bind nucleolin, and, therefore, glycosylation of Int_{261} does not appear to block interaction with this host-cell receptor for intimin (Sinclair and O'Brien, 2002). Unfortunately, for technical reasons, the results of the Far Western analysis of Tir binding to Int_{261} were inconclusive (data not shown).

Because the glycosylation of the Int_{261} molecule in CSP20 adversely affected the immune response to this antigen, even though the antigen appeared to maintain the

Figure 13. Western blot analysis of Int_{261} from CSP20 before and after treament with

trifluoromethanesulfonic acid (TFMS) to deglycosylate the protein. Int_{261} purified by

nickel affinity chromatography (from CSP20) migrated as two visible intimin-specific

bands that were of higher molecular weight than Int_{261} from pMW103 (Gansheroff *et al.*,

1999). After treatment of Int_{261} purified from CSP20 with TFMS, which non-selectively

removes N and O-linked glycans from proteins, the higher molecular weight intimin-

specific bands disappeared and a band consistent with the size of control Int_{261} (~35 kD,

75 ng protein loaded in well) appeared.
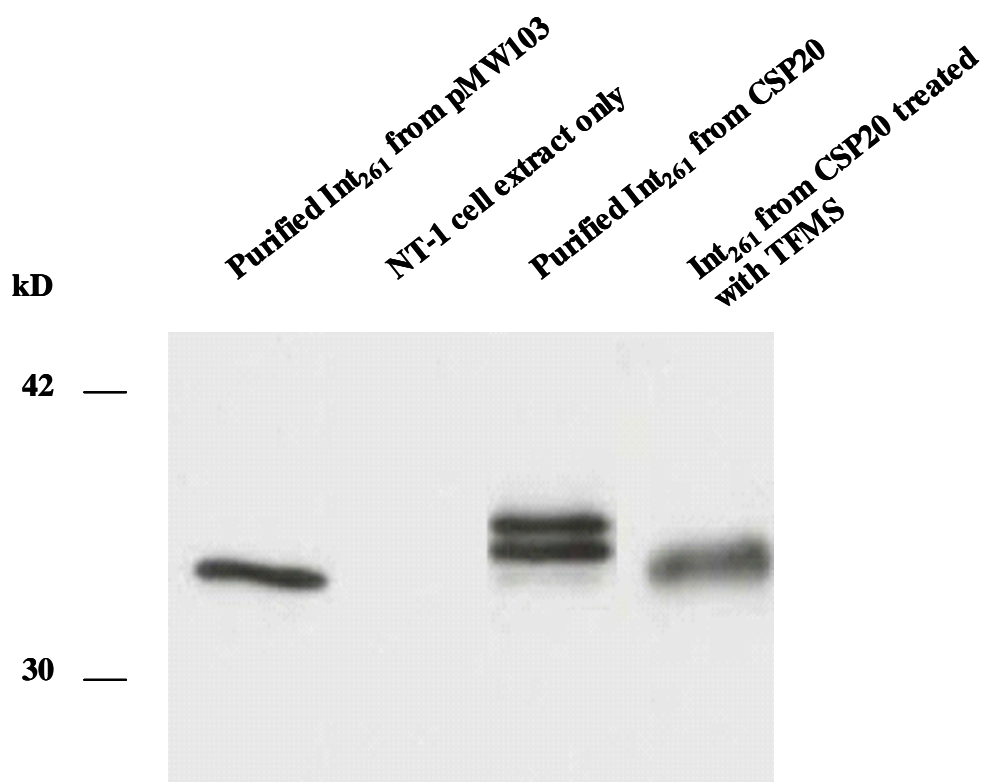
Figure 14. Theoretical location of potential sites of N-linked glycosylation in Int_{261} based on a ribbon crystal structure of the EPEC intimin- (in white) bound to the translocated intimin receptor (Tir, in bright blue) (Luo *et al*., 2000). The carboxy-terminal domain of EPEC intimin shown consists of amino acid residues 858-939. The Tir binding domain is located at residues 904-909. Based on the amino acid sequence of EHEC $intimin_{O157}$, there are two potential sites for N-linked glycosylation with the C-terminus of $intimin_{O157}$, shown on the EPEC ribbon structure in green and highlighted with arrows. Glycosylation at either or both of these sites would not appear, based on this structure, to interfere with Tir or nucleolin binding (Luo *et al*., 2000; Sinclair and O'Brien, 2002).
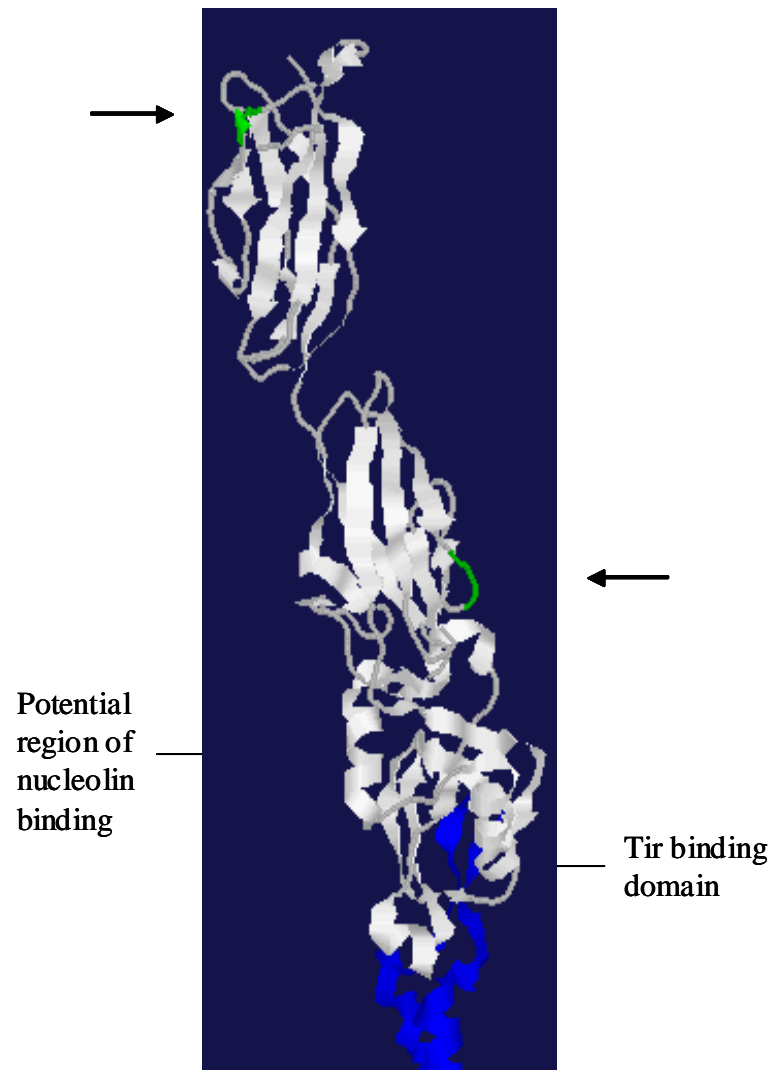
Ribbon drawing adapted from (Luo *et al.*, 2000).

Figure 15. Far Western blot analysis of Int_{261} from CSP20 to assess whether it bound to nucleolin. HEp-2 cell protein extracts electroblotted to nitrocellulose were cut into strips and incubated with various $intimin_{O157}$ proteins. Lanes 1 and 2 were incubated with PBS and no $intimin_{O157}$. Lanes 3 and 4 were incubated with 6 µg $intimin_{O157}$ from pEB313 (McKee and O'Brien, 1996). Lanes 5 and 6 were incubated with 6 µg Int_{261} from pMW103 (Gansheroff *et al.*, 1999). And Lanes 7 and 8 were incubated with 6 µg Int_{261} from CSP20. The blot of lanes 1 and 2 was then probed with a nucleolin monoclonal antibody and the bands that resulted were each ~110 kD protein, sizes consistent with that of nucleolin. The other blots were probed with $intimin_{O157}$ polyclonal antibodies to detect the sizes of the bands to which the intimin proteins were bound. All of the intimin proteins appeared bound to a protein(s) in the HEp-2 cell lysates of ~110 kD, sizes consistent with that of nucleolin (arrow).
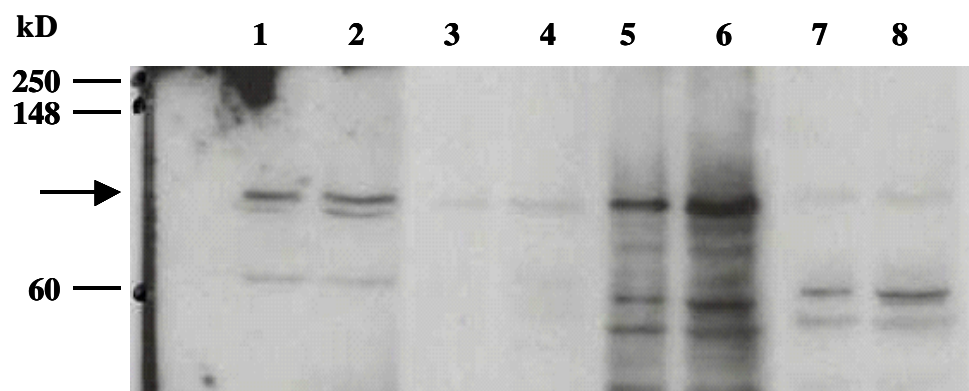
function of binding to nucleolin, we elected to discontinue experiments with CSP20. Instead, we used an NT-1 cell clone transformed with pNR50, C34, that expressed Int_{261} without the addition of a signal peptide and in an apparently unglycosylated state (Fig. 16). C34 expressed ~3 μg Int_{261} per gram of total plant material.

