Journal of Microbiological Methods 116 (2015) 1–7

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Purification and characterization of lipopolysaccharides from six strains of non-O157 Shiga toxin-producing *Escherichia coli*

Loreen R. Stromberg [a,b,c], Zachary R. Stromberg [d], Afsheen Banisadr [b], Steven W. Graves [a,c], Rodney A. Moxley [d], Harshini Mukundan [b,c,*]

[a] The University of New Mexico, Center for Biomedical Engineering, MSC01 1141 1 University of New Mexico, Albuquerque, NM 87131, USA
[b] Chemistry Division, Los Alamos National Laboratory, MS J567, C-PCS, Los Alamos, NM 87545, USA
[c] The New Mexico Consortium 4200 West Jemez Rd., Suite 301, Los Alamos, NM 87544, USA
[d] University of Nebraska-Lincoln, School of Veterinary Medicine and Biomedical Sciences, P.O. Box 830905, East Campus Loop and Fair Street Lincoln, NE 68583, USA

ARTICLE INFO

Article history:
Received 3 April 2015
Received in revised form 15 June 2015
Accepted 16 June 2015
Available online 18 June 2015

Keywords:
Antibody specificity
E. coli
Lipopolysaccharide (LPS)
O-antigen (O-ag)
Serotyping
Shiga toxin-producing *E. coli* (STEC)

ABSTRACT

Certain Shiga toxin-producing *Escherichia coli* (STEC) are virulent human pathogens that are most often acquired through contaminated food. The United States Department of Agriculture, Food Safety and Inspection Service has declared several serogroups of STEC as adulterants in non-intact raw beef products. Hence, sensitive and specific tests for the detection of these STEC are a necessity for implementation in food safety programs. *E. coli* serogroups are identified by their respective O-antigen moiety on the lipopolysaccharide (LPS) macromolecule. We propose that the development of O-antigen-specific immunological assays can facilitate simple and rapid discriminatory detection of STEC in beef. However, the resources (antigens and antibodies) required for such development are not readily available. To overcome this, we extracted and characterized LPS and O-antigen from six STEC strains. Using hot phenol extraction, we isolated the LPS component from each strain and purified it using a series of steps to eliminate proteins, nucleic acids, and lipid A antigens. Antigens and crude LPS extracts were characterized using gel electrophoresis, immunoblotting, and modified Western blotting with commercially available antibodies, thus assessing the serogroup specificity and sensitivity of available ligands as well. The results indicate that, while many commercially available antibodies bind LPS, their activities and specificities are highly variable, and often not as specific as those required for serogroup discrimination. This variability could be minimized by the production of antibodies specific for the O-antigen. Additionally, the antigens generated from this study provide a source of characterized LPS and O-antigen standards for six serogroups of STEC.

Published by Elsevier B.V.

1. Introduction

Certain STEC are virulent human foodborne pathogens that are most often acquired through contaminated food. These organisms are the primary cause of hemolytic-uremic syndrome in children, and a leading cause of gastroenteritis worldwide (Quiñones and Swimley, 2011). In 2012, the United States Department of Agriculture, Food Safety and Inspection Service began testing beef manufacturing trimmings for six serogroups of non-O157 STEC, in addition to STEC O157:H7. All seven serogroups have been declared adulterants in raw, non-intact beef (USDA-FSIS Rules and Regulations, 2012), and the six non-O157 serogroups include O26, O45, O103, O111, O121, and O145. Additionally, the newly emergent O104:H4 strain has raised concerns about additional STEC serogroups contaminating food.

The emergence of STEC O157:H7 in the 1980's led to the development of a number of commercially available reagents and detection methods for this organism and more recently, several have become available for non-O157 STEC as well. These include differential agar for isolation of bacteria (Church et al., 2007; March and Ratnam, 1986); latex agglutination for O-antigen (O-ag) group testing of isolates (Karmali et al., 1999); immunomagnetic separation for targeted enrichment in broth culture (Drysdale et al., 2004; Fu et al., 2005; Varela-Hernández et al., 2007); enzyme-linked immunosorbent assay (ELISA) for detection of target antigens (Fach et al., 2003; Ge et al.,

