Investigation of Enterotoxigenic *Escherichia coli* (ETEC) Vaccine Candidates and Identification of Inhibitor of Enterohemorrhagic *Escherichia coli* (EHEC) Type III Secretion System Effector NleB

by

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Abstract

Enterotoxigenic *Escherichia coli* (ETEC) is the most common cause of diarrhea in travellers and young children in developing countries. We previously characterized three vaccine candidates (MipA, Skp, and ETEC_2479) which effectively protected mice in an intranasal ETEC challenge model after immunization. However, these proteins are conserved not only in multiple ETEC isolates, but also in commensal bacteria. In this study, we examined the potential of these antigens to affect the host intestinal microbiota and subsequently found no significant impact on healthy of host after vaccination. In addition, we also optimized the types of adjuvants and forms of antigens and evaluated the efficacy in a mouse intranasal challenge model.

Enterohemorrhagic *Escherichia coli* (EHEC) is an emerging zoonotic pathogen that cause global public health threads. EHEC possesses the potential to cause gastroenteritis, hemorrhagic colitis and hemolytic uremic syndrome (HUS), which may lead to renal failure. Type III secretion system (T3SS) is a hallmark of EHEC, characterized by the needle-like structure and a variety of effectors injected into host cells. NleB, one of T3SS effectors, is a glycosyltransferase with the ability to catalyze the transfer of *N*-acetyl-D-glucosamine (N-GlcNAc) to host proteins to suppress the activation of NF-κB signaling pathway. In this study, we employed luminescence-based glycosyltransferase assay and high-throughput screening using a chemical library of various compounds. A total of 128 chemicals was selected with significant inhibition on NleB glycosyltransferase activity for further pharmaceutical study as novel therapy against EHEC infection.

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Chapter 1 - Literature Review

Escherichia coli and classification

Escherichia coli (*E. coli*) has been extensively studied as the best characterized microorganism since its discovery in 1885 by Theodor Escherich. *E. coli* is capable to adapt to proliferate in various environments and lifestyles, not only in gastrointestinal (GI) tract and extraintestinal sites of most warm-blood animals. It can also withstand harsh environmental conditions outside of hosts for extended period, such as surface of plants or in the soil. Typically, *E. coli* resides in niches of lower intestinal tract of mammals, birds, and reptiles as an innocuous commensal bacterium. However, some have evolved by improving its robustness and efficient colonization to carry the potential to cause diverse diseases in hosts and render challenges to the health of human beings. Pathogenic variants of *E. coli* possess the virulence traits to cause a wide spectrum of human diseases ranging from the GI tract such as diarrhea, to extraintestinal sites, mostly the bloodstream, urinary tract, and central nervous system[1]. Diarrheagenic *E. coli* (DEC) is a particular collection of pathotypes involved in diarrheal diseases. Although varying significantly in virulence mechanisms, colonization sites and clinical symptoms, diarrheagenic *E. coli* have been traditionally categorized into six pathotypes: enterotoxigenic *E. coli* (ETEC), enterohemorrhagic (Shiga toxin-producing) *E. coli* (EHEC/STEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), diffuse adherent *E. coli* (DAEC) and enteroaggregative *E. coli* (EAEC)[2]. New pathotypes, which may have evolved to combine different characteristics, such as a Stx-producing EAEC strain, are still emerging[3].

Enterotoxigenic *E. coli* (ETEC) is the most common cause of diarrhea in travellers and farm animals[4]. The infection of ETEC is typically initiated through intake of contaminated water or food. ETEC can persist in contaminated water and adhere firmly to surface of fresh vegetables, both increasing the risk of transmission[5,6]. Two types of virulence factors produced by ETEC contribute to its pathogenesis. Adhesins, also named colonization factor (CF) antigens including various fimbriae, fibrillae, and filamentous protein structures, are expressed to facilitate intestinal colonization by binding to specific receptors on host cells[7]. Once established, ETEC produces enterotoxins which consist of heat-labile toxin (LT) and heat-stable toxin (ST), both responsible for fluid secretion. LT is arranged as an AB_5 where the B subunits mediate binding to intestinal cells and the A subunit possesses an ADP-ribosylation activity. LT B subunits bind non-covalently to subunit A and attach to enterocytes via ganglioside GM1 receptors on the surface[8]. Activation of adenylate cyclase increases cyclic AMP to induce dehydration of enterocytes. LT-B subunit is a potent immunogen and is widely considered as a vaccine candidate or adjuvant. While ST is a nonimmunogenic polypeptide and responsible for increase of cyclic GMP levels within intestinal cells. As a result, chloride secretion is increased, and sodium chloride adsorption is inhibited, leading to fluid secretion into lumen of the intestine[8].

Enterohemorrhagic *E. coli* (EHEC) is an emerging zoonotic pathogen that has been the subject of considerable research because of severe public health problem by its high morbidity and mortality[9]. EHEC serotype O157:H7 is capable to cause acute gastroenteritis and hemorrhagic colitis in infants and younger children, as well as hemolytic uremic syndrome leading to kidney failure and chronic post-infection

sequelae. The hallmarks of its pathogenesis are the attaching and effacing (A/E) lesions for intimate adherence to host cells and utilization of type III secretion system (T3SS) to translocate virulence proteins into infected cell.

Pathogenesis

ETEC Type II secretion system

Type II secretion system (T2SS) is used by many Gram-negative bacteria, including ETEC, to export diverse proteins across the bacterial membrane to extra-cellular milieu. Overall, the T2SS is composed of 12 to 15 proteins and assembles as three parts: the inner membrane complex providing energy through NTP hydrolysis, the pseudopilus which is a pilus-like polymer spanning across the periplasmic compartment, as well as a large, pore-forming outer membrane complex responsible for secretion specificity[10-12]. The process of secretion through T2SS is accomplished in two distinct steps. The proteins are first oriented by signal peptides to Sec or Tat machinery to transport into periplasm from cytoplasm. Then after removal of signal peptides, proteins are fully folded for recognition and translocation across the outer membrane to extracellular environment[13].

As delivery machinery, aside from exporting fimbrial components, outer membrane cytochromes and other surface-associated proteins, T2SS also mediates the release of virulence factors, such as LT[14]. Elucidation of assembly and function of T2SS machinery contributes the identification of novel ways to block the delivery of virulence factors, which are of great importance in application. For instance, mutation of T2SS,

which fails to secrete T2SS associated proteins, is a practical strategy to screen outer membrane proteins as potential antigens for vaccine development[15].

EHEC Type Ⅲ secretion system and effectors

A/E pathogens, such as EHEC, have evolved an effective delivery system of virulence effectors to manipulate host signaling pathway. An important role of the type Ⅲ secretion system is to inject the host cell with a variety of effectors via a needle-like structure spanning the inner and outer bacterial membranes. The T3SS consisting of more than 30 different proteins is regarded one of the most complicated secretion systems[16]. The hallmark of T3SS structure is composed of three main parts in general: extracellular appendages, basal body, and cytoplasmic components[17]. Extracellular appendages consist of translocation pore inserted to the host membrane, needle-shaped structure protruding above the outer membrane of bacteria, as well as filament structure on the top of the needle[18,19]. Three ring structures spanning the inner and outer membrane are connected with a periplasmic inner rod to compose the basal body[20]. The cytoplasmic components contain the C-ring, the ATPase complex, and the gatekeeper proteins which serve as sorting platform[21,22]. The precise assembly and regulation of T3SS, ensure the substrates are secreted strictly in proper time. In addition to structural proteins, proteins involved in T3SS can be divided into chaperones and effectors. The chaperones are some proteins essential for efficient secretion of effectors and structural proteins by improving protein stability and recognition[23].

The purpose of effective secretion system assembly is to manipulate the function of host cell for bacterial benefit and is fulfilled by injection of various effectors[24,25]. The effectors, as many as 39 in certain EHEC strains[16], are classified into two groups, LEE

(locus for enterocyte effacement) encoded and non-LEE effectors (Nle) encoded by genetic elements located outside the LEE. Tir is a receptor protein encoded by LEE and secreted to the plasma membrane of host cell and acts as a receptor for intimin expressed on the surface of bacteria. Binding between Tir and intimin facilitate the tight attachment and play an essential role in A/E lesion formation and bacterial establishment. Additional non-LEE effectors also contribute to the disruption of host signaling and subversion of host innate immune response. For instance, NleB is a translocated N-acetyl-D-glucosamine (N-GlcNAc) transferase which mediates N-GlcNAcylation of mammalian glycolysis enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to inhibit its interaction with the TNF receptor associated factor 2 (TRAF2) and suppress the activation of downstream NF-κB pathway[26,27]. NleC and NleD, which are Zn-dependent proteases, disrupt the host NF-κB signaling and AP-1 activation by inactivation of RelA (p65) and c-Jun N-terminal kinase (JNK) pathway[28,29]. NleE functions as a methyltransferase that binds to TAB2 and TAB3 to block the NF-κB pathway of host[30]. NleF was reported to dampen caspase-4-mediated inflammatory epithelial cell death and prevent the production of IL-1β[31]. NleH1, not NleH2, is capable to alter the nuclear translocation of ribosomal protein S3 (RPS3) to inhibit NF-κB pathway[32]. The mechanisms of action of T3SS effectors contribution to EHEC pathogenesis warrant further investigation.

Treatments

ETEC vaccinology

ETEC remain leading causes of infantile and traveler's diarrhea in the developing world. Prevention by vaccination is advantageous due to high public health impact, cost-effectiveness and feasibility of immune protection. Many vaccine developments during last decade have largely focus efforts on the best-studied antigens of ETEC, involving inactivated whole cell vaccines, live attenuated vaccines and subunit vaccines targeting on enterotoxin or surface CFs[33,34]. However, the vaccines were not efficacious tested in clinical trials by the insufficient protections or risk of side effects[33,34]. Also, the overall protection yielded from these vaccines has varied due to the diversity of serotypes from ETEC pathovar[34]. Even more than 25 different CF have been described to be antigenically distinct, 40-50 % of ETEC do not produce a recognizable CF[36-38]. One killed whole celll vaccine (Dukoral), which is licensed to prevent cholera, has been proved to only provide short-term protection against ETEC due to the high antigenic similarity of cholera toxin and LT[39]. Therefore, to date neither licensed vaccine nor a broadly protective vaccine is available[40]. Currently, studies showed that pathogenesis of ETEC is much more complex than previously appreciated and additional virulence proteins of surface molecules may be targeted in vaccines[15]. We have previously described three ETEC surface proteins, which were screened out using T2SS mutant, as protective antigens. Vaccination with these three proteins (MipA, Skp, and ETEC_2479) protected mice in an intranasal challenge[15]. Further study is still needed to select novel vaccine candidates to prevent disease from diverse ETEC.

