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(Citation)
Current Microbiology,54(1):14-19

(Issue Date)
2007-01

(Resource Type)
journal article

(Version)
Accepted Manuscript

(URL)
https://hdl.handle.net/20.500.14094/90000319

O Side Chain Deficiency Enhances Sensitivity of *Escherichia coli* to Shiga Toxin 2-Converting Bacteriophages

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Running title: The role of O side chain to lytic and lysogenic infection of Stx2 phage

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Abstract

We investigated the relationship between expression of the O side chain of outer membrane lipopolysaccharide (LPS) and infection by a Shiga toxin 2

(Stx2)-converting phage in normal and benign strains of *Escherichia coli*. Of 19 wild-type *E. coli* strains isolated from the feces of healthy subjects, those that displayed low-molecular-weight LPS showed markedly higher susceptibility to lytic and lysogenic infection by Stx2 phages than those with high-molecular-weight LPS. All lysogens produced infectious phage particles and Stx2. The Stx-negative *E. coli* O157:H7 strain ATCC43888 with an intact O side chain was found to be resistant to lysis by an Stx2 phage and lysogenic infection by a recombinant Stx2 phage whereas a *rfbE* mutant deficient in the expression of the O side chain was readily infected by the phage and yielded stable lysogens. The evidence suggests that an O side chain deficiency leads to the creation of new pathotypes of Shiga toxin-producing *E. coli* (STEC) within the intestinal microflora.

Introduction

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) has been recognized as a serious food-borne pathogen that causes hemorrhagic colitis and hemolytic-uremic syndrome [10, 15, 18]. To date, the most epidemic STEC serogroup has been O157, but more than 200 other STEC O:H serotypes have been described [19, 21]. Stx produced by STEC is considered to be a major virulence factor causing these illnesses [3]. Stx is classified into two broad types, Stx1 and Stx2, and the structural genes encoding these toxins are located in the genomes of temperate lambdoid bacteriophages [3, 23, 27]. The genes for Stxs are encoded in the late gene region of lysogenic phages [22, 24]. Late phage genes are expressed only when the phages are engaged in the replicative or lytic cycle, releasing free phage particles as well as producing

the toxins [17, 31]. The released phages may lysogenize non-toxin-producing *E. coli*, leading to the emergence of new STEC pathotypes [16, 26].

Schmidt et al. [26] and James et al. [16] demonstrated that a recombinant Stx2 phage could infect and lysogenize a variety of enteric *E. coli* and *Shigella* strains. More recently, Gamage et al. [9] reported the presence of wild-type *E. coli* strains in the human intestine that were susceptible to Stx2 phages. In gram-negative bacteria, one major component of the outer membrane is lipopolysaccharide (LPS), which consists of lipid A, the core oligosaccharide, and an O side chain extending from the cell surface. It was reported that Stx-converting phages could infect only strains of *E. coli* and *Shigella sonnei* of rough colonial appearance [28], which was indicative of deficient O side chain expression. In this context, van der Ley et al. [30] and Bradley [4] suggested that O side chains of *E. coli* normally shield the outer membrane receptors for various bacteriophages (thereby preventing the access of some phages to the receptors). The accumulating evidence suggests that strains deficient in O side chain expression are candidates for new STECs, producing the toxin and phage particles in the host intestine. We here describe that an O side chain deficiency markedly enhances sensitivity to Stx2 phages in non-STEC strains.

Materials and Methods

Bacterial strains, plasmids and bacteriophages. The bacterial strains, plasmids and phages used in this study are listed in Table 1. Nineteen wild-type *E. coli* strains were isolated from fecal samples of healthy human individuals, and identified biochemically as *E. coli* by using an API 20E test (Biomerieux, Lyon, France). These isolates were negative by PCR for *stx1* and *2* (data not shown). Serotyping was performed using a slide agglutination assay with antisera prepared at the National Institute of Infectious Diseases,

Tokyo, Japan. Four isolates could be classified as serogroups O2 (E21 and E128), O44 (E27) and O91 (E148). Fifteen isolates could not be classified into any of the known serotypes. The *E. coli* K-12 strain MC1061 [33] was used as a host for the propagation of phages. High-titer phage stocks (approximately 10^9 PFU/ml) were prepared from plaques formed on overlay plates, as described previously [23].