Abbreviations: APS, amino methylpersulfate; AP, alkaline phosphatase; BSA, bovine serum albumin; K, capsular antigen; CTAB, cetyltrimethylammonium bromide; cm, centimeters; °C, degrees Celsius; ELISA, enzyme-linked immunosorbent assay; *E. coli*, *Escherichia coli*; H, flagellar antigen; h, hours; LPS, lipopolysaccharide; μL, microliters; μm, micrometers; mg, milligrams; mL, milliliters; mm, millimeters; min, minutes; NBT–BCIP, nitro-blue tetrazolium/5-bromo-4-chloro-3′-indolyphosphate; O-ag, O-antigen; PBS, phosphate buffered saline; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride; rpm, revolutions per minute; RT, room temperature; STEC, Shiga toxin-producing *Escherichia coli*; NaCl, sodium chloride; SDS, sodium dodecyl sulfate; TEMED, tetramethylethylenediamine; TBS, tris-buffered saline; Tris HCl, tris hydrochloride; TSB, Tryptic soy broth; v/v, volume/volume; w/v, weight/volume.

* Corresponding author at: Chemistry Division, Los Alamos National Laboratory, MS J567, C-PCS, Los Alamos, NM 87545, USA.
E-mail address: harshini@lanl.gov (H. Mukundan).

http://dx.doi.org/10.1016/j.mimet.2015.06.008
0167-7012/Published by Elsevier B.V.

2002; Hegde et al., 2012); and polymerase chain reaction (PCR) for detection of virulence factor genes, including targeted amplification of the O-antigen gene (Bai et al., 2012; Blanco et al., 2004; Settanni and Corsetti, 2007; Varela-Hernández et al., 2007). In general, the reagents and detection methods for STEC O157:H7 are relatively sensitive and specific, but in contrast, those for non-O157 STEC are not (Carroll et al., 2003; Gerritzen et al., 2011; Hegde et al., 2012; Orskov et al., 1977). Cross-reactivity, batch-to-batch variability, excessive cost, limited shelf life, and lag time to available results are some of the immediate issues associated with such assays. PCR assays, while very sensitive, are laboratory intensive, may detect residual nucleic acid contaminants, and cannot discriminate whether target genes originated from one or more bacterial cells or types. All of the above are factors that can potentially lead to false positive results.

Historically, identification of serotype has been an important part of the *Escherichia coli* (*E. coli*) diagnostic repertoire, and is part of the requirement for identification of STEC as adulterants. Serotyping is performed by identifying the unique chemical structures of O-, capsular (K), and flagellar (H) antigens, and combinations thereof present on the bacterial cell surface (Orskov et al., 1977; Wang et al., 2010). Due to the frequent problems associated with their identification, K-antigen testing is now only infrequently done. Current procedures primarily focus on identification of the O- and H-antigens or their associated genes. The detection of adulterant STEC in food products and distinguishing them from non-pathogenic species is problematic. Further, the number of existing O-, H-, and K-antigens is estimated to be 180, 60, and 80 respectively, resulting in hundreds of serotypes of *E. coli* (Eisenstein and Zaleznik, 2000; Griffin and Tauxe, 1991; Johnson et al., 1996; Stenutz et al., 2006), more than 200 of which are known to possess Shiga toxin (Kaper et al., 2004).

Lipopolysaccharide (LPS) makes up approximately 70% of the outer membrane of Gram-negative bacteria (Nikaido, 2003; Raetz et al., 2008; Whitfield, 1995). It is a virulence factor produced by several bacteria including *E. coli* (Alexander and Rietschel, 2001; Raetz and Whitfield, 2002) and released by the bacterial cells during an infection. The LPS molecules of various pathogens are structurally different. The contribution of LPS to virulence, and the differences in this biomarker between bacterial species contributes, not only to pathogenesis, but also makes LPS an ideal target for pathogen detection (Bosshart and Heinzelmann, 2007) and serogroup identification. The biochemical differences in LPS between different bacteria are attributable to three major components of the LPS macromolecule (Whitfield, 1995). Specifically, the molecule contains a highly conserved lipid A group attached to a core polysaccharide and a hypervariable O-antigen (O-ag) polysaccharide chain (Raetz et al., 2008).