EHEC therapies

EHEC can cause bloody diarrhea and hemolytic uremic syndrome (HUS) leading to acute kidney failure. Recently several devastating EHEC epidemics in Europe have urged the need to offer effective therapies[41]. Current treatment strategy is a multitargeted approach. The general supportive treatments, such as fluid and electrolyte balance, have been shown to reduce risk of HUS developing[42,43]. Utilizations of antimicrobials, particularly β-lactams, are avoided due to the potential risk of endotoxin release from dead bacterial cells[44]. Antibodies targeting lipopolysaccharide (LPS) or A subunit of Stx1 have proved to be protective in cell or animal experiment[45,46]. Even with potential of therapeutic use, human trials are still needed to prove clinical effectiveness. Artificial probiotics are designed to express proteins to mimic receptors on host cell, so as to neutralize the deceived pathogen[47]. However, these proteins decorated probiotic need to survive the adverse condition encountering digestive enzymes and the trial data is still awaited. Several vaccine strategies include virulence proteins (Stx1/2), recombinant fimbrial proteins, DNA vaccine, live attenuated, or avirulent bacteria[48]. But the immunogenicity varies in different vaccines and the administration modes. Moreover, toxin binder such as cell-permeable peptide binds to Stx2 and cause increased survival in a baboon model and outcomes of human trials are awaited[49]. It takes time for human trial, but the frequent outbreaks of EHEC, as well as no special effective therapy, focus the world's attention onto development of novel treatments.

Purpose of study

Investigation of Enterotoxigenic *Escherichia coli* (ETEC) vaccine candidates

Enterotoxigenic *Escherichia coli* (ETEC) causes hundreds of millions of cases of diarrhea annually, particularly in developing countries[50]. In addition to their acute impact on human health, repeated infections also contribute to delayed growth and malnutrition[50]. Many vaccine strategies have focused on ETEC colonization factors (CFs). These heterogeneous surface structures function in attachment by binding to host glycoprotein conjugates[51]. Numerous CFs have been described, but many ETEC strains do not produce a recognizable CF[34]. A need therefore exists to identify new vaccine targets that are independent of strain-specific CFs.

We previously characterized three ETEC H10407 proteins as protective antigens in a mouse model involving intranasal bacterial challenge[15]. Antisera raised against the ETEC MipA, Skp, and ETEC_2479 proteins protected HCT-8 cells from attachment by multiple ETEC strains[15]. Immunization with these antigens also protected mice from an otherwise lethal challenge with intranasally administered ETEC H10407[15]. Skp is a molecular chaperone that rescues misdirected outer membrane proteins[52]. MipA is an immunoreactive protein that belongs to a family of proteins involved in remodeling peptidoglycan[53]. ETEC_2479 is predicted to function as an outer membrane porin involved in long chain fatty acid transport[54]. The intranasal challenge model is a useful alternative for ETEC vaccine studies because mice do not naturally develop diarrheal disease after oral ETEC challenge[55-57]. This model also permits the quantification of mouse survival, bacterial clearance, and host immune responses, and stimulates

mucosal immune responses, especially secretory IgA (sIgA) responses that are important to blocking bacterial adherence to mucosal surfaces.

Identifying broadly conserved, protective antigens is important to vaccine development. However, MipA, Skp, and ETEC_2479 are conserved not only among pathogenic ETEC strains, but also among the commensal *Proteobacteria*. This may be an important issue because it is known that alteration of commensals can influence susceptibility to gastrointestinal disease and vaccine efficacy[58,59]. Several previous studies have begun to address this issue. Rotavirus vaccination of humans did not have a major impact to infant microbiomes[60]. A challenge of cynomolgus macaques with an oral-live attenuated *Shigella* strain found a possible protective role for the microbiota and highlighted the importance of considering host genetics in vaccine studies[61]. Oral immunization with the live-attenuated typhoid vaccine strain Ty21a did not cause significant perturbation of the fecal microbiota related to vaccine administration[62]. Thus, while the impact of inactivated viral vaccines and live-attenuated bacterial vaccines on the host microbiota have been examined, the potential impact of using subunit vaccines consisting of antigens that are also encoded by commensal organisms has not been investigated.

In addition, our previous work used GST-tagged forms of MipA, Skp, and ETEC_2479 to demonstrate vaccine efficacy, but an antigen lacking the epitope purification tags will be appropriate for further study. Although cholera toxin (CT) is an effective adjuvant, it is not suitable for future vaccine trials due to its enterotoxicity. So we employed a novel vaccine strategy based on outer membrane vesicles (OMVs). OMVs are released by both pathogenic and non-pathogenic Gram-negative bacteria

which is a ubiquitous process during normal growth[63,64]. The artificial presentation of target antigens in complex with the natural presence of LPS as an adjuvant makes OMVs an effective means to induce host immune response[65-67].

We hypothesized that using the conserved antigens MipA, Skp, and ETEC_2479 as subunit vaccine candidates could negatively impact the health of the host by affecting the intestinal microbiota. We also assumed that both untagged vaccine candidates and employment of OMVs could yield effective protection in mice challenge model. Herein, we tested and subsequently failed to reject these hypotheses.

Identification of inhibitor of Enterohemorrhagic *E. coli* (EHEC) type Ⅲ secretion system effector NleB

Enterohemorrhagic *E. coli* (EHEC) have been a major cause of gastroenteritis and hemorrhagic colitis as well as hemolytic uremic syndrome (HUS), which present great challenges to public health. They carry an enormous potential to cause acute renal failure which is detrimental to human health. However, no special effective therapy exists to date, giving motivation to develop a promising medication with protective efficacy.

EHEC is distinctively characterized by the attaching and effacing (A/E) lesions for intimate adherence to host cells and utilization of type Ⅲ secretion system (T3SS) to translocate virulence proteins into infected cell. A plethora of effector proteins are injected by T3SS to modulate the innate immune system via several different mechanisms. T3SS effector NleB is described to be highly conserved among A/E pathogens enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), and *Citrobacter rodentium*, which is a mouse pathogen using similar strategies with *E. coli*[68].

EHEC carries two copies of NleB, NleB1 and NleB2, which are encoded in separate pathogenicity islands. NleB1 and NleB2 are highly related and share 60 to 62 % amino acid identity and ~80 % similarity. NleB1 from EHEC and EPEC share ~98 % amino acid identity with each other and have ~89 % amino acid identity and ~96 % similarity with NleB of *C. rodentium* which is its only copy. In addition, *Salmonella enterica* strains also possess three NleB orthologs, SseK1, SseK2, SseK3[69]. NleB is a bacterial glycosyltransferase with the ability to modify the arginine residues by translocating *N*-acetyl-D-glucosamine (N-GlcNAc), which is an atypical post-translational modification (PTM) not observed in eukaryotic cells. Glycosylation of the mammalian glycolysis enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by NleB disrupts its interaction with TNF receptor associated factor 2 (TRAF2) and subsequently suppress TRAF2 polyubiquitination and NF-κB activation[27]. Other host proteins which are substrates of NleB orthologs have been reported, such as Fas-associated via death domain (FADD), TNFRSF1A-associated via death domain (TRADD), and receptor-interacting serine/threonine kinase 1 (RIPK1)[70]. However, the preferences of substrate in different NleB orthologs vary and mechanisms are still under investigation (detail in Table1)[71-74]. NleB is also essential for virulence of *C. rodentium* in mice. NleB deficient *C. rodentium* strains exhibited attenuated ability to colonize in mice model[27]. NleB is exemplified as a virulence strategy how pathogens employ T3SS and its effectors to inhibit NF-κB dependent host innate immune responses.

Table 1 NleB orthologs

NleB ortholog	Effect on NF-κB pathway	substrate			
		GAPDH	FADD	TRADD	RIPK1
EHEC NleB1	block	yes	Yes	*	*
EHEC NleB2	--	no	No	*	*
EPEC NleB1	block	no	Yes	yes	yes
EPEC NleB2	--	no	No	*	*
***C. rodentium* NleB**	block	yes	Yes	*	*
***S. enterica* SseK1**	block	yes	Yes/no	yes	*
***S. enterica* SseK2**	--	no	Yes	*	*
***S. enterica* SseK3**	block	no	No	yes	*

Table1: the preference substrate of NleB orthologs. EHEC= enterohemorrhagic *E. coli*: EPEC= enteropathogenic *E. coli*: block=inhibit the activity; yes= substrate; no=not substrate; yes/no= conflicting reports; *= not tested.

Although EHEC cause severe outbreaks and present tremendous challenge to public health, no practical and effective therapy is available to date and research efforts are still needed[48]. Recently, some studies on toxin inhibitor or binder have shed light on investigation of potential therapy. For instance, a novel agent can bind to Stx directly as a neutralizer and inhibit its binding to receptor presented on target cells[75]. Another study

use cell-permeable peptide as a toxin binder to neutralize Stx2 and increase survival in a baboon model[49]. So in this study, we employed a luminescence-based glycosyltransferase assay to screen a potential inhibitor of NleB which contribute to EHEC pathogenesis. The UDP-Glo™ glycosyltransferase assay is a bioluminescent assay to determine the activity of UDP-GlcNAc glycosyltransferase. The released UDP from UDP-sugar in glycosyltransferase reaction is then converted to ATP and measured by light intensity using a luminometer. After using a variety of chemical compound in reaction, the inhibitor candidate can be screened out by significantly reduced luminescence. The potential inhibitors of NleB are worthy of further investigation as a novel therapeutic modality against EHEC infection.

Chapter 2 - Investigation of Enterotoxigenic *Escherichia coli* (ETEC) vaccine candidates

These data were published in 2015[35].