Colonization of mice by EHEC O157:H7 is dependent on intimin$_{O157}$. In anticipation of testing the *in vivo* efficacy of the transgenic plant vaccine in blocking bacterial colonization with EHEC O157:H7, we sought to develop a small animal, intimin$_{O157}$-dependent model of bacterial colonization. Previously our laboratory had reported that mice that are treated with streptomycin and then fed a-Str^R version of EHEC O157:H7 can be successfully colonized with the organism at high levels (Wadolkowski *et al*., 1990a). However, Dr. Marian McKee, while a graduate student in this laboratory, found no evidence of intimin$_{O157}$-dependent colonization of mice treated with streptomycin in their drinking water (unpublished finding), perhaps because the oral antibiotic enhanced bacterial colonization with EHEC O157:H7 strain 86-24 *eae*Δ10 Str^R to such a high level as to abrogate differences between this mutant and wild-type. Note that even an *E. coli K-12* strain colonizes streptomycin-treated mice well (Wadolkowski *et al*., 1990a; Wadolkowski *et al.*, 1990b). In this study, I confirmed Dr. McKee's observation.

Several other groups have reported that untreated mice or mice given cimetidine are transiently colonized with clinical strains of EHEC O157:H7 (Wadolkowski *et al*., 1990a; Nagano *et al.*, 2003; Conlan and Perry, 1998). However, none of these explored the intimin$_{O157}$-dependent nature of the bacterial colonization. Therefore, I evaluated the impact of intimin on the colonization of untreated mice with EHEC O157:H7. I found

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Figure 16. Western blot analysis of Int_{261} expressed by C34. NT-1 cell clone C34

transformed with pNR50 expressed Int_{261} (C34-plate culture and C34-suspension culture)

that did not appear to be glycosylated like CSP20. 75 ng of Int_{261} from pMW103 used as

a positive control (Gansheroff *et al*., 1999).
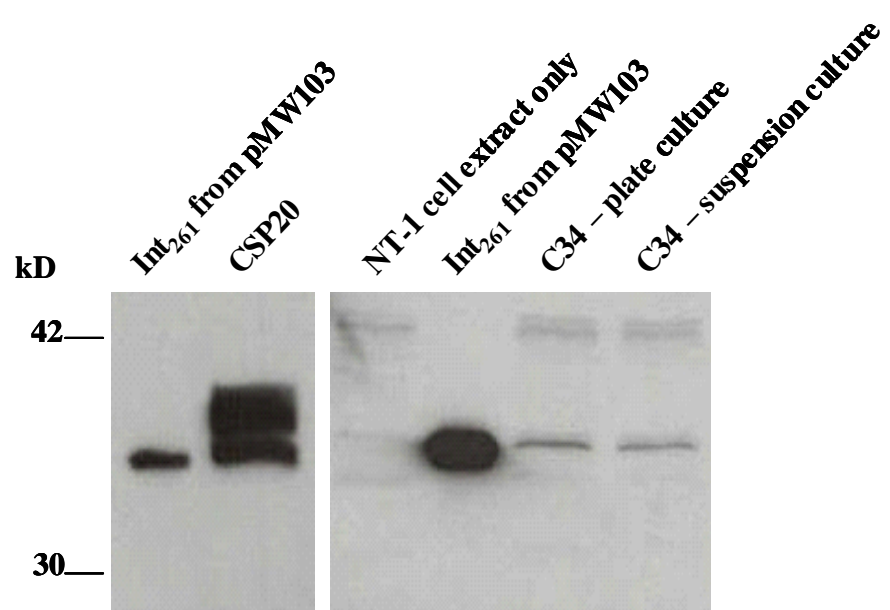
that untreated mice could be colonized with the strain at modest levels (approximately

1×10^3 CFU/g feces) for a mean of 10 days (range 6.4 to 13.4 days for unimmunized

mice), an observation consistent with our finding in ferrets (Melton-Celsa *et al*., 1998).

With this untreated mouse model of EHEC O157:H7 colonization, in an experiment

limited to 7 days, EHEC O157:H7 86-24 Str^R colonized BALB/c mice a mean of 6.4

days, whereas the intimin$_{O157}$-negative mutant EHEC O157:H7 86-24 Str^R *eae*Δ10

showed a statistically significant reduction in the duration of colonization (mean, 3.2

days; *P*<0.005; Log-rank test). Thus, in this model of EHEC O157:H7 infection, as in

the ferret, colonization was augmented by the presence of intimin$_{O157}$. In addition, two

different infectious doses of EHEC O157:H7 86-24 Str^R, 1×10^6 CFU or 1×10^9 CFU, were

tested to see if a lower infectious dose could also lead to bacterial colonization and might

therefore be a better model to ascertain the functionality of an anti-intimin$_{O157}$-specific

immune response. Mice (n=5) challenged with 1×10^9 CFU EHEC O157:H7 86-24 Str^R

were colonized a mean of 6.4 days in an experiment limited to 7 days, whereas mice fed

1×10^6 CFU were colonized a mean of 3 days (*P*=0.027, Log-rank test). The lower

infectious dose of 1×10^6 EHEC O157:H7 86-24 Str^R colonized mice (mean 3 days) to the

same degree as EHEC O157:H7 86-24 Str^R *eae*Δ10 (mean 3.2 days) thus did not function

as an intimin$_{O157}$-dependent model of bacterial colonization.

Generation of intimin$_{O157}$-specific serum and fecal antibodies. We tested several

different vaccination strategies with C34 in BALB/c mice. The vaccine protocols and the

serum IgG and fecal IgA and IgG ELISA results are summarized in Table 9. To

maximize the immune response to orally administered Int$_{261}$, we tried a prime-boost

protocol (Groups E and F) similar to that used to elicit an enhanced immune response to

Footnotes for Table 9.

[a]Serum and fecal pellet extracts were collected throughout the experiment and were evaluated by ELISA for the presence of antibodies to Int_{261}. The titer was defined as the geometric mean of the reciprocal of the highest dilution that gave an absorbance (A_{600}) above both pre-immune and background levels. Responders, in parenthesis, were defined as mice that had a detectible titer at the final sample collection point (7 days after the final feeding or injection). Mice with no titer or that lost titer by the last collection point were called non-responders. The titers reflect the geometric mean of responders only.

[b]Significantly higher serum IgG titer and responders compared to other groups ($P<0.0001$; two-sided t-test and $P<0.003$; Fisher's exact test)

[c]Fecal IgA titer compared to group E and F, $P=0.019$ and $P=0.013$, respectively (two-sided t-test). Number of fecal IgA responders compared to group E and F, $P=0.175$ and $P=1.000$, respectively (Fisher's exact test).

[d]CT – purified cholera toxin, 7.5 μg (Sigma)

Table 9. Immunogenicity in BALB/c mice of NT-1 cells that express Int_{261}

Group/Immunization	Vaccination Method	Serum[a] IgG titer (responders)	Fecal[a] titer (responders)
A: Int_{261} from pMW103 given i.p.	20 μg Int_{261}+TiterMax® i.p. day 0; 10 μg Int_{261}+TiterMax® i.p. days 10 and 20	300000 (10/10)[b]	560 (8/10)[c] [IgA only]
B: Fed NT-1 cells only	5g NT-1 cells fed on days 0, 7, 14	<50 (0/10)	<50 (0/10)
C: Fed C34 cells only	5 g C34 (~15 μg Int_{261}) fed on days 0, 7, 14	<50 (0/10)	340 (4/10) [IgG and IgA]
D: Fed C34 cells with CT[d]	5 g C34 (~15 μg Int_{261})with CT fed on days 0, 7, 14	<50 (0/10)	280 (6/10) [IgG and IgA]
E: Int_{261} (Ni-affinity purified) from C34 given i.p.; Fed NT-1 cells only	~15 μg Int_{261} from C34+TiterMax® i.p. on day 0; 5 g NT-1 cells fed on day 7 and 14.	50 (1/10)	150 (5/10) [IgA only]
F: Int_{261} (Ni-affinity purified) from C34 given i.p.; Fed C34 cells with CT[d]	~15 μg Int_{261} from C34+TiterMax® i.p. on day 0; 5 g C34 (~15 μg Int_{261}) with CT fed on day 7 and 14.	80 (3/10)	180 (7/10) [IgA only]

the B subunit pentamers of heat-labile enterotoxin of *E. coli* (LT-B) (Lauterslager *et al*.,
2001). Five of 10 mice primed by injection of purified Int_{261} from C34 and then fed non-
transgenic plant material developed an intimin-specific fecal antibody response. These
results suggested that even a single inoculation with Int_{261} from C34 can prime for a
subsequent mucosal antibody response to intimin. Seven of 10 mice primed by injection
of Int_{261} from C34, then boosted by feeding C34 (with CT as an oral adjuvant) produced
an intimin-specific fecal IgA response. In addition, 3 out of 10 mice developed a serum
IgG response, and serum samples from these mice blocked adherence of wild type EHEC
O157:H7 to HEp-2 cells compared to pre-immune serum controls (Fig. 17). Although
the fecal IgA titer for group A was significantly higher than both groups E and F
(P=0.019 and 0.013, respectively; two-sided t-test), the number of mice making an Int_{261}-
specific fecal IgA response in both groups E and F did not differ significantly from the
number responding from group A (P= 0.175 and 1.000, respectively; Fisher's exact test).

