Video Article

Purification and Visualization of Lipopolysaccharide from Gram-negative Bacteria by Hot Aqueous-phenol Extraction

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Abstract

Lipopolysaccharide (LPS) is a major component of Gram-negative bacterial outer membranes. It is a tripartite molecule consisting of lipid A, which is embedded in the outer membrane, a core oligosaccharide and repeating O-antigen units that extend outward from the surface of the cell[1, 2]. LPS is an immunodominant molecule that is important for the virulence and pathogenesis of many bacterial species, including *Pseudomonas aeruginosa, Salmonella* species, and *Escherichia coli*[3-5], and differences in LPS O-antigen composition form the basis for serotyping of strains. LPS is involved in attachment to host cells at the initiation of infection and provides protection from complement-mediated killing; strains that lack LPS can be attenuated for virulence[6-8]. For these reasons, it is important to visualize LPS, particularly from clinical isolates. Visualizing LPS banding patterns and recognition by specific antibodies can be useful tools to identify strain lineages and to characterize various mutants.

In this report, we describe a hot aqueous-phenol method for the isolation and purification of LPS from Gram-negative bacterial cells. This protocol allows for the extraction of LPS away from nucleic acids and proteins that can interfere with visualization of LPS that occurs with shorter, less intensive extraction methods[9]. LPS prepared this way can be separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and directly stained using carbohydrate/glycoprotein stains or standard silver staining methods. Many anti-sera to LPS contain antibodies that cross-react with outer membrane proteins or other antigenic targets that can hinder reactivity observed following Western immunoblot of SDS-PAGE-separated crude cell lysates. Protease treatment of crude cell lysates alone is not always an effective way of removing this background using this or other visualization methods. Further, extensive protease treatment in an attempt to remove this background can lead to poor quality LPS that is not well resolved by any of the aforementioned methods. For these reasons, we believe that the following protocol, adapted from Westpahl and Jann[10], is ideal for LPS extraction.

Video Link

The video component of this article can be found at https://www.jove.com/video/3916/

Protocol

1. Preparation of Bacteria for LPS Extraction

1. Start an overnight culture in 5 mL of Luria Broth (LB), supplemented with antibiotics if necessary. Grow culture overnight (12-18 hours) in a shaking incubator at 37 °C and 200 rpm.
2. Dilute the culture 1:10 with LB and take an OD_{600} reading in a spectrophotometer. Based on the OD_{600} reading, make a 1.5 mL suspension of your bacteria to an OD_{600} of 0.5.
3. Pellet the bacteria in a microcentrifuge at 10,600x *g* for 10 minutes. Remove and discard the supernatant. The pellet can be stored at -20 °C, if LPS is not going to be extracted immediately.

2. Extraction of LPS

1. First, prepare 2x SDS buffer. Make a 50 mL solution of 4% β-mercaptoethanol (BME), 4% SDS and 20% glycerol in 0.1 M Tris-HCl, pH 6.8. Add a pinch of bromophenol blue to dye the solution. Make a 1x SDS-buffer stock by diluting 2X stock 1:1 in sterile distilled H_2O. This can be stored at room temperature.
2. Make three separate 10 mg/mL solutions of DNase I, RNase, and Proteinase K in sterile distilled H_2O.
3. Resuspend the pelleted bacteria from step 1.3 in 200 µl of 1x SDS-buffer. Ensure that the pellet is completely resuspended by pipetting the solution up and down slowly. Do not vortex.
4. Boil the suspended bacteria in a water bath for 15 minutes. Allow the solution to cool at room temperature for 15 minutes.
5. Add 5 µl of both DNase I and RNase solutions prepared in 2.2. Incubate the samples at 37 °C for 30 minutes. *This step is optional; there is minimal difference in the quality of LPS between nuclease-treated and non-treated samples.

6. Add 10 µl of the Proteinase K solution prepared in 2.2. Incubate the samples at 59 °C for 3 hours. This step can be performed overnight, if there are time constraints.
7. To each sample, add 200 µl of ice-cold Tris-saturated phenol (water-saturated phenol may be used as a substitute if Tris-saturated phenol is not available). Ensure the caps on the tubes are closed tightly, and vortex each sample for approximately 5 to 10 seconds.
8. Incubate the samples at 65 °C for 15 minutes, vortexing occasionally. After incubating cool to room temperature, then add 1 mL of room-temperature diethyl ether to each sample and vortex for 5 to 10 seconds. Be sure to perform handle ether in a fume hood, as it is volatile.
9. Centrifuge the samples at 20,600x *g* for 10 minutes. Carefully remove the samples from the centrifuge and extract the bottom blue layer. Be sure to avoid the upper, clear layer. Leaving behind a small amount of the blue layer is preferable to contamination with the upper layer.
10. Re-extract the samples by repeating steps 2.7 - 2.9. Two extractions are usually sufficient. If the samples appear cloudy, more extractions may be performed. Add 200 µl of 2x SDS-buffer to each of the extracted samples before separating by SDS-PAGE. Samples can be run on 8%-15% SDS-polyacrylamide gels. Five to fifteen µl of LPS prepared using this method is usually sufficient for visualization.

3. Representative Results

LPS samples prepared as above can be visualized by direct staining following separation on SDS-PAGE using a standard silver-staining protocol or a commercially available LPS staining kit. Alternatively, LPS separated on a polyacrylamide gel may be transferred to a nitrocellulose membrane and subjected to Western immunoblotting using LPS-specific anti-sera. For this protocol, we used the Pro-Q Emerald 300 Lipopolysaccharide Gel Stain Kit (Molecular Probes), and followed the manufacturer's instructions.