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is the leading cause of diarrhea of young children in developing countries and adults and children traveling to endemic areas[50]. Antigenic and structural heterogeneity among colonization factors of ETEC pathovar has complicated vaccine development efforts. Identifying and characterizing conserved ETEC antigens that induce broadly protective immunity is therefore of interest. We previously characterized three proteins (MipA, Skp, and ETEC_2479) that protected mice in an intranasal ETEC challenge model after vaccination[15]. However, these proteins are conserved not only in multiple ETEC isolates, but also in commensal bacteria. While the impact of inactivated viral vaccines and live-attenuated bacterial vaccines on the host microbiota have been examined, the potential impact of using subunit vaccines consisting of antigens that are also encoded by commensal organisms has not been investigated. We addressed this issue by characterizing changes to mouse intestinal microbiomes as a function of vaccination. We hypothesized that using the conserved antigens MipA, Skp, and ETEC_2479 as subunit vaccine candidates could negatively impact the health of the host by affecting the intestinal microbiota. Herein, we tested and subsequently refuted this hypothesis.

Materials and Methods

Ethics statement

Animal experiments were performed according to Kansas State University Institutional Animal Care and Use Committee-approved protocols (IACUC #3648). This institution complies with all applicable provisions of the Animal Welfare Act and other Federal statutes and regulations relating to animals.

Antigen purification

Escherichia coli BL21 (DE3) strains expressing individual antigens were grown overnight as described[15]. A single colony of each expressing strains was cultured in 4 ml Luria-Bertani (LB) broth supplemented with 50 μg/ml Kanamycin shaking at 37 °C overnight. 2 ml of the overnight culture was transferred to 200 ml LB medium containing 50 μg/ml Kanamycin. Once optical density (OD) value of the culture reached 0.4~ 0.6, I mM Isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma, MO) was added to into culture to induce protein expression for 5 h shaking at 37 °C. Culture were collected and proteins were purified by using nickel-affinity chromatography. After purification, proteins were dialyzed into glycerol in Pierce Slide-A-Lyzer dialysis cassettes. Protein concentrations were quantified by using the Precision Red Advanced Protein Assay (Cytoskeleton, Inc.).

Polyclonal antisera production

Female BALB/c mice of matched age (6 weeks at initial vaccination) were obtained from the Jackson Laboratory (Bar Harbor, Maine) and handled as described previously[15]. Mice were housed (5 per group) in microisolator cages (1 cage per group)

and provided with food and water ad libitum. Antigens (20 μg/dose) were mixed with 2.5 μg of cholera toxin in 25 μl phosphate-buffered saline (PBS) and then administered intranasally to the external nares of mice that had been anesthetized with isoflurane[15]. Two booster doses were administered, at 2- and 4-weeks after the initial vaccination. Mice were euthanized 2 weeks after the final immunization and exsanguinated. The blood was processed into serum using centrifugation for 2 min at 2500*g* in a BD microcontainer serum separator tube. Control serum samples were also obtained from mice immunized with PBS or with EHEC EspB.

Immunoassays

Enzyme-linked immunosorbent assays (ELISAs) were performed as previously described[15], using serial dilutions of mouse serum samples and polystyrene 96-well, flat bottom plates (Whatman) coated with purified antigens or with bovine serum albumin (BSA; 0.5 μg/ml). Plates were developed with 1-StepTM Ultra TMB-ELISA (Thermo) and quenched with 3 N H_2SO_4. Absorbance was read at 450 nm.

Fecal DNA extraction

A fecal pellet from each mouse was collected weekly, with the initial collection prior to the first vaccination and the final collection 2 weeks after the final vaccination. After collection, the fecal pellets were stored at −80 °C until DNA extraction could be performed. Prior to DNA extraction using QIAamp DNA Stool Mini Kits (Qiagen), 1.4 ml of Buffer ASL was added to fecal samples on ice. Fecal pellets were vortexed until completely resuspended and DNA was extracted by following the manufacturer's protocol.

Library construction and sequencing

Library construction and sequencing was performed essentially as described[76]. Prior to PCR, DNA concentrations were determined via fluorometry (Qubit dsDNA BR assay, Life Technologies, Carlsbad, CA) and normalized to a standard concentration. Bacterial Microbial 16S rRNA amplicons were generated via amplification of the V4 hypervariable region of the 16S rRNA gene using single-indexed universal primers as described previously [U515F/806R; [77]] flanked by Illumina standard adapter sequences. Primer sequences are available at proBase [[78]; http://www.microbial-ecology.net/probebase/]. PCR was performed using the following parameters: 98 °C$^{(3:00)}$ + [98 °C$^{(0:15)}$ + 50 °C$^{(0:30)}$ + 72 °C$^{(0:30)}$] × 25 cycles +72 °C$^{(7:00)}$. Following PCR, amplicons were pooled for sequencing using the Illumina MiSeq platform and V2 chemistry with 2 × 250 bp paired-end reads.

Informatics analysis

Informatics analysis was performed essentially as described[76]. FLASH software was used to assemble contiguous DNA sequences[78]. Sequences were culled if determined to be short after trimming for a base quality less than 31. Reference-based and *de novo* chimera detection and removal was conducted using Qiime v1.8 software[79]. Remaining contiguous sequences were assigned to operational taxonomic units (OTUs) via *de novo* clustering with a criterion of 97 % nucleotide identity as described[76]. Annotation of selected OTUs was performed using BLAST [80] against the Greengenes database[81] of 16S rRNA sequences and taxonomy. Principal component analysis was performed using ¼ root-transformed OTU relative abundance data via a non-linear iterative partial least squares (NIPALS) algorithm, implemented using an

open access Excel macro available from the Riken Institute (http://prime.psc.riken.jp/Metabolomics_Software/StatisticalAnalysisOnMicrosoftExcel/index.html). Sequence data were deposited in the NCBI Sequence Read Archive (SRA) under the BioProjectID PRJNA320839.

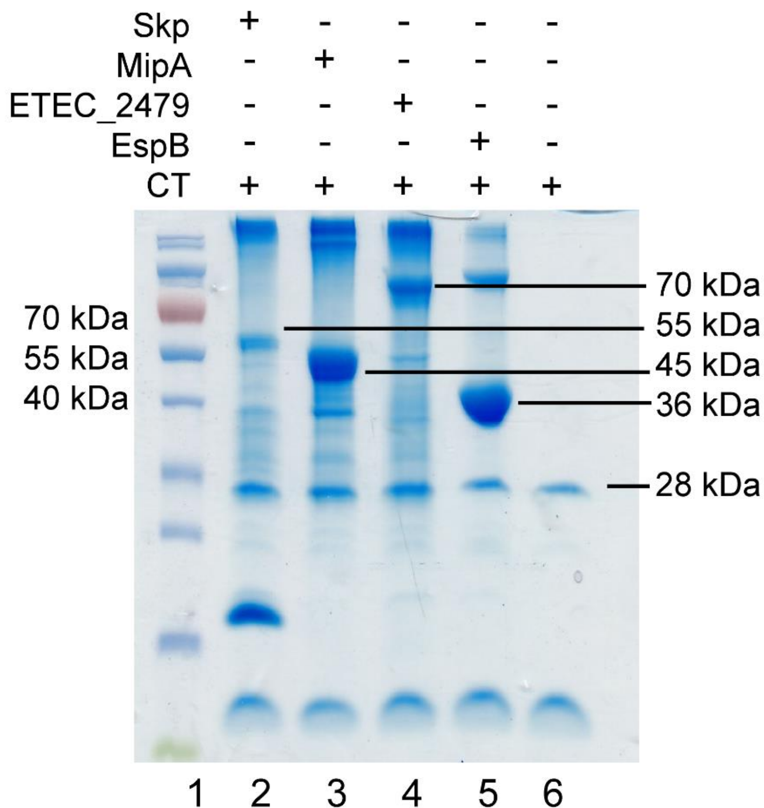
Statistical analyses

Statistical analysis was performed using Sigma Plot 12.3 (Systat Software Inc., Carlsbad, CA). Interactions and differences between treatment groups and time-points in Chao1 indices were determined using 2-way analysis of variance (ANOVA). Analysis of molecular variance (AMOVA) was implemented in a general linear model using SPSS software, version 23 (IBM, Armonk, NY). Results were considered statistically significant for p values ≤ 0.05.

Results

Mice were vaccinated intranasally with purified, recombinant forms of ETEC MipA, Skp, and ETEC_2479[15], as well as with a purified, recombinant form of E. coli O157:H7 EDL933 EspB (Fig. 1)[82]. EspB was used as an external control because it is immunogenic but is not expressed by either ETEC or by commensal bacteria[83].

Figure 1 Antigen purification and vaccine mixture with cholera toxin.



Coomassie stained SDS-PAGE gel of vaccine mixtures. Lane legends are 1, protein marker; 2, purified Skp protein and cholera toxin; 3, purified MipA protein and cholera toxin; 4, purified ETEC_2479 protein and cholera toxin; 5, purified EspB protein and cholera toxin; and 6, cholera toxin control.

Mouse health and weight gain were monitored during the vaccination regimen. Neither obvious changes to mouse health or behavior, nor changes in the rate of weight gain were observed. The weights of the mice after vaccination were 21.1 ± 1.0 g for the PBS control group, 20.7 ± 1.0 g for EspB, 21.2 ± 0.9 g for MipA, 21.2 ± 2.0 g for Skp, and 21.3 ± 0.9 g for ETEC_2479 ($p > 0.05$) (Fig. 2). After 3 immunizations, mice were sacrificed and their serum was used in ELISAs to quantify antibody titers. All mice (5/group) produced detectable IgG titers (Fig. 3a). Fecal IgA responses for mice immunized with either MipA, Skp, or ETEC_2479 ranged from a 13.2 ± 2.2, 13.0 ± 3.4, and 26.8 ± 4.6 fold-increase as compared with control groups, respectively[15].