Challenge of vaccinated mice with EHEC O157:H7. With this intimin-dependent
mouse model of EHEC O157:H7 colonization, we orally infected the same groups of
immunized mice described in Table 9 with EHEC O157:H7 strain 86-24 Str[R]. We found
that mice primed with an intraperitoneal (i.p.) injection of Int_{261} from C34, then fed
homologous C34 plant material showed a statistically significant (P<0.002; Log-rank
test) reduction in the duration of colonization (as measured by fecal shedding) compared
to the negative control mice fed non-transgenic NT-1 cells only (Fig. 18). These mice
primed and boosted with Int_{261} from transgenic plant material also exhibited a statistically

Figure 17. Serum from mice immunized with Int_{261} from C34 blocked bacterial adherence to HEp-2 cells. Panel A shows adherence of EHEC O157:H7 strain 86-24-GFP to HEp-2 cell monolayers in the presence of non-immune serum. Panel B shows reduced adherence of EHEC O157:H7 strain 86-24-GFP to HEp-2 cell monolayers in the presence of serum from a mouse that was primed i.p. with Int_{261} from C34, then boosted with oral feedings of C34 plant material with cholera toxin as an oral adjuvant. Reduced adherence determined by visual, qualitative analysis of the digital images.
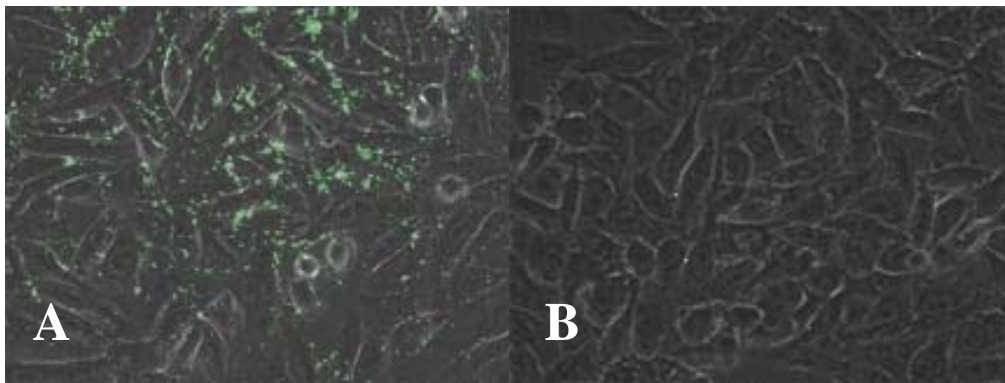
Figure 18. Duration of EHEC O157:H7 colonization in mice immunized with Int_{261}. All

mice from the same groups as shown in Table 9 (p. 91) were challenged with wild type

EHEC O157:H7. Mice in both groups A and F (*) showed a statistically significant

(*P*<0.002, Log rank test) reduction in the duration of colonization compared to group B.

No other groups demonstrated a statistically significant reduction compared to group B.

Mice from group F (**) also showed a statistically significant decrease in the duration of

bacterial colonization compared to mice from group E (*P*=0.033; Log-rank test). The

Int_{261} material used to immunize mice in group A was nickel-affinity purifed from

pMW103 (Gansheroff *et al*., 1999). Purified cholera toxin (7.5 µg; Sigma) was used as

an oral adjuvant in groups D and F.
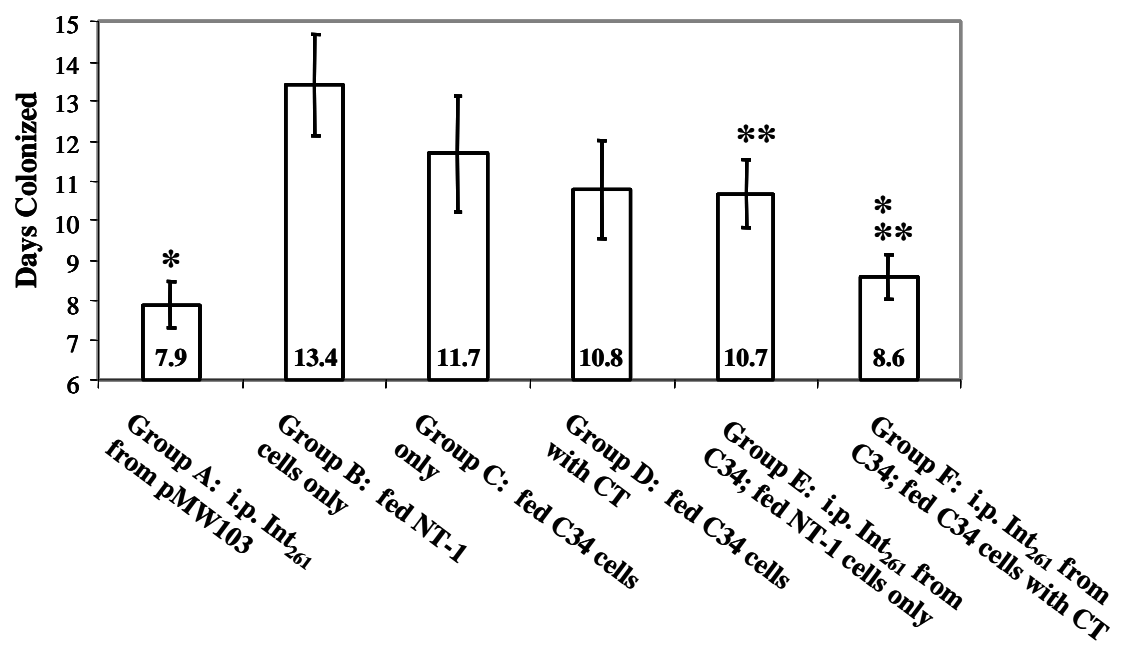
significant decrease in the duration of bacterial colonization compared to mice only

immunized i.p. with Int_{261} from C34, then boosted with non-transgenic plant material

(P=0.033; Log-rank test). These results suggest that the feeding of transgenic, Int_{261}-

expressing plant material plays a significant role in boosting the immune response to

Int_{261} and in reducing the duration of bacterial colonization. The positive control mice,

injected i.p., 3 times with highly purified Int_{261} from pMW103 (Gansheroff *et al.*, 1999),

also displayed a statistically significant reduction in the duration of colonization versus

the negative control (P<0.002; Log-rank test). There was no difference in the duration of

shedding between the positive control immunizations and the prime-boost protocol with

oral delivery of transgenic plant material that expressed Int_{261} as a boost. This latter

observation suggests that immunization with a plant-based oral vaccine can induce an

immune booster response sufficient to provide protection from a challenge with wild-type

EHEC O157:H7.

Expression of Int_{261} in a whole plant model: *Arabidopsis thaliana*. As a consequence

of our success in the expressing Int_{261} in NT-1 cells and in collaboration with Boyce

Thompson Institute for Plant Research in Ithaca, NY, we proceeded to transform a whole

plant model, *Arabidopsis thaliana*, with *Agrobacterium* strains that carried the $intimin_{O157}$

and Int_{261} expression constructs. We used the floral dip method (as described in

Materials and Methods, p. 35). *Arabidopsis* leaf material grown from transformed seeds

was sent to Uniformed Services University for Western blot analysis. Leaf material

proteins extracted directly into SDS-Page loading buffer proved to be the optimal

preparation method for visualizing Int_{261} expression. *Arabidopsis* plants transformed with $intimin_{O157}$ expression constructs (pNR12 or pNR14) did not produce any $intimin_{O157}$-specific proteins. However, 3 out of 6 plant clones transformed with pNR49 (Int_{261} gene with a plant-specific signal peptide sequence) expressed Int_{261}-specific protein. In addition, 1 out of 4 plant clones transformed with pNR50 (Int_{261} gene without signal peptide sequence) produced Int_{261}-specific protein (Fig.19). These results show that Int_{261} can be expressed in a more complex, whole plant model. Furthermore, these results suggest that the plant-optimization of the Int_{261} gene may improve the efficiency of the antigen's expression in plants.

Figure 19. Western blot analysis of Int_{261} expression in *Arabidopsis* leaf material. The

Arabidopsis leaf proteins were extracted directly into SDS-Page loading buffer prior to

SDS-Page and subsequent Western blot analysis. *Arabidopsis* clones 7, 8 and 10,

transformed with pNR49, expressed Int_{261} specific protein (~35 kD). Clone 21,

Arabidopsis transformed with pNR50, expressed Int_{261}-specific protein (~35 kD).

II.   Construction of a Shiga toxin type 2 toxoid for expression in plants.

Rationale for initiating experiments on Stx2 toxoids rather than Stx1 toxoids. The

majority of isolates of EHEC O157:H7 produce both Stx1 and Stx2 (Obrig, 1998). We

elected to first focus on the construction of a Stx2 toxoid, rather than a Stx1 toxoid, for

eventual expression in plant cells. Our reasons for this choice were based on several

published observations. First, Stx2 is more toxic than Stx1 when injected into mice or

when expressed by STEC and fed to these animals (Melton-Celsa and O'Brien, 1998;

Melton-Celsa *et al*., 1998). Second, expression of Stx2 by O157:H7 appears to be more

likely to lead to development of HUS in an infected patient, although Stx1-only

producing strains have also been associated with cases of HUS (Ostroff *et al*., 1989;

Scotland *et al*., 1987). Third, Stx2 is more toxic for human renal and intestinal capillary

endothelial cells than is Stx1 (Louise and Obrig, 1995) even though Stx 1 is more toxic

per antigen unit to Vero cells than is Stx2 (unpublished data, O'Brien laboratory).

Evaluation of an A_2B subunit antigen as a Stx2 toxoid. Our first approach for

construction of this Stx2 toxoid was to try to express the A_2 subunit with the Stx2 B

pentamer. Recall that Stx2, like Shiga toxin from *Shigella dysenteriae* and Stx1, has an

A1:B5 noncovalently associated subunit structure (Fig. 2, p. 19). The Stx2 A subunit

(~35 kDa) is cleaved to produce A_1 (~28 kDa) and A_2 (~4 kDa) portions. The A_2 subunit

remains associated with the A_1 subunit via a disulfide bond and serves to link the A_1

portion of the subunit to the B pentamer. Shiga toxin type 1 (Stx1) B subunits, when

expressed from a recombinant plasmid, can form functional pentamers that elicit toxin

neutralizing antibodies (Ramotar *et al*., 1990; Acheson *et al.*, 1993; Acheson *et al*.,

1995). Stx2 B subunits do not form functional pentamers or elicit Stx2 neutralizing

antibodies when expressed in a recombinant form, unless the subunits are expressed with

the Stx1 B leader sequence (Acheson *et al*., 1995; Marcato *et al.*, 2001). Since the A_2

portion of Stx1 is required to noncovalently bind the A_1 subunit to the B pentamer and is

very important for full toxicity of the molecule (Austin *et al.*, 1994), we hypothesized

that the A_2 portion of Stx2 may be critical for B subunit pentamerization. Therefore, we

decided to express the Stx2 B subunit in conjunction with its A_2 portion with the hopes

that this construct would produce functional Stx2 B subunit pentamers.