Construction of the *rfbE* mutant and plasmid. The gene *rfbE* encodes a putative perosamine synthetase, which is required for expression of the O157 antigen [2]. A deletion mutant of *rfbE* was constructed from the Stx-negative *E. coli* O157:H7 strain ATCC43888 using a lambda red system [6]. PCR products amplified from pKD4 with primers 5'-GAAACTATATTCAGAAGTTTGAAAATAAATTTGCGGAACAAAACCGTGTA GGCTGGAGCTGCTTC-3' and 5'-GATCCTCAGCTATAGGGTGCTTTTGATATTTTTCCGAGTACATTGCATATG AATATCCTCCTTAGT-3', which contains kanamycin resistance cassette and Flp recombinase target site (FRT) flanked by 45 bp of homology to the 5' and 3' termini of *rfbE* were electroporated into strain ATCC43888 carrying pKD46. Recombination into the chromosome and loss of the pKD46 plasmid were simultaneously selected for by growing the cells on Luria-Bertani (LB) (Difco, Detroit, Mich.) agar plates containing 50 μg of kanamycin per milliliter at 37・C. The FRT-flanked kanamycin cassette was removed after transformation with pCP20. For complementation experiments, full-length *rfbE* was amplified from ATCC43888 by PCR with the primers 5'-AGCCATTTTGGGTTAACTGTT-3' and 5'-TAAAATCAATTCCACCGCCC-3', and cloned into pGEM-T-Easy (Promega, Madison, Wis.) to yield pTH512.

Construction of a recombinant Stx2 phage. The lambda red system was also used to generate a recombinant Stx2 phage (ϕ927) that was inserted with a chloramphenicol resistance gene (*cat*). The chloramphenicol resistance cassette with FRT sites flanked by 45 bp of homology to the 5' and 3' termini of *stx2A* was amplified from pKD3 using the primers 5'-ATGAAGTGTATATTATTTAAATGGGTACTGTGCCTGTTACTGGGTGTGTAG GCTGGAGCTGCTTC-3' and 5'-TGTTCAGAAACGCTGCAGCTGTATTACTTTCCCATAATGTATTGTCATATG AATATCCTCCTTAGT-3', and electroporated into Sakai strain to yield Sakai-12. On Sakai-12, the Stx2A gene was replaced with *cat*, but the Stx2B gene remained intact.

Susceptibility of *E. coli* strains to lytic infection. Cultures of strains were grown overnight in LB broth at 37·C. One hundred milliliters of cultures was added to 3 ml of soft agar (0.7% agar) and the mixture was poured on LB agar plates. To determine the susceptibility of stool *E. coli* strains to lytic infection by ϕSKI2, ϕ19V2 and ϕCDC2, 10 μl of phage lysates (10^8 PFU/ml) were spotted onto the top agar overlay. In the case of ATCC43888 and its derivatives, sensitivity to ϕSKI2 was tested by spotting 10 μl of serial dilutions of phage lysate. After 22 h of incubation at 37·C, lysis of bacteria was recorded.

Susceptibility of *E. coli* strains to lysogeny. Aliquots (100 μl) of log-phase cultures of test strains (approximately 5 x 10^8 CFU/ml, as determined by measuring the optical density at 600 nm of 0.4) were mixed with 100 μl of ϕ927 stock solution (1 x 10^9 PFU/ml). After 30 min of incubation at 37· , 2.5 ml of top agar was added to the infection mix, and spread onto LB agar plates containing 25 μg of chloramphenicol per milliliter. Single colonies grown on selective agar

plates after incubation overnight were further subcultured to single colonies on new selective plates and colonies isolated on the plates were confirmed to be lysogens by PCR with the ϕ927–specific primers 5’-TCAAAAAATACGCCCGGTAG-3’ and 5’-TTCTTTCCCGTCAACCTTCA-3’, which were designed to amplify the Δ(*stx2A*)::*cat–stx2B* fusion region in the genome of ϕ927.