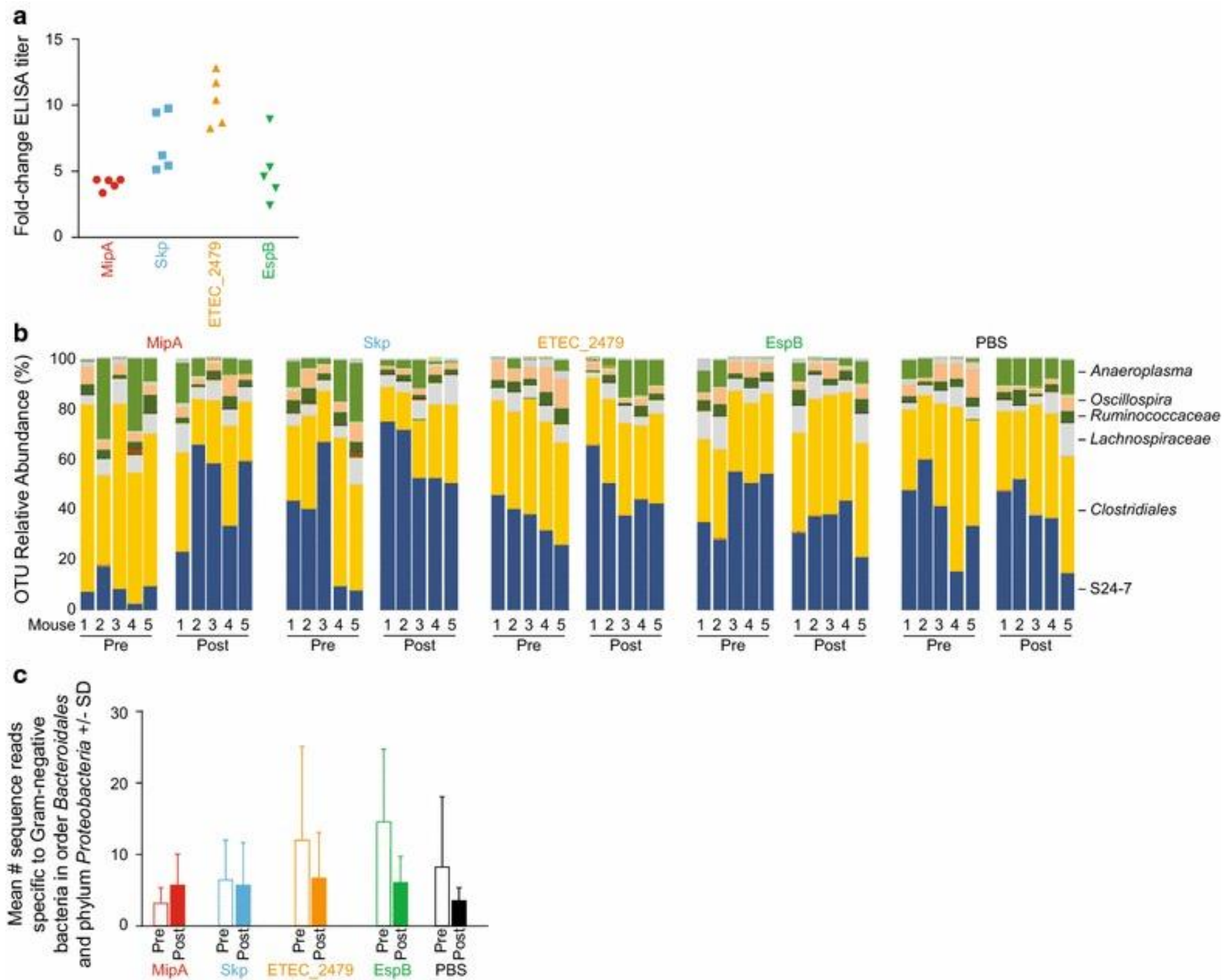
Figure 2 Mice weights during vaccination.

Mice weights during vaccination. Skp1-5: Five mice immunized with Skp+CT; MipA1-5: Five mice immunized with MipA+CT; ETEC_24791-5: Five mice immunized with ETEC_2479+CT; EspB1-5: Five mice immunized with EspB+CT; PBS1-5: Five mice immunized with PBS+CT.

To determine whether vaccination affected the diversity and overall composition of the mouse intestinal microbiota, 16S rRNA amplicon sequencing was performed using DNA extracted from feces collected prior to vaccination, from the mid-point of the vaccination regimen (after the second vaccination), and two weeks after the final vaccination, as template. Following vaccination, there were no apparent differences in the microbial profiles when resolved to the level of operational taxonomic unit (OTU), and the same six OTUs (families S24-7, *Lachnospiraceae*, *Ruminococcaceae*, order *Clostridiales*, *Oscillospira* sp., and *Anaeroplasma* sp.) dominated the pre- and post-vaccination microbiota of all groups (Fig. 3b). Regarding the effect of vaccination on other Gram-negative taxa that potentially express the targeted antigens, i.e., microbes in the order *Bacteroidales* or phylum *Proteobacteria*, no differences were detected between pre- and post-vaccination samples in the relative abundance of these bacteria (Fig. 3c). The Chao1 index, a measure of α-diversity (i.e., within samples), was also compared between groups to determine if vaccination affected the richness or distribution of microbes. Two-way ANOVA detected no significant differences between groups, or between pre- and post-vaccination samples (Fig. 4a). Samples obtained from the mid-point of the vaccination regimen (after the second vaccination) were also analyzed and were not significantly different from pre-or post-vaccination samples (Fig. 4a). Similarly, neither the Shannon diversity index, nor the raw number of unique

sequences detected in each group, were significantly different among groups (Fig. 4b, c).

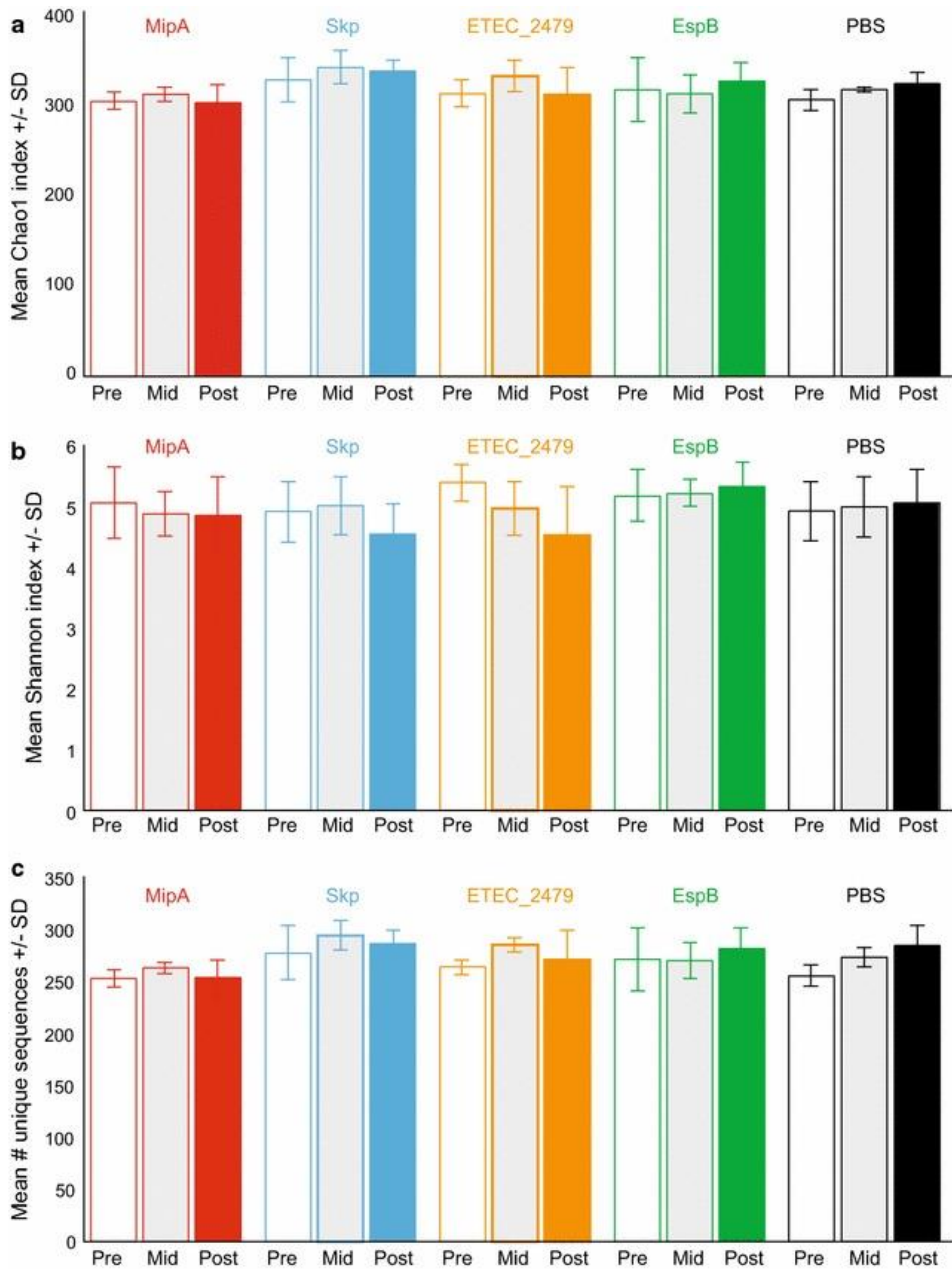
Figure 3 Vaccination with ETEC MipA, Skp, and ETEC_2479.



Vaccination with ETEC MipA, Skp, and ETEC_2479. **a** Serum IgG responses in mice. Data are plotted as the fold-change in serum IgG after immunization with the indicated antigens, n = 5/group. **b** Bar chart showing relative abundance of all operational taxonomic units (OTUs) detected in the feces of mice prior to (pre) and 6 weeks after (post) vaccination with the indicated antigens, as detected using 16S rRNA amplicon sequencing. The identities of

dominant taxa are shown at the right. **c** Mean number + standard deviation (SD) of sequence reads that were specific to *Bacteriodales* or *Proteobacteria* in indicated treatment groups.