The construct used was EHEC O157:H7 *stx2* ΔA_1kan::A_2B, where the A_1 subunit

of the toxin was replaced by a kanamycin resistant cassette (made by Dr. Clare Schmidt;

unpublished). This construct was not cytotoxic and appeared to produce B subunit

pentamers when the material was cross-linked with dimethyl suberimidate (DMS), as

seen in Figures 20 and 21. Unfortunately, when this material was injected i.p. into

female BALB/c mice, they (n=5) did not make Stx2-neutralizing antibodies (Fig 22).

One possible explanation for these results is that Stx2 B pentamers expressed from this

recombinant construct is not stable and readily dissociates when not crosslinked. We

speculate that the multimeric Stx2 B subunit structures express the Stx2 toxin

neutralizing epitopes whereas the immunodominant epitopes recognized on the Stx2 B

subunit monomers do not elicit such antibodies. Indirect evidence in support of this

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Figure 20. Expected Stx2 B subunit multimerization pattern upon crosslinking of the

EHEC O157:H7 *stx2* ΔA_1kan::A_2B sonic lysate with dimethyl suberimidate (DMS).

Recombinant Stx2 B subunits are predicted to form into multimers of differing sizes

when crosslinked with increasing concentrations of DMS.
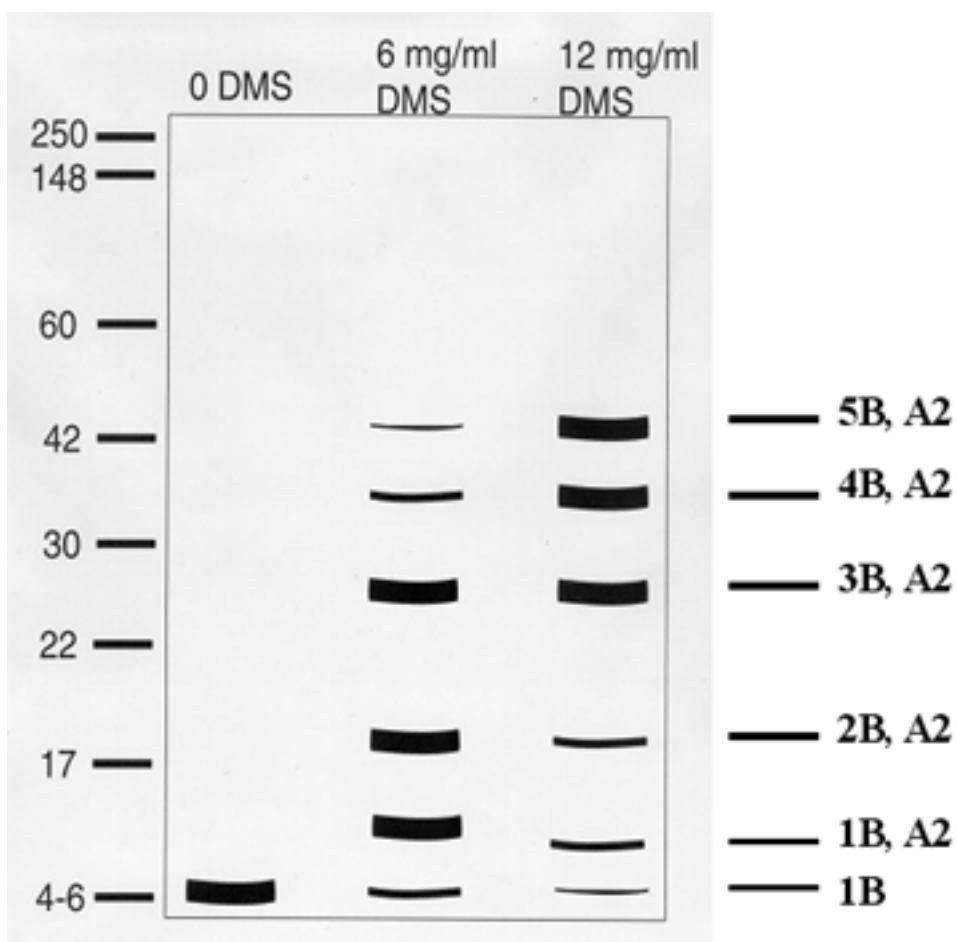
Figure 21. Stx2 B subunit multimers expressed from EHEC O157:H7 *stx2*

ΔA_1kan::A_2B. Stx2 toxin or Stx2 B subunits from EHEC O157:H7 *stx2* ΔA_1kan::A_2B

were crosslinked with 0, 12, 24, or 48 mg/ml DMS, separated by SDS-Page, transferred

to nitrocellulose, and probed with BC5, a Stx2 B subunit-specific MAb (Zieg et al.,

1978). Without DMS, both wild-type and recombinant B subunits existed as monomers

(~7 kD). With increasing concentrations of DMS both wild-type and recombinant B

subunits exhibited similar patterns of multimerization. The higher molecular weight

bands present in the wild type Stx2 toxin lanes are most likely due to the presence of the

complete A_1 subunit.
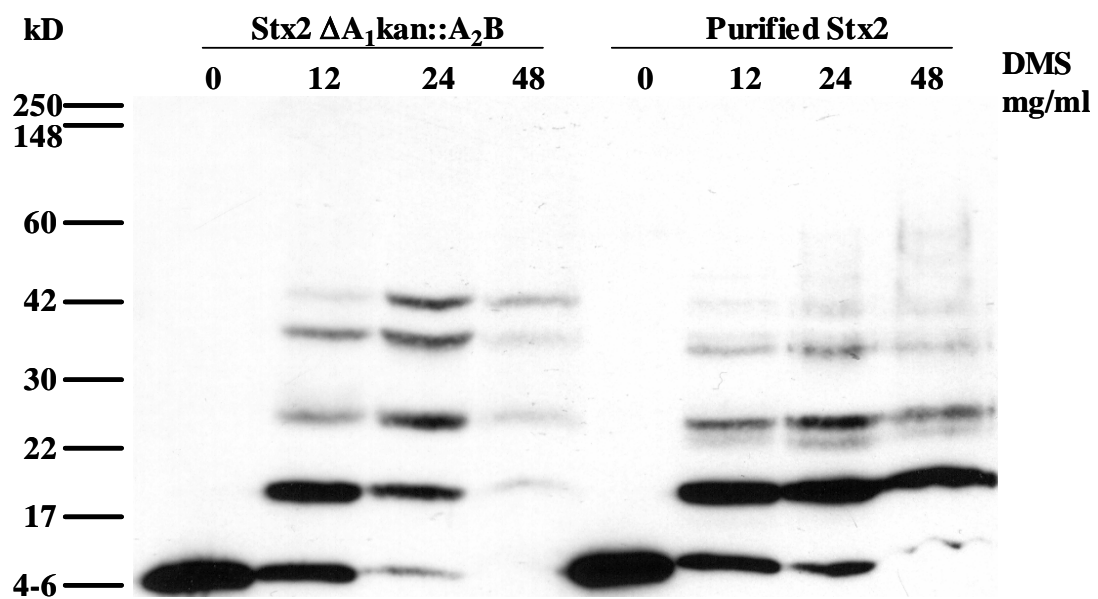
Figure 22. Toxin neutralization assay using serum from a mouse immunized with a lysate of a sonically disrupted culture of EHEC O157:H7 *stx2* ΔA_1kan::A_2B. The OD values were obtained by measuring the absorbance at 600 nm in each well with the ELx800 microtiter plate reader (Bio-Tek Instruments, Inc.). The cytotoxic titer (CD50 – the dose of toxin where 50% of the cells die) of culture lysate was expressed as the reciprocal of the highest dilution required to kill 50% of the cells in a well. Therefore, the lower the OD_{600} reading, the higher the cytotoxic dose. Neutralizing serum antibodies prevent Vero cell death, and, thus one expects an increase in the OD_{600} reading for wells with immune sera plus toxin compared to wells that contain toxin alone or toxin plus prebleed sera. Ten serial two-fold dilutions were prepared in Vero cell tissue culture medium starting at a dilution of 1:2. These serum dilutions were tested with both 2x CD50 and 20x CD50. Serum from mouse #1 (M1) could not neutralize either 2xCD50 or 20xCD50 of purified Stx2, as the presence of this serum did not result in higher OD_{600} readings from the 2xCD50 or 20xCD50 controls. The data shown here are from mouse #1 and are representative of all mice that were immunized with EHEC O157:H7 *stx2* ΔA_1kan::A_2B.