Since many structural elements of LPS are largely conserved between strains of *E. coli*, antibodies directed against the intact macromolecule exhibit cross-reactivity between different serogroups of STEC. Serogroups of LPS primarily differ in the chemical nature of the O-ag, while the other components remain relatively conserved (Meredith et al., 2006; Whitfield, 1995). Thus, to ensure serogroup discrimination, it is important that antibodies are targeted against the variable O-ag of LPS, and not the conserved regions of the molecule. It naturally follows that some research groups have developed antibodies against the specific O-ag epitopes of LPS (Clark et al., 2006; Westerman et al., 1997) to facilitate serogroup-specific detection of *E. coli*. However, in the absence of purified and well-characterized STEC LPS from different serogroups, the evaluation of these antibodies is difficult. To overcome this limitation, the objective of this work was to isolate and purify the O-ag from strains of non-O157 STEC. These antigens can also be used to develop targeted and specific monoclonal antibodies, and consequently discriminatory detection assays for specific serogroups of STEC LPS. Isolation and characterization of LPS and O-ag from non-O157 STEC, including a comprehensive evaluation of commercially available antibodies against the same is presented herein.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all chemicals and reagents were obtained from Thermo Fisher Scientific™ Inc. (Rockford, IL). STEC strains DEC10B (O26:H11), B8227-C8 (O45:H2), MT#80 (O103:H2), 0201 9611 (O111:H11), and GS G5578620 (O145:NM) were obtained from Dr. Shannon Manning (STEC Center, Michigan State University, Lansing, MI). Serogroup was confirmed using latex agglutination kits (Abraxis Inc., Warminster, PA) and PCR according to Bai et al. (Bai et al., 2012). TY-2482 (O104:H4) was obtained from Dr. John Luchansky (USDA, Agricultural Research Service, Eastern Regional Research Center, Wyndmoor, PA) and serogroup was confirmed by PCR as previously described (Paddock et al., 2013). MDCH-4 (O113:H21) was obtained from the STEC Center at Michigan State University and serogroup was confirmed by PCR according to Bai et al. and subsequently retyped at the *E. coli* Reference Center at Pennsylvania State University (University Park, PA) (Bai et al., 2012; Son et al., 2014). Twelve separate antibodies were used for characterization studies, and the identifying numbers used throughout this paper, as well as supplier information are listed in Table 1. LPS O111:B4 phenol extract, cetyltrimethylammonium bromide (CTAB), phenol (purified by re-distillation), eosin B, acetic acid, and bovine serum albumin (BSA) were from Sigma-Aldrich (St. Louis, MO). Precision Plus Dual Xtra Protein™ Standards, 40% acrylamide solution (19:1), polyvinylidene difluoride (PVDF) membrane, and nitrocellulose 0.2 μm membrane, were from Bio-Rad® Laboratories (Hercules, CA). Regenerated cellulose dialysis tubing (molecular weight cutoff, 12–14,000) was from VWR Scientific (Radnor, PA), LPS O157:H7 was obtained from List Biological Labs (Campbell, CA), and sheep blood agar plates were from Remel (Lenexa, KS). Nitro-blue tetrazolium/5-bromo-4-chloro-3′-indolyphosphate (NBT-BCIP) came from Pierce™ Thermo Fisher Scientific™ (Grand Island, NY), and Tryptic soy broth (TSB), amino methylpersulfate (APS), and tetramethylethylenediamine (TEMED) were from Thermo Fisher Scientific™ Inc. (Rockford, IL). FreeZone6® Freeze Dry System and accompanying freeze drying flasks and accessories were from LabConco® (Fort Scott, KS). Water unless specified otherwise was 18.2 MΩ-cm, filtered through a 0.22 μm membrane from a Barnstead™Nanopure™ system (Thermo Fisher Scientific Inc., Rockford, IL).

2.2. Growth of STEC

Pure cultures from each STEC strain were streaked for isolation onto sheep blood agar plates. Isolated colonies were inoculated into 20 milliliters (mL) of TSB (125 mL flasks) and incubated on a shaking incubator at 220 revolutions per minute (rpm), 37 degrees Celsius (°C), overnight (approximately 18 hours (h)). Broth cultures were swabbed for confluency on 150 millimeters (mm) TSB with agar plates using sterile cotton swabs (Jann and Westphal, 1985; Westerman et al., 1997). Plates were allowed to dry for 3 minutes (min), then incubated for 24 h at 37 °C. Bacterial paste was harvested with a sterile cell scraper, and suspended in water at an estimated 100% weight to volume (w/v) ratio. Theoretical bacterial yields for 150 mm plates, based on data previously collected by our lab (Clark et al., 2006), was used to approximate an equivalent w/v ratio of wet bacterial cultures to water (Limjuco et al., 1978). Bacteria were dried under vacuum with −20 °C acetone using a Büchner funnel apparatus (Jann and Westphal, 1985; Limjuco et al., 1978). Repeat washes were applied, and the bacteria were continually stirred and crushed using a glass rod.

2.3. Phenol extraction

Hot phenol–water extraction, following a modified procedure for high molecular weight polysaccharides was performed as outlined by Jann et al. (Clark et al., 2006; Darveau and Hancock, 1983; Jann et al.,