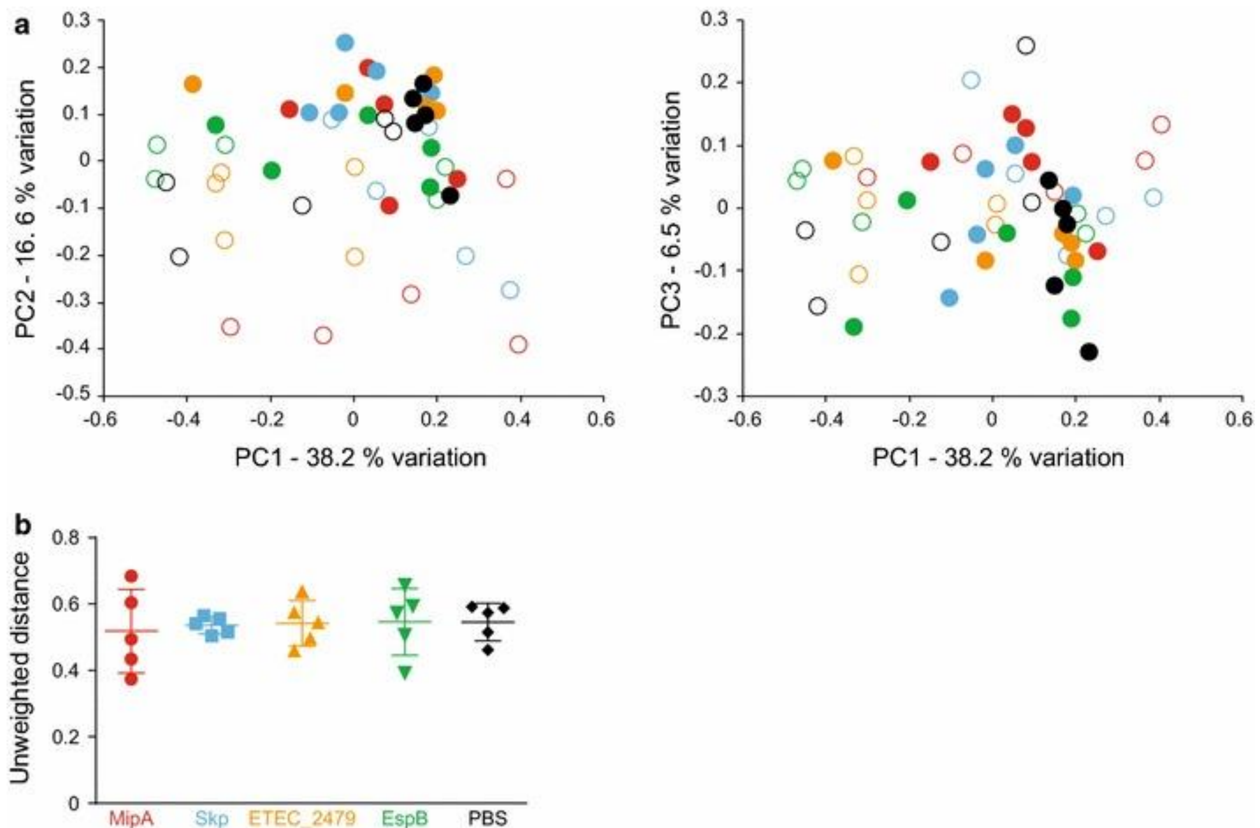
Figure 4 Sequence diversity among treatment groups.



Sequence diversity among treatment groups. **a** Bar chart showing mean + standard deviation (SD) Chao1 a-diversity index of fecal microbiota in mice pre- and post-vaccination with the indicated antigens, as detected using 16S rRNA amplicon sequencing. **b** Mean Shannon diversity indices. **c** Mean number of unique sequences in treatment groups.

Principal component analysis (PCA) was performed both to assess β-diversity (i.e., between samples) and to determine if vaccination induced changes in the overall composition of the microbiota. No clustering of post-vaccination samples was observed in plots of principal component 1 (PC1) against PC2 or PC3, which accounted collectively for over 60 % of the variability between samples, suggesting that there were negligible shifts in the bacterial community composition of any treatment group (Fig.5a). A comparison of the mean intragroup unweighted UniFrac distance between pre- and post-vaccination samples detected no greater dissimilarity between the two samples in treated groups as compared to the control group, as determined by performing ANOVA with post hoc Dunnett's tests (Fig. 5b). Supporting our prior analysis, AMOVA detected no significant effect of vaccination treatment ($p = 0.053$; F value = 3.302), and also no significant effect of time-point ($p = 0.359$; F value = 4.517), using the first and last samples as time-points.

Figure 5 Principal component analyses and UniFrac distances.



Principal component analyses and UniFrac distances. **a** Unweighted principal component analyses showing β-diversity of fecal microbiota in mice pre- and post-vaccination with the indicated antigens, as detected via 16S rRNA amplicon sequencing. Principal component 1 (PC1) versus PC2 (*left*) and PC1 versus PC3 (*right*) are shown. *Color-coding* is identical to Fig. 4, with *open symbols* representing pre-vaccination and *closed symbols* representing post-vaccination samples. **b** Mean intragroup unweighted UniFrac distances between pre- and post-vaccination samples.

Discussion

The discovery and characterization of broadly conserved ETEC vaccine antigens that are independent of strain-specific CFs is of emerging interest. However, an important consideration is whether targeting antigens also expressed by commensal flora will negatively impact host health or vaccine efficacy. We have characterized the ETEC proteins MipA, Skp, and ETEC_2479 for their protective efficacy in an intranasal challenge model. These proteins are highly conserved not only in diverse ETEC isolates, but also in commensal *Proteobacteria* and other *E. coli* and *Shigella* strains, sharing ~99 % identity with the corresponding *Shigella* proteins. Because altering commensal abundance and diversity may affect host health and vaccine efficacy, we were therefore interested in determining whether using these antigens in a subunit vaccine would affect the mouse microbiota. Commensal *E. coli* strains may contribute to colonization resistance against pathogens[84]. For example, *E. coli* Nissle 1917 has been extensively characterized as a probiotic agent[85] and could potentially function by competing for nutrients that are required by pathogens[84]. They also play important, though incompletely defined roles in maintaining intestinal homeostasis[86].

We did not observe changes to mouse health, behavior, or rate of weight gain following intranasal vaccination with either MipA, Skp, or ETEC_2479. We also observed no significant differences among the microbial profiles when resolved to the level of operational taxonomic unit (OTU), as determined by performing 16S rRNA amplicon sequencing. Analysis of the Chao1 index, the Shannon diversity index, and the raw number of unique sequences detected in each treatment group, also failed to

reveal any significant differences. PCA analysis and comparison of UniFrac distances also suggested negligible shifts in the bacterial community composition of any treatment group.

While no consistent shifts in the composition of the fecal microbiota were detected following vaccination against the three conserved candidate antigens, a closer examination of the effects of vaccination on the relative abundance of taxa expected to express these antigens, i.e., bacteria within order *Bacteroidales* and phylum *Proteobacteria*, was performed. While no vaccination-dependent differences were detected in the relative abundance of these groups, we recognize that both *Bacteroidales* and *Proteobacteria* are rare taxa in the current samples. This is not however unique to this cohort of mice, as we have demonstrated very comparable levels of these taxa in multiple genetic backgrounds of mice purchased from the same vendor and the overwhelming majority of gut bacteria in most research mice are Gram-positive[87]. Regardless, the low numbers of Gram-negative bacteria present in the present cohorts were not affected by vaccination. It will be important in future studies to validate these studies using diarrheal disease models for ETEC in either mice or piglets.

Overall, despite the conservation of MipA, Skp, and ETEC_2479 among Gram-negative bacteria, vaccination with these antigens fails to alter significantly the host intestinal microbiota and suggests that their inclusion in future ETEC vaccine preparations may be efficacious.

Chapter 3 - Investigation of Enterotoxigenic *Escherichia coli* (ETEC) vaccine candidates

Introduction

Enterotoxigenic *E. coli* (ETEC) is a leading cause of diarrhea in travelers and farm animals[50]. Vaccines have been considered as an efficacious way to prevent ETEC infection. Typically, vaccines targeting on surface antigens on bacteria can yield effective protection by inducing immune response. However, the antigenic and structural heterogeneity among various ETEC pathovar has posed a great challenge to vaccine development efforts. In our previous study, three proteins (MipA, Skp, and ETEC_2479) were identified and characterized as vaccine candidates due to the ability to protect mice in an intranasal ETEC challenge model after vaccination[15]. In addition to antigens, adjuvants also play essential role in induction of immune response. Delivery of antigens via outer membrane vesicles (OMVs) is a novel and effective means due to the artificial presentation of antigens in complex with the natural presence of LPS as an adjuvant. In this study, we employed OMV for antigen presentation and adjuvantation and then evaluated the protection from OMV delivered antigens in a mouse pulmonary challenge model. We also refined the vaccine candidates in the absence of cholera toxin (CT) and epitope purification tags to investigate the impact of adjuvants and epitope tags of antigens on vaccine efficacy for potential clinical trial.

Materials and Methods

Ethics statement

Animal experiments were performed according to Kansas State University Institutional Animal Care and Use Committee-approved protocols (IACUC #3900). This institution complies with all applicable provisions of the Animal Welfare Act and other Federal statutes and regulations relating to animals.

Antigen purification

Escherichia coli BL21 (DE3) strains expressing individual antigens were grown overnight as described[15]. A single colony of each expressing strains was cultured in 4 ml Luria-Bertani (LB) broth supplemented with 50 µg/ml Kanamycin shaking at 37 °C overnight. 2 ml of the overnight culture was transferred to 200 ml LB medium containing 50 µg/ml Kanamycin. Once optical density (OD) value of the culture reached 0.4~ 0.6, I mM Isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma, MO) was added into culture to induce protein expression for 5 h shaking at 37 °C. Cultures were collected and proteins were purified by using nickel-affinity chromatography. After purification, proteins were dialyzed into 10 % glycerol in Pierce Slide-A-Lyzer dialysis cassettes. N-terminal epitope tag were removed by TEV protease if necessary. Protein concentrations were quantified by using the Precision Red Advanced Protein Assay (Cytoskeleton, Inc.).

Polyclonal antisera production

56 BALB/c female mice of matched age (3-4 weeks of age) were obtained from Jackson Laboratory and divided randomly into 14 groups of 4 mice. The mice were

housed in microisolator cages and provided with food and water ad libitum. Individual proteins (20 μg) will be combined with 2.5 μg cholera toxin (CT) and mixed in sterile phosphate-buffered saline (PBS) to a final volume of 25 μl. OMVs were obtained from Abera Bioscience and are purified from *S. enterica* serovar Typhimurium at a concentration of 8 optical density 660 nm (OD660) equivalents per 5 μl. Inoculum of purified proteins (25 μl) or OMVs (5 μl) or PBS (25 μl) will be administered intranasally to the external nares of mice that have been lightly anesthetized with isoflurane. Two identical booster doses will be administered 2- and 4-weeks subsequent to the initial vaccination. Fecal samples were collected before the immunization and challenge respectively to monitor the production of mucosal antibody. Fresh stool pellets (5-6 pellets) of each mice were collected and added to 1 ml of fecal reconstitution buffer. The fecal reconstitution buffer includes 50 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mg/ml soybean trypsin inhibitor, 1.39 μg/ml phenylmethylsulfonylfluoride (PMSF) and homogenized. The fecal samples were homogenized thoroughly and centrifuged (5 min, 5,000 g) to remove insoluble material. The supernatants were stored at -4 °C.