Toxin Neutralization Assay: Mouse immunzied with recombinant Stx2 B
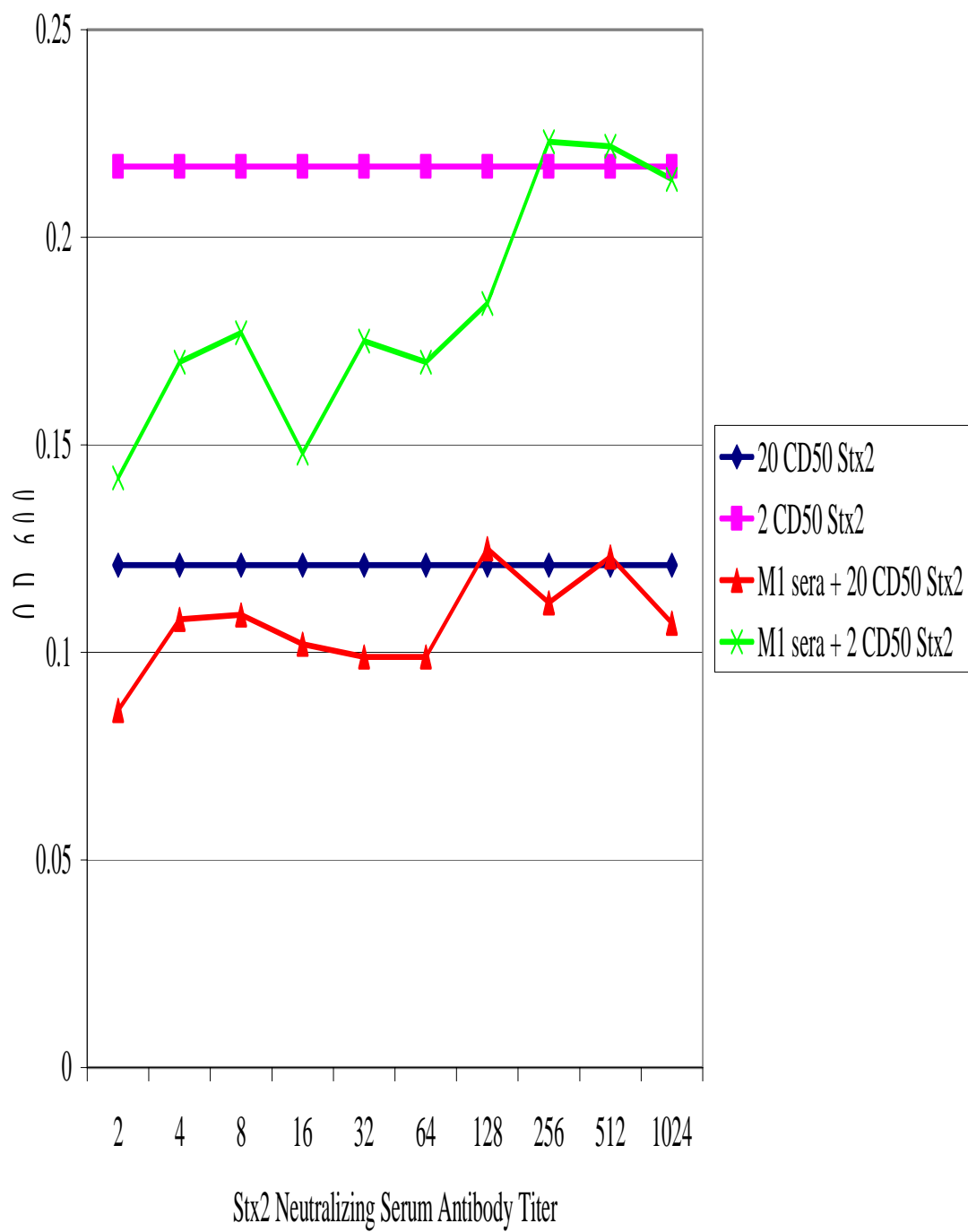
premise is the fact that Donohue-Rolfe *et al*. were able to generate a Stx2 B monoclonal

antibody (4D1) that was raised against holotoxin that did not recognize Stx2 B monomers

on Western blot (Donohue-Rolfe *et al.*, 1989; Acheson *et al*., 1995). Another explanation

for the findings of these investigators is that the epitope is present on the Stx2B monomer

but is conformationally dependent and not detected on Western blot analysis.

Rationale and strategy for construction of a site-directed Stx2 toxoid. Our next

approach for creation of a Stx2 toxoid was to attempt to alter, by site-directed

mutagenesis, certain codons for amino acids that are required for enzymatic activity of

Stx2. We reasoned that mice immunized with such a toxoid would generate antibodies to

both the Stx2 A and B subunits. We also hypothesized that the Stx2 B subunit would be

more likely to pentamerize in a holotoxoid molecule than when expressed alone on a

recombinant plasmid, and, therefore, that Stx2 toxin neutralizing epitopes would be

conserved. Finally, we rationalized that expression and assembly of multiple subunits in

a plant system was not an unreasonable expectation based on previous research that

showed that functional antibodies can be expressed in plant systems (Ma *et al*., 1995; Ma

et al., 1998).

The first step in Stx2 toxoid construction was to identify amino acid residues in

the Stx2 A subunit that abrogate cytotoxic activity. The enzymatic function of Stx2 is the

inhibition of protein synthesis by depurinating an adenine residue from 28S ribosomal

RNA of the 60S ribosome. The A_1 subunit contains the N-glycosidase activity (Endo *et*

al., 1988; Saxena *et al*., 1989). The glutamic acid at position 167 of the A subunit is the

active site and is critical for enzymatic activity (Hovde *et al*., 1988), though other amino acid residues in the A subunit (such as the tyrosine at position 77) are also important for toxicity (Deresiewicz *et al*., 1992).

The modification of Stx2 to create a toxoid. We used the QuikChange™ Site-directed Mutagenesis Kit (Stratagene) to change the glutamic acid at position 167 to glutamine and the tyrosine at position 77 to a serine. We decided to change both residues, even though each individual residue successfully abrogated Vero cell cytotoxicity (Fig. 23), to prevent spontaneous reversion to a toxic molecule.

Analysis of toxoid capacity to elicit Stx2-neutralizing antibodies. The resulting recombinant plasmid was named pNR100. That plasmid was transformed into XL-1 Blue (Stratagene), and the Stx2 toxoid was expressed and purified by affinity chromatography over a column to which the Stx2A MAb, 11E10, had been coupled (Perera *et al*., 1988). This purified material was emulsified one to one with TiterMax® and used to immunize mice as detailed in the Materials and Methods section (p. 45). These immunized mice remained healthy, made Stx2 toxin neutralizing antibody, and were able to survive purified Stx2 toxin injections of up to 1 μg toxin (Fig. 24).

Optimization of the Stx2 toxoid A and B subunit genes for expression in plants. We further applied the strategy of site-directed mutagenesis to make three additional changes to the A subunit of the catalytically-inactivated Stx2 toxoid so as to optimize expression

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Figure 23. Vero cell cytotoxicity assay of Stx2 toxoid purified from pNR100. The OD

values were obtained by measuring the absorbance at 600 nm in each well. The CD50 of

culture lysate was expressed as the reciprocal of the highest dilution required to kill 50%

of the cells in a well, as represented by the straight line (blue diamonds). Therefore, the

higher the OD_{600} reading, the lower the cytotoxic dose. The CD50 of the Stx2 toxin

control was approximately $5x10^3$ CD50/ml (purple squares). Purified Stx2 toxoid, from

three different preparations, purified on the dates shown, had no evidence of cytotoxicity.

Stx2 Toxoid Vero Cell Cytotoxicity
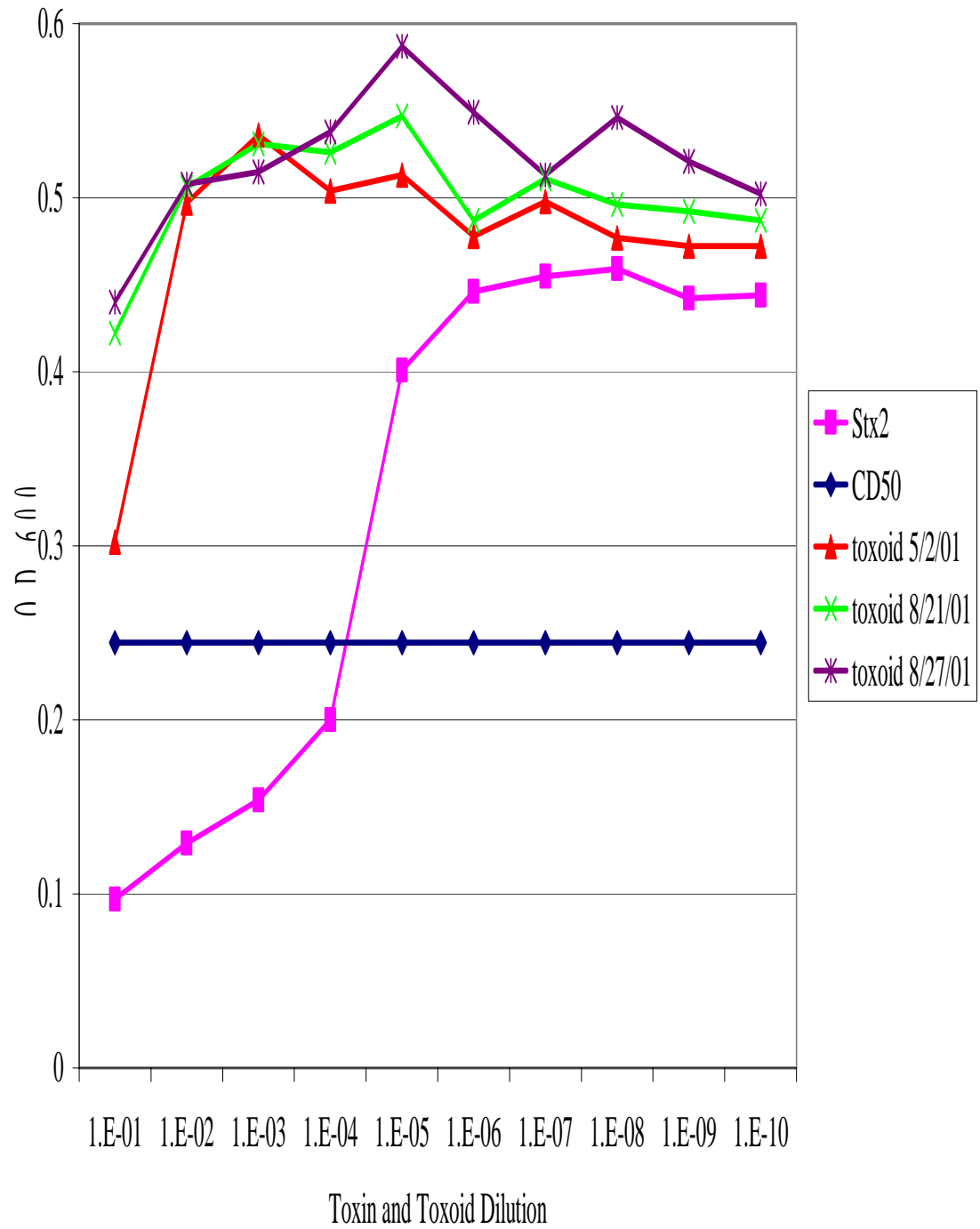
Figure 24. Stx2 toxin neutralization assay with serum from a mouse immunized with Stx2 toxoid purified from pNR100. The OD values were obtained by measuring the absorbance at 600 nm in each well. The CD50 of culture lysate was expressed as the reciprocal of the highest dilution required to kill 50% of the cells in a well, therefore the higher the OD_{600} reading, the lower the cytotoxic dose. Neutralizing serum antibodies prevent Vero cell death, and, thus one expects an increase in the OD_{600} reading for wells with immune sera plus toxin compared to wells that contain toxin alone or toxin plus prebleed sera.. Ten serial two-fold dilutions were prepared in Vero cell tissue culture medium starting at a dilution of 1:6. These serum dilutions were tested with both 2xCD50 and 20xCD50. Serum from mouse #5 (M5) had a Stx2 toxin neutralizing titer for both 2xCD50 and 20xCD50. The data shown here are from one mouse and are representative of all mice that were immunized with Stx2 toxoid purified from pNR100.