LPS analysis. LPS from *E. coli* was analyzed by the method of Hitchcock and Brown [13]. The LPS preparations were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by silver staining. To confirm the absence of the O157 antigen, a companion gel was blotted onto a Hybond-P membrane (Amersham, Buckinghamshire, United Kingdom) and probed with a 1:10,000 dilution of anti-O157 antibody (a gift from Dr. Tamura K.). The binding of secondary anti-rabbit immunoglobulin G antibody conjugated to horseradish peroxidase was detected using ECL Western blotting detection reagents (Amersham).

Detection of the production of phage particles and recombinant Stx2 by lysogens. To determine the ability of lysogens to produce ϕ927 particles, MC1061 was used as a host strain. Overnight LB broth culture filtrates of lysogens were mixed with the MC1061 culture and spread onto LB agar plates containing chloramphenicol. Colonies isolated from the plates were confirmed to be lysogens with ϕ927 by the ϕ927-specific PCR, as described above. Production of the recombinant Stx2 in which Stx2B was left intact but Stx2A was deleted, was determined with an immunochromatographic assay kit (Wako Pure Chemical Ind. Ltd., Osaka, Japan) that used colloidal gold-labeled monoclonal antibodies to detect Stx1B or Stx2B.

Results and Discussion

During our studies characterizing the recipient *E. coli* for the infection of Stx-converting phages, we isolated and examined 19 *E. coli* strains from fecal samples of healthy human individuals for their susceptibilities to Stx2 phages such as, ϕSKI2, ϕ19V2 [24] and ϕCDC2 [24] by spot test. These phages produced turbid clearing zones in the lawn of strains E109, E143, E147, E150 and E157, suggesting that these were susceptible to lytic infection by Stx2-phages. Susceptibility profiles to ϕSKI2, ϕ19V2 and ϕCDC2 were the same: all the strains except five described above showed insensitive to all the stx2-paheges examined (data not shown). The LPS samples were prepared from 19 *E. coli* isolates and analyzed by SDS-PAGE followed by silver staining (Fig 1). We found that four isolates, E109, E143, E150 and E157 displayed low-molecular-weight LPSs, which did not express the O side chains. Strain E147 showed longer O side chains than E109, E143, E150 and E157 but apparently shorter than those of other phage-insensitive strains. Although the LPS of strain E34 had a similar size of O side chain as that of E147, it was insensitive to stx2-phage infection. These results revealed that the loss of high-molecular-weight LPS may increase the sensitivity to lytic infection by Stx2 phages.

In order to further examine the role of O side chain of LPS in the infectivity by Stx2 phages, we systematically constructed *rfbE* mutant of *E. coli* O157:H7 strain ATCC43888 (designated ATCC43888-LD), which did not possess *stx1* and *stx2* genes. ATCC43888 displayed a high-molecular-weight ladder representing O side chains, which reacted with the anti-O157antibody, while the *rfbE* mutant ATCC43888-LD did not express any O side chains (Fig. 2). The expression of the O side chain in ATCC43888-LD complemented with

pTH512 plasmid had almost the same LPS profile as that in ATCC43888. ATCC43888 was found to be resistant to lytic infection by ϕSKI2, while ATCC43888-LD was clearly susceptible (Table 2).

In addition, the ability of Stx2 phages to lysogenize was investigated by using a recombinant Stx2 phage, ϕ927, which carry the *stx2A*::*cat*. The number of lysogens isolated through three independent assays is summarized in Table 3. It was found that ϕ927 yielded more stable lysogens in ATCC43888-LD than in ATCC43888. Consistent with this observation, Muniesa et al. [20] reported that the infectivity of the Stx2 phages was lower in ATCC43888 than *E. coli* laboratory strains, and failed to isolate stable ATCC43888 lysogens with Stx2 phages. Although we have been able to isolate a stable lysogen of ATCC43888 with ϕ927, that has been shown to be spontaneous mutants with low-molecular-weight LPSs (data not shown). Subsequently, we examined the susceptibility of 19 wild *E. coli* strains to lysogeny by using ϕ927. Lysogeny with toxin-encoding phages has an important implication for the evolution of new pathogenic strains. Lysogens were obtained in 17 human isolates (Table 3). Four fecal isolates, E109, E143, E150 and E157, produced significantly more lysogens than the others, especially E143 which showed greater susceptibility to lysogenic infection.