Immunoassays

IgA concentrations in mouse feces were quantified and analyzed by Enzyme-Linked Immunosorbent Assay (ELISA). ELISAs were performed in polystyrene 96-well, flat bottom plates (Whatman) which were coated with 0.5 μg/ml of each purified protein or bovine serum albumin (BSA) and incubated overnight 4 °C. Plates were washed 3X in PBST (PBS with 0.1 % Tween-20) and blocked with 5 % milk in PBS, 0.1 % Tween-20 for 1 h at room temperature. After wash 3X with PBS, plates were inoculated with 50 μl of supernatant from each fecal sample in triplicate at 4 °C overnight. Rabbit anti-

mouse IgA HRP detection antibody (Sigma) was diluted 1:4,000 in PBST and added to the well to incubate at 37 °C for 1 h. Plates were developed with 1-StepTM Ultra TMB-ELISA (Thermo) following the manufacturer'protocol and quenched with 3 N H_2SO_4. Absorbance was read at 450 nm to quantify the antibody titers. For statistical test, antibody titers were first transformed logarithmically and the Student's *t* test was employed to compare the average antibody titer values of different groups of mice with those of non-immunized mice. Any difference in *P* values of < 0.05 was considered significant.

Intranasal challenge assays

Two weeks after the final immunization, mice were lightly anesthetized with isoflurane in a VetEquip RC2 isoflurane and challenged intranasally with $5x10^8$ CFUs of ETEC H10407 which were cultivated on Colonization Factor Antigen (CFA) agar at 37 ˚C for 18 h. The mice were observed every 8 h after challenge. Changes to mouse clinical signs of illness were quantified and recorded as a function of ETEC challenge and immunization. Clinical signs of illness were categorized into lack of responsiveness to stimulation, hunched posture, ruffled hair coat, dehydration. Mice with any clinical signs of illness or at the end of the study (7 d) were euthanized, necropsied. Data were analyzed statistically using log-rank tests. The lungs were extracted aseptically and dissected into halves. One half of each lung was homogenized for quantification of ETEC colonies. Serial dilutions of lung homogenates were prepared and plated on MacConkey agar (MAC) for quantification of ETEC concentrations. The other half of the left lung was immersed in formalin for subsequent histopathology studies. The other half

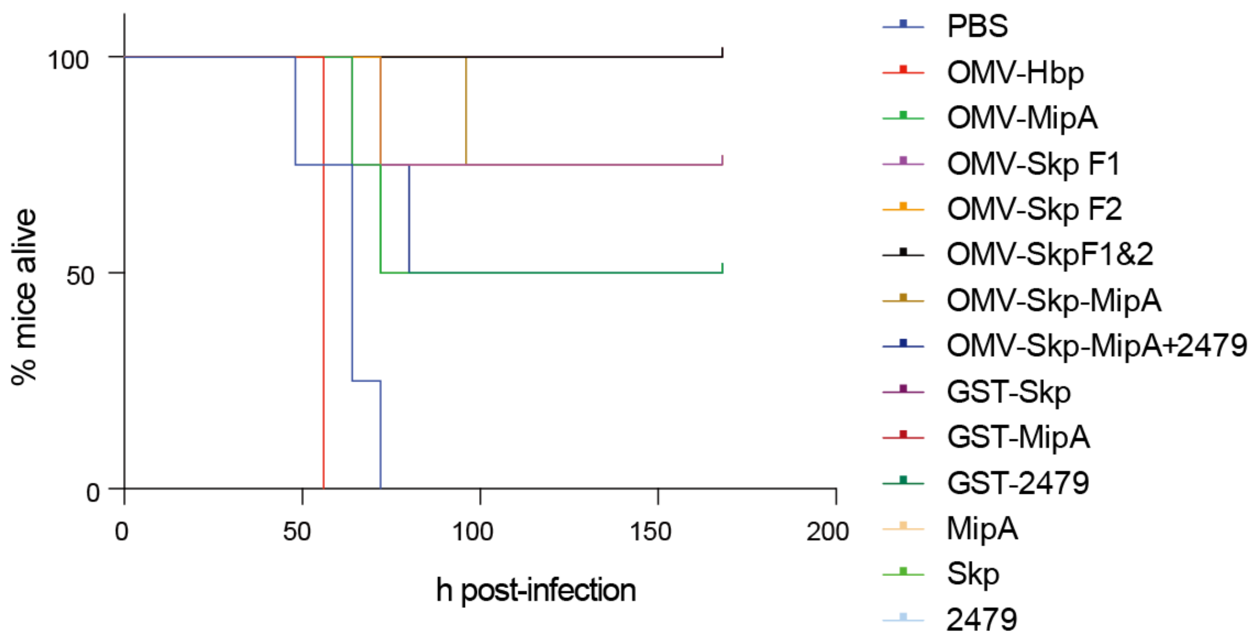
of the right lung was flash frozen in liquid nitrogen and stored at -80 ˚C for subsequent biochemical assays.

Results

Three ETEC H1047 proteins were previously characterized as protective antigens in a mouse model with intranasal challenge[15]. In this study, the impacts of OMV delivery of antigens as well as purification epitope tag of proteins on the vaccine efficacy are of interest. We employed a well-developed hemoglobin protease (Hbp) autotransporter platform for presentation of heterologous polypeptides on the surface of *Salmonella* OMVs. Antigens without epitope tags were obtained using TEV protease and nickel-affinity chromatography to eliminate cleaved epitope tags. Antigens regardless of the presence of epitope tags were immunized with cholera toxin (CT) which serves as a potent adjuvant. While OMVs display of antigens processes the ability to induce immune response with the nature presentation of LPS as an adjuvant. To investigate whether the EHEC antigens (MipA, Skp, and ETEC_2479) with absence or presence of epitope tag and OMVs have any difference in protection from EHEC infection, we adapted an established intranasal mouse challenge model. The mice were divided into 14 groups (4 mice/group) and immunized three times at two-week intervals with antigens combined with or without OMVs. Two weeks after the final immunization, mice were challenged intranasally with $5x10^8$ CFUs of ETEC H10407, which has been cultivated on Colonization Factor Antigen (CFA) agar to facilitate the expression of colonization factors. Clinical signs were observed every 8h over a 7-day period and evaluated as a criteria of protected or not. Compared with the mice immunized with PBS or OMV without packaging antigens, which did not survive longer than 72 h after

infection, all antigen combinations were protective against the infectious challenge to variable degrees (Fig. 6). All mice immunized with three antigens containing epitope tags survived after 7 days, which is similar to our previous study[15]. OMVs delivery of both Skp fragment 1 and fragment 2, as well as MipA in absence of epitope tags also provided remarkable protection to survive the infection. Another OMVs presentation of three antigens, which were Skp fragment 1, Skp fragment 2 and MipA combined with Skp, yielded significant degrees of protection with 75 % survival of the mice. In contrast, OMVs delivery of MipA, untagged Skp and untagged ETEC_2479 were less effective with only 50 % protection of immunized mice.

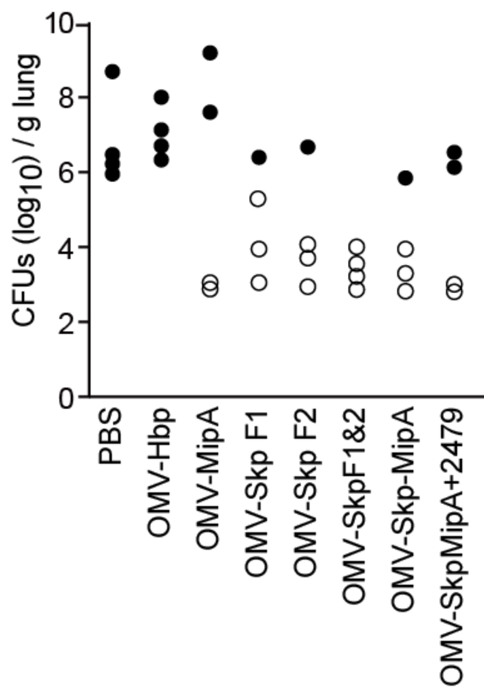
Figure 6 Survival curve of intranasal challenge.



Survival curve of intranasal challenge. Mice were challenged with ETEC H10407 after immunization with the indicated antigens. The survival of mice is plotted as a function of time.

Bacterial Loads of lung tissues were quantified after euthanization and dissection. ETEC loads were related to the survival of mice (Fig. 7). Significantly high loads of ETEC, which were about ~10^{6-10} CFUs/g, were cultured from lungs of mice that didn't survive the infection. Whereas, bacterial loads were relatively lower in lungs of surviving mice, all less than ~10^6 CFUs/g.

Figure 7 Bacterial loads in lung tissues.