Toxin Neutralization Assay: Mouse immunized with Stx2 toxoid
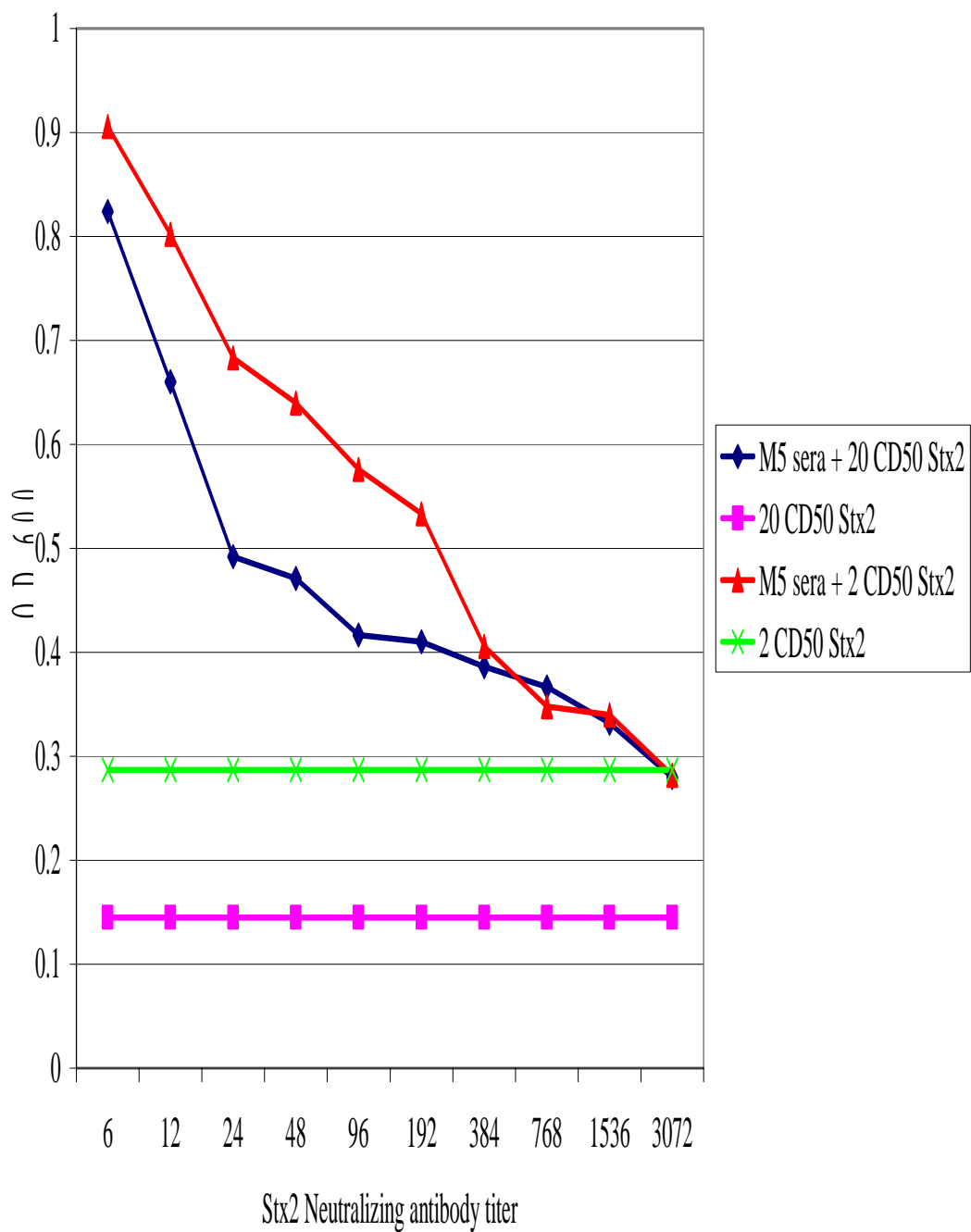
of the molecule in plants. The first and second changes were designed to disrupt RNA

destabilization motifs located from base 253 to 257 and 528 to 532. The third change

disrupted a polyadenylation motif from base 1115 to 1120. The Stx2 A subunit that

contained the two toxoid changes and the additional three plant-expression optimization

changes was cloned into pIBT210 to create pNR65.

To optimize the Stx2 B subunit for expression in plants, extensive changes to the

nucleotide sequence of the gene were required. It was not feasible to make the 37

nucleotide changes necessary to codon-optimize the gene for plant-expression by site-

directed mutagenesis, so the entire Stx2 B subunit gene was re-synthesized. We initially

tried to synthesize the Stx2 B subunit by an assembly PCR method as described by

Stemmer *et al.* (Stemmer *et al.*, 1995). However, we could not successfully re-synthesis

the Stx2 B plant-optimized gene by this technique. Therefore, we chose to try the ligase

chain reaction (LCR) as an alternate approach (Chalmers and Curnow, 2001). The

oligonucleotides used for LCR are outlined in Table 5 and the sequence of the plant-

optimized Stx2 B subunit gene is provided in Figure 3. The resulting LCR product (~360

bp) (Fig. 25) was amplified three times using primers 2B1 and 2B10 (Table 5). The final

amplified LCR fragment (Fig. 26) was digested with appropriate restriction enzymes and

cloned into pBTI210.3 to create pNAJ73.

Figure 25. Agarose gel of Stx2 B synthetic gene. PCR amplification of neat (assembly

#1) and a 1:10 dilution (assembly #2) of the Stx2 B subunit assembly PCR product

should be approximately 360 base pairs (bp); however, no such product was visible in the

assembly PCR lanes. PCR amplification of neat (LCR #1) and a 1:10 dilution (LCR #2)

of the Stx2 B subunit ligase chain reaction (LCR) show LCR products of approximately

360 bp are faintly visible in the LCR #1 lane. The LCR fragment was purified from the

LCR #1 lane and subjected to three additional rounds of PCR amplification with primers

2B1 and 2B10 (Table 5).
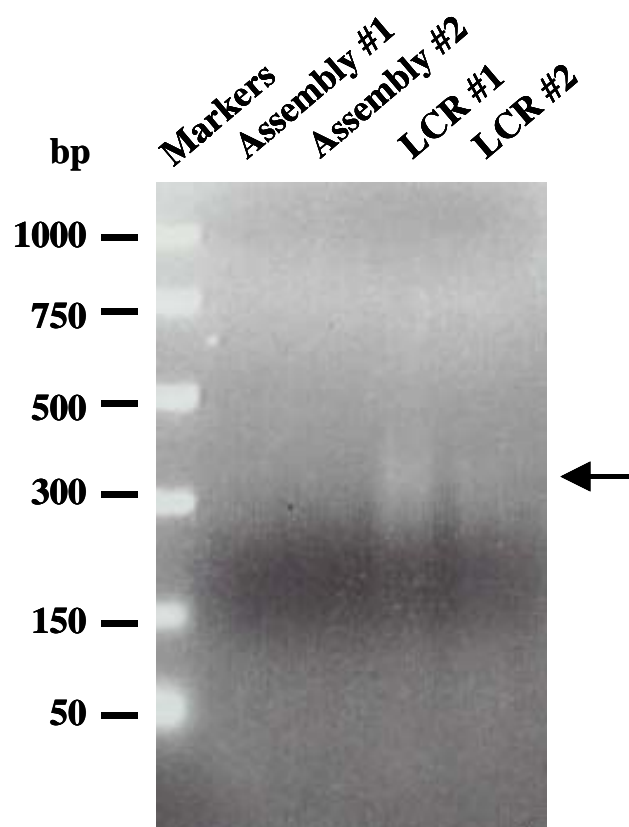
Figure 26. Agarose gel of amplified Stx2 B ligase chain reaction (LCR) product. LCR #1 and LCR #2 show LCR bands (~360 bp) amplified from the LCR product first synthesized in figure 25.
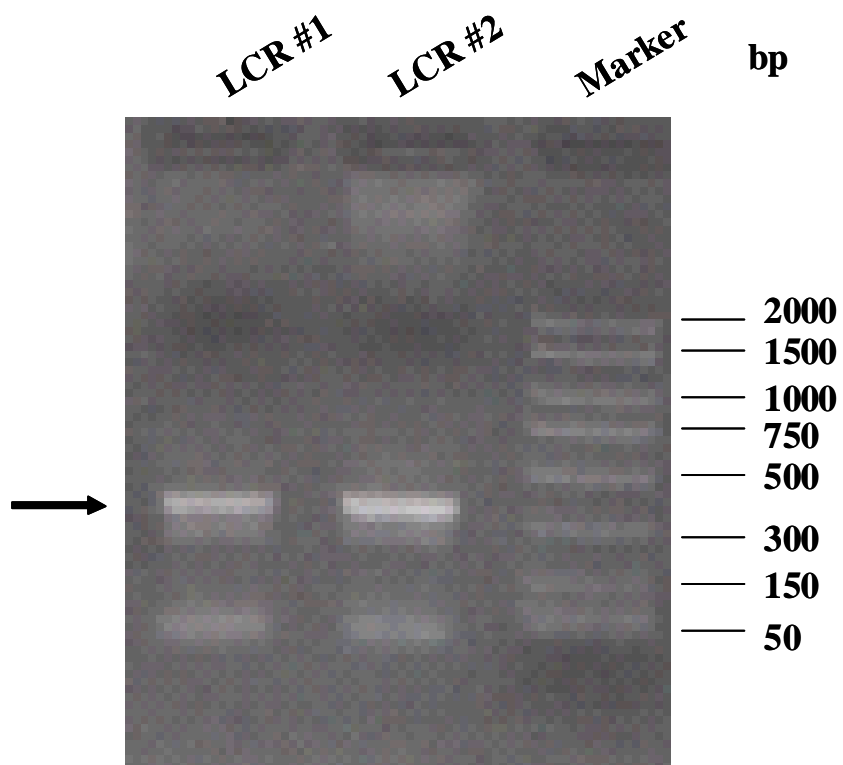
Discussion