Bacterial outer membrane proteins are known to function as receptors for many bacteriophages: the porin proteins such as OmpF and OmpC are receptors of phage T2 and T4, respectively [11, 43]; Stx2 phages recognize outer membrane proteins FadL and LamB as receptors [32]. Bradley [4] has shown that long O side chains of *E. coli* are able to prevent LPS-core and outer membrane proteins-specific bacteriophages from infecting. Camprubi et al. [5] have demonstrated that the presence of the O-antigen of the LPS in *Klebsiella pneumoniae* caused a significant reduction in the frequency of

establishment of P1 phage lysogeny. Although it is not clear whether which receptor(s) is responsible for infecting of ϕSKI2 or ϕ927, our study demonstrates that the O side chain has an important role in protecting *E. coli* from infection by Stx2 phages.

In this study, we isolated lysogens from ATCC43888, ATCC43888-LD (+pTH512) and fecal *E coli* parent strains with high-molecular-weight LPS. These lysogens were subjected to a SDS-PAGE-based analysis of LPS. It was found that a lysogen from ATCC43888 did not express the O side chain (Table 3). Furthermore, 15 of 22 lysogens from fecal *E coli* parent strains lost their high-molecular-weight LPSs. The number of this type of lysogens indicated in parentheses in table 3. Meanwhile there are actually 7 lysogens that still possessed the high-molecular-weight LPS (E25 - 1 lysogen, E34 - 2 lysogens, E144 - 1 lysogen, and E145 - 3 lysogens) (Table 3). These results suggested that spontaneous mutants lacking the O side chain became the target of infection by ϕ927. Previous epidemiological studies reported that some of the STEC strains isolated from patients with bloody diarrhea and HUS displayed O rough [1, 7, 8]. Beutin et al. [1] characterized 677 human STEC strains from patients in Germany, and found O rough in 86 (12.7%). These findings indicated that STEC strains without O side chains are present in the environment. In ATCC43888 lysogens, ϕ927 was integrated into *wrbA* (data not shown), which is the same site for the Stx2 phage as in the Sakai strain [12].

Lysogens were investigated for their ability to produce infectious ϕ927. The production of infectious phage particles was detected with the strain MC1061. All of the lysogens produced infectious phage particles. Furthermore, the lysogens were tested for the ability to produce recombinant Stx2. Production of the toxin was detected in all of the lysogens (data not shown). The evidence suggests that loss of the high-molecular-weight LPS induces the spread of *stx2*

genes among non-pathogenic *E coli* strains, leading to the creation of new STEC pathotypes

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Fig. 1. LPS analysis of *E. coli* strains isolated from stools of healthy human. LPSs of E19 (lane 1), E21 (lane 2), E24 (lane 3), E25 (lane 4), E27 (lane 5), E29 (lane 6), E33 (lane 7), E34 (lane 8), E109 (lane 9), E128 (lane 10), E143 (lane 11), E144 (lane 12), E145 (lane 13), E147 (lane 14), E148 (lane 15), E150 (lane 16), E152 (lane 17), E157 (lane 18), or E222 (lane 19) were separated by sodium dodecyl sulfate-polyacrylamide gel, and visualized with silver stain. Molecular masses are indicated on the left.

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel, visualized with silver stain (A) and Western blot (B) of bacterial LPS. Lanes: 1, ATCC43888 ; 2, ATCC43888-LD; 3, ATCC43888-LD (+pTH512); 4, ATCC43888-LD (+pGEM-self). Blot was probed with anti-O157 serum. Molecular masses are indicated on the left.