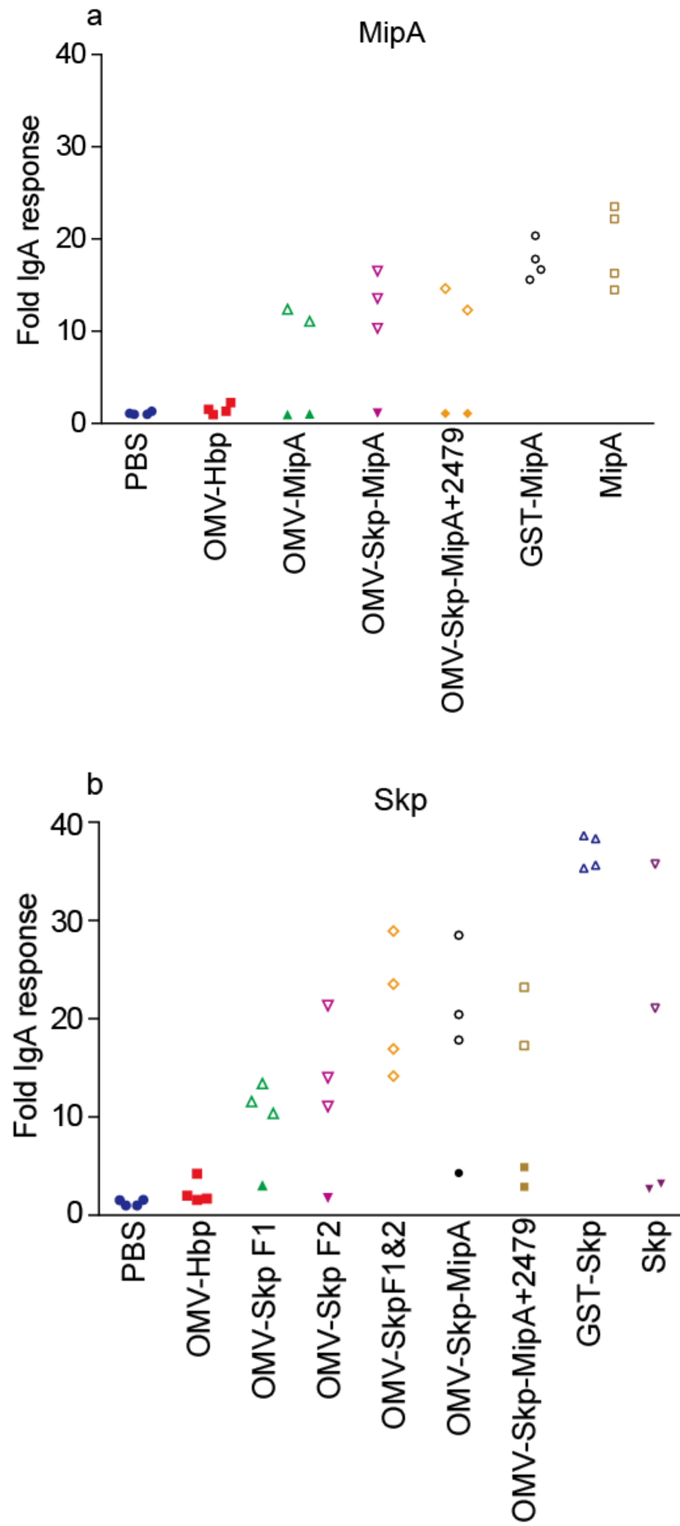


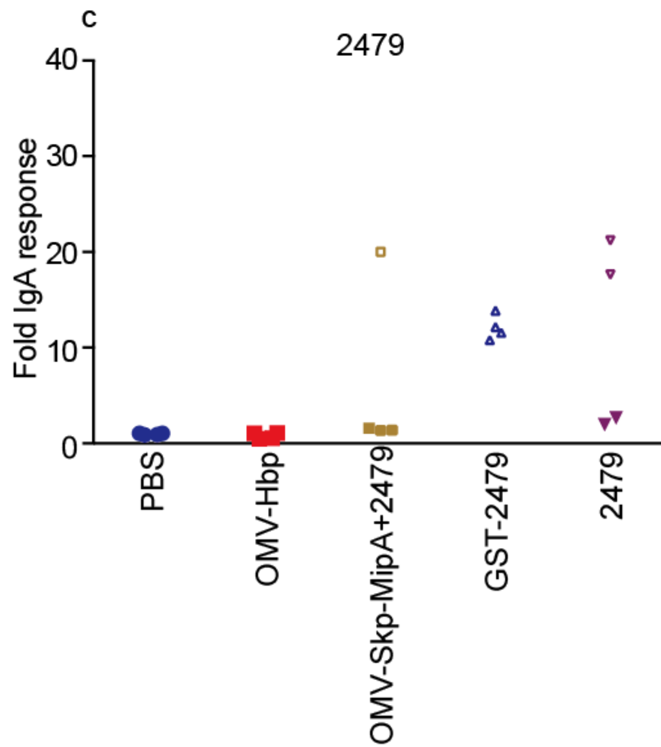
Bacterial load in lung tissues of mice inoculated with 5 × 10^8 CFU of ETEC H10407. After euthanization, half of lung tissues were collected and homogenized. Serial dilutions were placed on MacConkey agars. Colony-forming units (CFU) were determined 24 h after plating and were expressed as log CFU. Individual animals are represented by individual symbols. Open symbols

indicate mice that survived for the duration of the study. Closed symbols indicate mice that were euthanized due to their display of clinical signs of illness.

Fecal IgA response against different antigens evaluated using ELISA were more variable but highly correlated to mouse survival and inversely related to the ETEC loads (Fig. 8). No effective IgA response was detected in mice immunized with PBS or OMVs without antigens. MipA without OMVs regardless of the presence of epitope tags have the highest fecal IgA response to MipA (Fig. 8a). Three mice in OMV-Skp-MipA group and two mice in both OMV-MipA and OMV-SkpMipA+2479 yield significantly higher IgA response (more than 10-fold). In contrast, IgA responses against Skp were more variable also with the most effective response in tagged Skp group (Fig. 8b). Only 2 mice in untagged Skp and also in OMV-SkpMipA+2479 developed IgA response more than 10 folds. But mice in groups of other antigen combination has at least 3 mice with IgA response more than 10 folds. However, ETEC_2479, which failed to be packaged into OMV platforms, yield relatively lower IgA response (Fig. 8c). Even in tagged ETEC_2479 antigen group, all mice were effectively protected but still with lower IgA compared to tagged MipA and Skp. 2 mice in untagged ETEC_2479 antigen group and only 1 mice in OMV-SkpMipA+2479 group were able to produce effective response (more than 10-fold).

Figure 8 Fecal IgA response.





Fecal IgA responses in mice. Data are plotted as the fold-change in fecal IgA against MipA(a), Skp (b), or ETEC_2479 (c) after immunization with the indicated antigens, n = 5/group. Open symbols indicate mice that survived for the duration of the study. Closed symbols indicate mice that were euthanized due to their display of clinical signs of illness.

Discussion

Enterotoxigenic *E. coli* (ETEC) causes diarrhea in traveler and farm animals in developing countries. Developments of efficacious vaccines against ETEC infection are of interest. We have identified three surface proteins (MipA, Skp, and ETEC_2479) as vaccine candidates due to their effective protection from ETEC infection in an intranasal mouse challenge model. Outer membrane vesicles (OMVs) shedding from Gram-negative bacteria are used in vaccine development due to its ability to carry heterologous antigens and natural presentation of LPS as adjuvant[88]. In this study, ETEC antigens (MipA, Skp) were targeted to the surface of *Salmonella* OMVs after fusion to hemoglobin protease (Hbp). However, the efficacy of OMVs vaccine is less than expected compared to the traditional combination of antigens and adjuvants. Some potential reasons may lead to these results. The antigen concentration in OMVs is less than the traditional antigens. These protein antigens are sensitive to the protease digestion in the host respiratory mucosa. So excessive amount of antigens were used for immunization[89]. While antigens presented on surface of OMVs still have the same risk of being degraded due to absence of surface protective structure, such as pilus[64]. Despite antigens are more concentrated carried by OMVs, the total amount of antigens per dose is much less than the excessive amount of antigens alone. So the effective antigens which succeed to approach the host cell and induce immune response are less in OMV vaccines. To optimize, instead of display on surface, package of antigens in lumen of OMVs has potentially better protection of antigens and can be incorporated into future vaccine designs[90]. In addition, other OMV-enriched proteins, such as autotransporters, regulatory proteins involved in iron and zinc acquisition, and two-

partner secretion systems, which are selectively packaged into OMVs, may be a better candidate to fuse heterogeneous antigens[64,91,92].

In addition, our study showed the antigens with epitope tags have better performance on induction of immune response and protection of infectious mice than antigens in absence of epitope tags. This indicates that the purification epitope tags, which are His-tag and GST-tag, of these proteins contribute to its antigenicity. It is commonly known that the fusion tags play an essential role in enhancing the protein solubility when expression in engineering *E. coli* as well as facilitating purification of proteins[93]. However, it is reported that fusion tags also contribute to the immunogenicity of purified antigens, which supports the results in our study[94]. In contrast to expectation, existence of purification tags seems to increase the vaccine efficacy. Efforts should be taken to better understand the mechanism for further vaccine developments.

Chapter 4 - Identification of inhibitor of Enterohemorrhagic *E. coli* (EHEC) type III secretion system effector NleB

Introduction

Enterohemorrhagic *E. coli* (EHEC) have presented great challenges to public health by its potential to cause gastroenteritis, hemorrhagic colitis, and hemolytic uremic syndrome (HUS), which may lead to renal failure. EHEC is a prototypical member of attaching and effacing (A/E) pathogens, which is characterized by utilization of type III secretion system (T3SS). T3SS is a needle-like structure which enables the pathogen to inject the host cell directly with a variety of virulence proteins (effectors) to modulate the immune system. NleB, one of those effectors, is a glycosyltransferase with the ability to translocate *N*-acetyl-D-glucosamine (N-GlcNAc) to the arginine residues of its substrate proteins to suppress the activation of host NF-κB pathway. Therefore, the identification of potential inhibitors of NleB are worthy of investigation as a novel therapeutic modality against EHEC infection. In this study, we employed a luminescence-based glycosyltransferase assay to quantify the enzyme activity of NleB. After optimizing the glycosyltransferase assay condition, an array of chemical compounds were tested to screen for inhibitor candidates with the criteria of significantly decreased luminescence.

Materials and Methods

Protein purification

Escherichia coli BL21 (DE3) strains expressing NleB or mutant were grown overnight as described[15]. A single colony of each expressing strains was cultured in 4 ml Luria-Bertani (LB) broth supplemented with 50 μg/ml Kanamycin shaking at 37 °C overnight. 2 ml of the overnight culture was transferred to 200 ml LB medium containing 50 μg/ml Kanamycin. Once optical density (OD) value of the culture reached 0.4~ 0.6, 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma, MO) was added to into culture to induce protein expression for 5 h shaking at 37 °C. Cultures were collected and proteins were purified by using nickel-affinity chromatography. Protein concentrations were quantified by using the Precision Red Advanced Protein Assay (Cytoskeleton, Inc.).

N-GlcNAcylation assays

NleB proteins were soluble in His-tag purification elution buffer which was 50 mM NaH_2PO_4 pH 8.0, 600 mM NaCl, 280 mM imidazole, 10 % glycerol. The proteins were added into wells of a solid white 96-well plates and diluted with His-tag purification elution buffer. Glycosyltransferase reaction buffer (10 μl, 2.5X) supplemented with UDP-GlcNAc was added into each well in which the final concentration of UDP-GlcNAc was 0.2 mM. The 1X glycosyltransferase reaction buffer contains 125 mM Tris-HCl pH 7.5, 2.5 mM DTT, 25 mM $MnCl_2$. The glycosyltransferase assay was performed in a total volume of 25 μl and incubated for 4 h at room temperature.