The major results of this dissertation research fulfilled the specific aims of the project as defined in the introduction. These findings were as follows. First, we constructed NT-1 transgenic plant cell clones and showed that these cells could express full-length, histidine-tagged or the carboxy terminal portion of intimin$_{O157}$ (Int$_{261}$). As a corollary, we demonstrated in a collaborative study with the Boyce Thompson Institute that plant-optimized Int$_{261}$ can also be produced in a whole plant model (*Arabidopsis thaliana*). Second, we found that mice fed plant cells that expressed full-length or Int$_{261}$ made intimin$_{O157}$-specific serum and fecal immune responses. Furthermore, we demonstrated that oral delivery of Int$_{261}$ in plant cells, as a boost for parenterally injected homologous antigen, enhanced the intimin$_{O157}$-specific immune response. Third, and perhaps most importantly, we discovered that this prime-boost strategy led to induction of a serum response that could inhibit EHEC O157:H7 adherence *in vitro* and that this immunization protocol also reduced the duration of colonization of mice with wild type EHEC O157:H7. Fourth, a Stx2 site-directed toxoid that we constructed could elicit Stx2 toxin neutralizing antibodies when injected i.p. into mice and that these mice could survive a likely fatal Stx2 toxin challenge.

I. Expression of intimin$_{O157}$ or a portion thereof in plant cells and a whole plant model.

Numerous bacterial toxin vaccine antigens have been successfully expressed in

and delivered using transgenic plants, for example, enterotoxigenic *E. coli* heat labile toxin B subunit and *Vibrio cholerae* toxin B subunit (Haq *et al*., 1995; Mason *et al*., 1998; Tacket *et al*., 1998; Lauterslager *et al*., 2001; Arakawa *et al*., 1997; Arakawa *et al*., 1998). Surface-expressed proteins from viral pathogens, such as Hepatitis B and Norwalk virus, have also been effectively expressed in transgenic plant systems (Kong *et al*., 2001; Mason *et al*., 1992; Mason *et al*., 1996; Richter *et al*., 2000; Tacket *et al*., 2000; Thanavala *et al*., 1995). The expression of intimin$_{O157}$ or Int$_{261}$ in a transgenic plant system is the first reported production of a bacterial surface protein as a transgenic protein in a genetically transformed plant cell and whole plant system.

Several obstacles had to be overcome to successfully express Int$_{261}$ in plant cells and plants. First, we had to optimize the gene for Int$_{261}$ for better expression in plant systems because plants favor specific codons for certain amino acids during protein translation that differ from those codons preferred by viral, bacterial, or mammalian systems. For example, Gram negative bacteria use the codon GCG 34.3% of the time to encode alanine whereas dicotyledonous plants (such as tobacco) use GCG only 6.6% of the time to encode the same animo acid and prefer GCU (44.6% usage) to code for alanine (Ausubel *et al.*, 1989). In addition, there are sequence motifs present in bacterial genes that when transcribed by plant systems can lead to RNA destabilization and gene splicing that can result in a reduction or abrogation of expression of the transformed gene. We found that the modified gene for Int$_{261}$ expressed with greater frequency (4 out of 5 clones expressed Int$_{261}$) than the genes for the non-optimized full length and his-tagged intimin$_{O157}$ proteins (6 total out of ~100 clones expressed intimin$_{O157}$).

Another challenge faced during the course of this project was contamination of the NT-1 cell culture with mold, yeast, and/or bacteria. Untransformed and transgenic plant cell stocks and clones must be kept in continuous culture because the plant cells can not be frozen in glycerol stocks like bacterial cells. In addition, the NT-1 cells in broth were cultured for 7-10 days and NT-1 cells on agar plates were cultured for 21-30 days. These extended culture times put the NT-1 cell cultures at risk for contamination and can increase alterations in protein expression (such as reduction, loss, or modification of protein expression) as the culture ages. A product from Phytotechnology Laboratories Inc. (Shawnee Mission, KS), Plant Preventative Mixture (PPM), is a broad-spectrum anti-microbial that can be used in plant tissue culture to prevent contamination, or, when used in higher concentrations, can be used to decontaminate plant cell cultures. Unfortunately, use of this product with the intimin$_{O157}$ and Int$_{261}$-expressing NT-1 cell clones, slowed plant cell growth and thus decreased overall production of intimin$_{O157}$ and Int$_{261}$ in PPM-treated cultures.

In addition to expressing Int$_{261}$ in NT-1 plant cells in culture, in conjunction with The Boyce Thompson Institute for Plant Research in Ithaca, NY, we were able to successfully express Int$_{261}$ in a whole plant model, *Arabidopsis thaliana*. Three out of 6 Arabidopsis plants transformed with pNR49 (includes plant-specific signal peptide sequence) and 1 out of 4 plants transformed with pNR50 expressed Int$_{261}$. These results suggest that Int$_{261}$ can be expressed in a whole plant system. Whole plant systems tend to be more problematic expression systems due to increased complexity and the difficulty of transforming some whole plant species.

II. Immune response to Intimin$_{O157}$ expressed in plant cells.

Many pathogens infect or invade via mucosal surfaces, so the capacity of plant-based vaccines to induce mucosal immunity is a great advantage. Plant cells act as a natural microencapsulation system to protect the vaccine antigens from being degraded in the upper digestive tract before they can reach the gut-associated lymphoid tissue (GALT). Recent studies suggest that plant-based oral vaccines can significantly boost mucosal immune responses primed by parenteral injection (Van der Heijden *et al*., 1989; Lauterslager *et al*., 2001). Parenteral priming of the immune system may allow the GALT to react successfully to the small amounts of antigen delivered during oral immunization and thus decrease the possibility of inducing oral immunotolerance to plant-based and other orally-delivered vaccine antigens. Our studies support these recent findings by demonstrating that parenteral priming of mice with intimin purified from transgenic plant cells can assist in the development of an intimin-specific fecal immune response when these mice are subsequently boosted with oral feeding of the same intimin-expressing transgenic plant material. Furthermore, in this study mice parenterally primed, then orally boosted, showed a statistically significant decrease in the duration of colonization with wild type EHEC O157:H7 upon challenge ($P<0.002$; Log-rank test). Mice immunized entirely by oral feeding did exhibit a reduction in the duration of colonization versus unimmunized mice, though the reduction was not statistically significant. These results suggest that a combination of vaccination strategies with a vaccine antigen produced in and delivered by transgenic plants can function in inducing beneficial, specific immune responses. In addition, further testing is needed to determine

if higher antigen levels in the plant material or additional doses of plant material, when delivered orally, could elicit sufficient, specific immune responses that would result in a reduction in the duration of bacterial colonization without the need for a prime by parenteral injection.

This study also explored the use of cholera toxin (CT) as an oral adjuvant to enhance the systemic and mucosal immune responses of mice co-administered intimin$_{O157}$-expressing NT-1 cell material. CT is considered a potent mucosal adjuvant (reviewed in Freytag and Clements, 1999; Corthesy and Kraehenbuhl, 1999). In fact, CT successfully augmented serum immune responses to Hepatitis B surface antigen expressed in transgenic potato plants (Kong *et al.*, 2001). In addition, CT can facilitate B cell switching to an IgA isotype (Lycke and Strober, 1989) and can promote long-term immunological memory to co-administered antigens (Vajdy and Lycke, 1992). Although the mice given CT in this study did not exhibit any side effects of CT administration (loose stool or diarrhea), the enterotoxicity of CT limits its utility as an adjuvant for the induction of mucosal immunity. One way to eliminate the enterotoxic activity is to construct non-toxic derivatives of the molecule. However, the basis for the adjuvanticity of CT is in some dispute. Investigators have linked this activity with the ADP-ribosylating activity catalyzed by the CT-A subunit (Lycke *et al.*, 1992) that is responsible for the enterotoxicity of the molecule. By contrast, other investigators have demonstrated successful adjuvant activity upon the administration of CT-B subunit, either co-administered with or coupled to the antigen of choice (Isaka *et al.*, 1998; Czerkinsky *et al.*, 1989). Therefore, we remain hopeful that non-enterotoxic derivatives of CT or the closely related entertoxigenic *E. coli* heat-labile toxin (LT) (Freytag and

Clements, 1999) can be used as mucosal adjuvants to enhance overall systemic and local immune responses to a variety of whole-cell killed bacteria or viruses or recombinant vaccine antigens such as transgenic plants.

III. Glycosylation of Int261 in plant cells when expressed with the addition of a plant-specific signal peptide.

Intimin$_{O157}$ is required for EHEC O157:H7 to colonize neonatal calves and adult sheep and cattle (Dean-Nystrom *et al*., 1998; Cornick *et al*., 2002) and antibodies to intimin$_{O157}$, especially to the carboxy-terminal third of the molecule, can block bacterial adherence to HEp-2 cells and prevent infection of suckling piglets. These findings suggest that intimin$_{O157}$ is an attractive candidate for an EHEC O157:H7 anti-colonization and anti-transmission vaccine for cattle, especially if the antigen was produced in and delivered by transgenic plants. Transgenic plant vaccine technology can offer several advantages for the production and delivery of vaccine antigens for humans and especially livestock. Transgenic plants offer the flexibility to function as low cost, efficient, and practical vaccine antigen oral delivery systems to stimulate mucosal immunity or to boost and shift initial immunity to a mucosal antibody response (Lauterslager *et al*., 2001; Van der Heijden *et al*., 1989). One of the challenges associated with transgenic plant vaccine technology can be relatively low antigen expression in plant tissues. In an effort to increase Int$_{261}$ expression in NT-1 cells, a plant-specific signal peptide was used. The signal peptide gene, from the soybean *vspA*, was cloned 5' to the start of the Int$_{261}$ gene. In theory, the addition of the signal peptide directs the translation of Int$_{261}$ into the plant

cell endoplasmic reticulum (ER) and thus removes the antigen from the presence of potentially damaging cytosolic proteases. The translation of Int_{261} in the plant cell ER also makes the protein available to chaperones within the ER to assist in proper folding of the molecule. While the addition of the signal peptide increased Int_{261} expression in NT-1 cells, the antigen appeared to be modified as visualized on Western blot. We demonstrated that this modification was due to glycosylation by chemically deglycosylating the plant expressed protein with TFMS.

Our discovery that non-glycosylated bacterial proteins can be glycosylated in transgenic plant systems highlights an issue not previously reported in other studies of vaccine antigen expression in transgenic plants. In fact, recently published studies found that the fusion of the same plant-specific signal peptide to the Hepatitis B surface antigen increases expression of the antigen and enhances the immunogenicity of the molecule (Sojikul *et al.*, 2003). We found that the addition of the signal peptide and the subsequent glycosylation of Int_{261} expressed in NT-1 cells resulted in an aberrant immune response that was unable to block adherence of EHEC O157:H7. Although the addition of a plant-specific signal peptide increased the vaccine antigen expression levels in the plant cells, the signal peptide may have caused the protein to be glycosylated in the plant expression system by trafficking the newly expressed protein directly into the ER, where glycosylation occurs co-translationally. The removal of the plant-specific signal peptide from the Int_{261} plant expression constructs resulted in decreased protein expression but appeared to produce an unglycosylated protein. Our experience emphasizes one of the problems involved in the successful production of bacterial vaccine antigens in transgenic plant systems.

IV.   The mouse model of intimin$_{O157}$-dependent bacterial colonization and immune response to Int$_{261}$ expressed in plants blocks bacterial adherence.

A mouse model of intimin$_{O157}$-dependent colonization was developed to test the efficacy of the Int$_{261}$ NT-1 cell oral vaccine. In our study, we used the same method to inoculate mice that we reported in a ferret model of EHEC O157:H7 infection (Woods *et al.*, 2002). We showed in an experiment limited to 7 days that EHEC O157:H7 86-24 Str^R colonized untreated BALB/c mice with a mean colonization time of 6.4 days, whereas the intimin-negative mutant 86-24 Str^R *eae*Δ10 displayed a statistically significant reduction in the duration of colonization (mean, 3.2 days; $P<0.005$; Log-rank test). Treatment of the mice with 5 g/L streptomycin in their drinking water raised the overall bacterial colonization level so high as to obscure any evidence of an intimin$_{O157}$-dependence of the colonization. With this untreated mouse model, we showed that mice immunized with an i.p. injection of Int$_{261}$ from NT-1 cells, then boosted by feeding the same plant material made an Int$_{261}$-specific immune response and demonstrated significantly lower levels of bacterial colonization upon challenge with EHEC O157:H7. These results suggest that intimin$_{O157}$ is an important bacterial colonization factor in the mouse model and that this model can serve as a tool to ascertain the effectiveness of an intimin$_{O157}$-based vaccine.

V.   Stx2 toxoid can elicit a Stx2 toxin neutralizing immune response in mice.

Epidemiological data suggest that Stx2 may play a more critical role in the development and severity of HUS than Stx1, although Stx1-only producing strains have also been associated with cases of HUS (Scotland *et al*., 1987; Ostroff *et al*., 1989). Because of these findings, we chose to focus initially on the production of a plant-based vaccine using a recombinant Stx2 B subunit antigen or a Stx2 holotoxoid to be delivered orally and that would provide protection from Stx2-mediated disease.

We found that a recombinant Stx2 B subunit antigen expressed without the Stx2 A subunit did not elicit Stx2 toxin neutralizing antibodies. This result supports the observation of Acheson *et al.* that Stx2 B subunits must pentamerize to elicit Stx2 toxin-neutralizing antibodies (Acheson *et al*., 1995).   Expression of the Stx2 B subunits out of context of the complete Stx2 A subunit may destabilize the pentamers and cause them to form Stx2 B monomers. By contrast, an Stx2 holotoxoid molecule that we created by site-directed mutagenesis appeared similar to the wild-type Stx2 toxin when visualized by Western blot analysis, but did not evoke a cytotoxic response when tested on Vero cells. When this material was injected into mice, the material was not toxic to the mice, and the mice made Stx2 toxin-neutralizing antibodies. The Stx2 toxin-neutralizing antibody response was sufficiently robust to protect the mice from a subsequent toxin challenge of up to 1 μg purified Stx2 toxin. The lethal dose of purified Stx2 toxin for mice is approximately 1 ng (Tesh *et al.*, 1993; Lindgren *et al.*, 1994). These results suggest that the Stx2 toxoid was indeed structurally similar to the Stx2 toxin and most likely was

comprised of stable Stx2 B multimers and/or pentamers capable of eliciting Stx2 toxin-neutralizing antibodies.

To facilitate the optimal expression of the Stx2 toxoid molecule in plants, we speculated that several changes to the nucleotide sequence of the Stx2 A and B subunits would be needed. Indeed, the changes to the Stx2 B subunit were extensive and several methods were tried to resynthesize the Stx2 B subunit gene. The first approach employed a variation of a gene assembly protocol originally described by Stemmer *et al.* (Stemmer *et al.*, 1995; Mason *et al.*, 1998). After several months of limited success with this method, this approach was abandoned. However, we were successful in synthesizing a plant-optimized Stx2 B subunit gene when we used the ligase chain reaction (LCR) (Chalmers and Curnow, 2001). LCR uses ligase to assemble the oligonucleotides that span the synthetic gene, rather than using the oligonucleotides as overlapping primers in a complicated PCR procedure (Chalmers and Curnow, 2001; Stemmer *et al.*, 1995).

VII. Future Directions

The ultimate goal of the intimin$_{O157}$ portion of this project was to create a transgenic plant that would function as an anti-transmission vaccine against EHEC O157:H7 for cattle. The antigen used in this vaccine is specific for EHEC O157:H7. We believe that this vaccine could also protect against colonization with other strains expressing gamma-type intimin, such as EHEC O55:H7 (Fig. 1; Table 1, p. 15-16) (Gansheroff *et al.*, 1999). However an intimin$_{O157}$-specific vaccine may have limited utility against other EHEC strains, such as O26:H11 (beta-type intimin), because

adherence of these organisms to HEp-2 cells are not blocked by $intimin_{O157}$ antibodies

(Gansheroff *et al*., 1999). Our finding that these transgenic cells synthesize the carboxy

terminal portion of EHEC $intimin_{O157}$ (Int_{261}) in a conformation immunologically

equivalent to the native protein underscores the feasibility of transgenic plant-based

systems for expression and delivery of oral vaccines. Plant expression systems are

flexible and have the potential to express and deliver numerous different vaccine

antigens, such as many different intimin types, within the same vaccine dose. Ongoing

research in the lab is investigating EPEC alpha-type intimin as a potential plant-based

vaccine candidate. For further $intimin_{O157}$ vaccine development, we plan to express a

signal sequence-free Int_{261} construct in a whole plant amenable to large-scale production,

perhaps by recently developed models of chloroplast transformation and expression

(Heifetz and Tuttle, 2001) that maximize protein expression and minimize protein

modification (such as glycosylation). The selected plant system will likely be alfalfa

because this plant is compatible with oral feeding to cattle and is amenable to both

nuclear and chloroplast transformation methods. In conjunction with Dr. Wayne Curtis

of Penn State University (State College, PA) and Dr. Evelyn Dean-Nystrom of the USDA

(Ames, Iowa), we scaled-up production of the NT-1 cell clone C34. Dr. Dean-Nystrom is

feeding this Int_{261}-expressing plant material to calves and testing the cattle for

$intimin_{O157}$-specific immune responses. Debate still exists as to whether feedlot food

type, holding pen cleanliness, or water quality management would offer the benefit of

reducing EHEC O157:H7 colonization levels in cattle (Diez-Gonzalez *et al.*, 1998;

LeJeune *et al*., 2001; Smith *et al.*, 2001). One might hypothesize that removing or

reducing environmental sources of EHEC O157:H7 infection, such as soiled bedding or

contaminated water sources, would reduce the prevalence of EHEC O157:H7 in cattle. Unfortunately, these hypotheses have not been adequately tested. However, the notion that reduction of sources of EHEC O157:H7 infection and contamination has strong intuitive appeal, that these measures would reduce not only prevalence of EHEC O157:H7 in cattle, but also reduce the risk of human infection through other environmental sources such as drinking or recreational water and fruits and vegetables. We believe the use of transgenic plants for the production and delivery of a EHEC O157:H7 vaccine for cattle, alone or in conjunction with other potential control methods (Brashears *et al.*, 2003), could lead to a significant decrease in the level of colonization or percent of cattle infected with pathogenic *E. coli* O157:H7. Such a reduction will likely translate into a decline in beef carcass and environmental contamination by EHEC O157:H7 and a decrease in transmission of the bacterium to humans.

Our laboratory is currently working on the assembly of a plant expression vector that will produce plant-optimized Stx2 B subunit and non-catalytically active A subunit (contains the Y77S and Q167E toxoid changes). The ultimate goal of this research will be to create a transgenic plant-based oral vaccine for humans comprised of Int_{261} and Stx2 toxoid. We propose that such a vaccine would reduce or eliminate EHEC O157:H7 infection in humans and concomitantly minimize the effects of Stx2-related pathology.

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