Luminescence assays

The Luminescence assays were performed according to the protocol from Promega UDP-Glo^{TM} glycosyltransferase assay. Briefly, 25 µl of UDP detection reagent was added into glycosyltransferase reaction in each well of solid white 96 well plates. A UDP standard curve was performed in the same assay plate to correlate luminescence to the UDP concentrations generated in each glycosyltransferase reaction. UDP serial dilutions were diluted with glycosyltransferase reaction buffer in duplicates, and 25 µl UDP serial dilutions were transferred into the standard curve-designated wells of the 96-well assay plate. 25 µl of UDP detection reagent was added and mixed thoroughly. The assay plates were incubated at room temperature for 1 h. Luminescence was measured and recorded using a luminometer. Luminescence assays were performed in triplicate using proteins from three independently purifications.

Validation screening for NleB inhibitor

The small molecule compound library screen for NleB inhibitor was performed by HTS lab in University of Kansas with diversity set containing 5,160 compounds synthesized by the Chemical Methodologies and Library Development Center. NleB (150 nM) and each compound (20 µM) was mixed in a 5 µl glycosyltransferase reaction buffer containing 0.5 % DMSO in wells of Corning® 384 Well Low Flange Black Flat Bottom Polystyrene NBS™ Microplate. After incubation at 30 °C for 30 min, 100 µM UDP-GlcNAc was added to incubate at 30 °C for 2 h. UDP Detection Reagent (5 µl) in each well was used for detection of luminescence intensity. Luminescent readout from reaction without NleB is denoted as minimum signal. Luminescent readout from reaction without compound is denoted as maximum signal. Luminescent readout from reaction

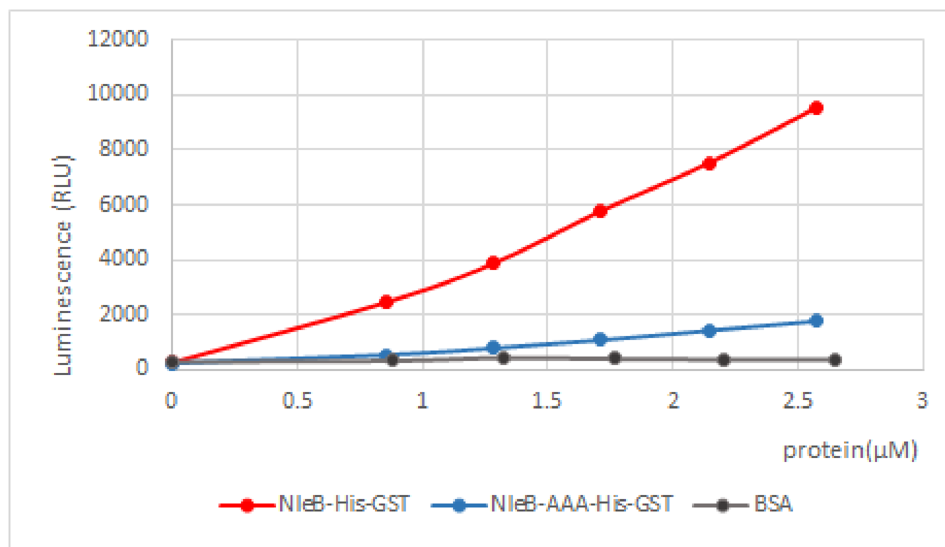
with both NleB and each compound is denoted as tested signal. The inhibition percentage is calculated as: $\% \text{ Inhibition} = 100 - \frac{Test-Min}{Max-Min} * 100$. Distribution of Z’ Scores was employed to estimate difference among plates. Inhibition percentages of 5,160 compounds were plotted as scattergram. Inhibition effect of compounds with scatter beyond plate median + 3 standard deviation were regarded as very significant. Inhibition effect of compounds with scatter between plate median + 2 to 3 standard deviation were regarded as significant. Reconfirmation of inhibition effect was performed with compounds at 10, 20, 40, 80, or 160 μM. Inhibition effect of compounds with scatter beyond plate median + 3 standard deviation and with more than 50 % max inhibition was regarded as very significant.

Results

NleB, the T3SS effector of EHEC, plays an important role in the virulence of A/E pathogens by modulating the host signaling pathway. NleB functions as a glycosyltransferase with the ability to translocate *N*-acetyl-D-glucosamine (N-GlcNAc) to the arginine residues of its target proteins, including both mammalian proteins, such as GAPDH, FADD, TRADD, and NleB itself.

In this study, we employed a luminescence-based glycosyltransferase assay to quantify the enzyme activity of NleB with NleB as its own substrate. We use bovine serum albumin (BSA) and constructed mutant NleB-AAA (NleB $_{221}$DAD-AAA$_{223}$ mutant), which failed to N- GlcNAcylate proteins, as controls. After optimizing the conditions for glycosyltransferase reactions, different amount of NleB, NleB-AAA, and BSA were incubated with UDP-GlcNAc. After translocating GlcNAc to arginine residues of NleB, UDP was released and transformed to ATP to generate light which was measured by a luminometer. As we expected, NleB exhibited a higher intensity of luminescence than NleB-AAA mutant and BSA (Fig. 9). The glycosyltransferase activity increased in a positively linear relation with the concentration of NleB.

Figure 9 N-GlcNAc Transferase Activity



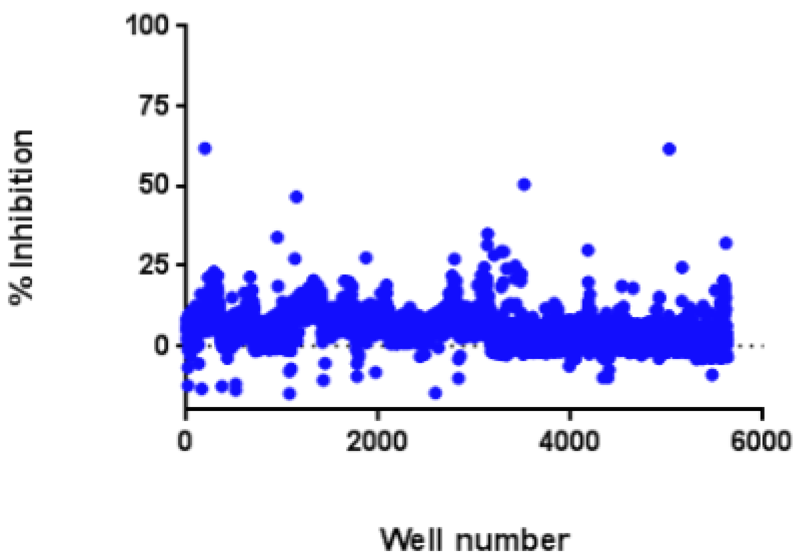
Detection of NleB activity with UDP-Glo™ Glycosyltransferase Assay. Recombinant His-GST-linked NleB (red) or NleB mutant (blue) or BSA (black) was added in 25 μl in glycosyltransferase reaction buffer in a solid white 96-well plate in the presence of 200 μM UDP-GlcNAc. After incubating 4 h at room temperature, the UDP-Glo™ Glycosyltransferase Assay was performed using 25 μl of UDP Detection Reagent. Luminescence was recorded 1 h after adding the UDP Detection Reagent using a 96 Microplate Luminometer. Values represent the mean of three replicates. RLU = relative light units.

Then the reaction was applied in a 384 well plates to screen potential inhibitors with diversity set containing 5,160 compounds, which was performed by HTS lab in University of Kansas. A variety of chemical compound (5,160) were incubated with NleB before reaction with UDP-GlcNAc. Percentage of inhibition were calculated and analyzed for valid screening. Distribution of Z' Scores (average Z' Score: 0.88 ± 0.048)

showed that there was no significant difference among plates, suggesting that all plates were equally treated.

The scattergram revealed that 52 hits were beyond the plate median + 3 standard deviation of inhibition percentage and 76 hits were in plate median + between 2 and 3 standard deviation, which means these 128 chemical compounds could be inhibitor candidates (Fig. 10).

Figure 100 Scattergram of percent inhibition



Scattergram of percent inhibition of 5,160 chemicals. Dashed lines indicate means 0 percent inhibition.

Discussion

Type III secretion system of attaching and effacing (A/E) pathogen has raised increasing attention in research these years because it can manipulate host immune response via its versatile effectors. NleB is distinctively characterized as an *N*-acetyl-D-glucosamine (N-GlcNAc) glycosyltransferase by modifying the arginine residues on target proteins. To execute its function, NleB is injected directly into mammalian cells via T3SS, which has a needle-like channel for translocation without being secreted into extracellular environment. This renders the advantage of evasion from immune system, but also leads to the predicament to target NleB for vaccine development. However, In the process of drug discovery, high-throughput screening is desirable and effective strategy to screen an inhibitor of certain virulence with a chemical or compound library. Chemicals or compounds in library are usually designed and synthesized by organic chemistry and medicinal chemistry. A systematic database is constructed to store the corresponding information of each chemical, including the chemical structure, purity, quantity, and physiochemical characteristics of the compound. In this study, we establish a stable reaction condition for high-throughput screening using a compound library of 5,160 chemicals. In total, 128 chemicals were selected with significant inhibition on glycosyltransferase activity of NleB and might be developed into a drug. All 5,160 chemicals from the diversity set in this study are safe to human cell lines and able to permeate the cell membrane. Subsequent investigations are needed involving determination of IC50s, chemical kinetics, and type of inhibition. However, a potential drawback also exists, as there are multiple glycosyltransferases with important function in host cells. For instance, O-linked N-acetylglucosamine (O-GlcNAc) transferase (OGT)

in human catalyzes intracellular glycosylation of serine and threonine residues with the addition of a single N-acetylglucosamine in O-glycosidic linkage. OGT plays critical important role in regulation of host biological function involving the resistance of insulin in muscle cells and adipocytes[95]. Therefore, the selected chemical for further therapy development should be tested the impacts on glycosyltransferases of host cells before clinical trial. In addition, NleB is characterized as highly conserved among A/E pathogens. Therefore, whether a global inhibiting effect exists for NleB orthologs is worthy of investigation in the future. In conclusion, we employed a luminescence-based glycosyltransferase assay using an array of chemical compounds. A total of 128 chemicals was screened out with significant inhibiting effects on NleB to be further studied as a novel therapeutic modality against EHEC infection.

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