Shown in Figure 1 is a Pro-Q Emerald 300 stained 12% SDS-polyacrylamide gel. Each lane contains 15 µl of LPS prepared from different strains of *Burkholderia dolosa* isolated from sputum samples of cystic fibrosis patients. Different LPS banding ladder patterns, reflective of different numbers O-antigen repeating units attached to core oligosaccharide, are evident using this method; for example, samples in lane 1 and 6 have similar banding patterns to each other (boxed in red).

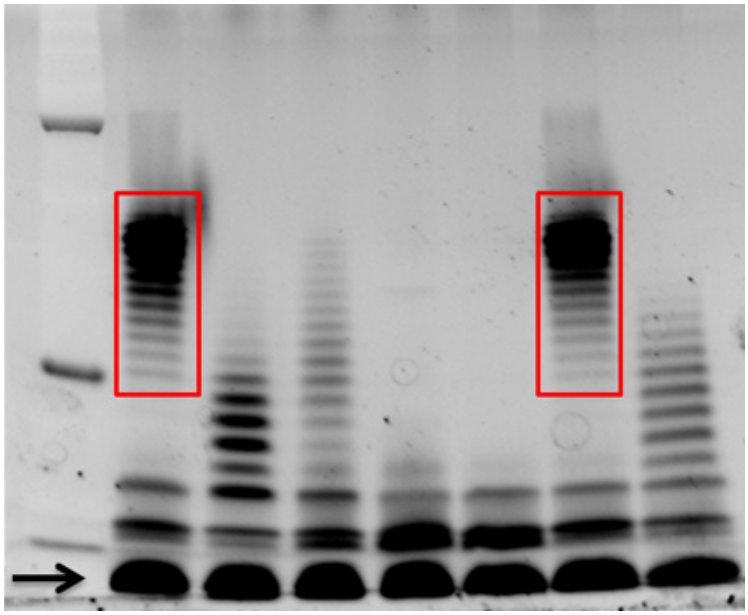

Figure 1. Pro-Q Emerald 300 stained LPS from *Burkholderia dolosa* clinical isolates. LPS from seven *B. dolosa* strains from an outbreak in cystic fibrosis patients were isolated as described in this protocol. Fifteen µl were separated on a 12% SDS-polyacrylamide gel, and stained with Pro-Q Emerald 300, per the manufacturer's instructions. LPS core is indicated an arrow, and LPS showing similar banding patterns of O-antigen repeats are boxed in red. M = Molecular weight marker.

Discussion

We have described a method of purifying LPS away from other cellular components, including nucleic acids and proteins. This method provides high-quality LPS that can be used in a number of different visualization methods, including carbohydrate staining of SDS-PAGE gels, as shown in Figure 1. This method can be used to serotype LPS from a variety of strains, using specific anti-sera, or to show relatedness between isolates by direct visualization. For example, a recent genome-wide sequencing project in combination with LPS characterization from an outbreak of *B. dolosa* led to the discovery of a single nucleotide polymorphism (SNP) that correlates with the presence and absence of LPS in these strains[11]. We believe that this method is preferable to the protease treatment of whole-cell lysates described by Hitchcock and Brown[9], as it is a relatively quick, yet rigorous enough to yield high-quality LPS for further analyses.

While we only show an example using the *Burkholderia dolosa*, this protocol can be adapted to other Gram-negative species as well. We have successfully used this protocol to extract and visualize LPS from other *Burkholderia* spp., *Escherichia coli, Helicobacter pylori, Pseudomonas aeruginosa* and *Salmonella* spp. If the protocol results in little or no LPS yield, it may be that the LPS fractionates to a different layer in the extraction steps, 2.7-2.9. To troubleshoot this, repeat the protocol and save a sample of each layer during the extraction steps, these can be visualized by staining an SDS-PAGE gel in order to determine where the LPS fractionates, and the protocol can be modified accordingly. It

should also be noted that this protocol, while ideal for analytical purposes, does not yield LPS that is appropriate for other applications, such as structural analyses.

Disclosures

No conflicts of interest declared.

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References

1. Raetz, C.R. & Whitfield, C. Lipopolysaccharide endotoxins. *Annu. Rev. Biochem.* **71**, 635-700 (2002).
2. Valvano, M.A. Export of O-specific lipopolysaccharide. *Front. Biosci.* **8**, s452-71 (2003).
3. Pier, G.B. *Pseudomonas aeruginosa* lipopolysaccharide: a major virulence factor, initiator of inflammation and target for effective immunity. *Int. J. Med. Microbiol.* **297**, 277-295 (2007).
4. Freudenberg, M.A., *et al.* Role of lipopolysaccharide susceptibility in the innate immune response to *Salmonella typhimurium* infection: LPS, a primary target for recognition of Gram-negative bacteria. *Microbes Infect.* **3**, 1213-1222 (2001).
5. Jann, K. & Jann, B. Polysaccharide antigens of *Escherichia coli. Rev. Infect. Dis.* **9 Suppl 5**, S517-26 (1987).
6. McCallum, K.L., Schoenhals, G., Laakso, D., Clarke, B., & Whitfield, C. A high-molecular-weight fraction of smooth lipopolysaccharide in *Klebsiella* serotype O1:K20 contains a unique O-antigen epitope and determines resistance to nonspecific serum killing. *Infect. Immun.* **57**, 3816-3822 (1989).
7. Hancock, R E., *et al. Pseudomonas aeruginosa* isolates from patients with cystic fibrosis: a class of serum-sensitive, nontypable strains deficient in lipopolysaccharide O side chains. *Infect. Immun.* **42**, 170-177 (1983).
8. Gupta, S.K., Masinick, S., Garrett, M., & Hazlett, L.D. *Pseudomonas aeruginosa* lipopolysaccharide binds galectin-3 and other human corneal epithelial proteins. *Infect. Immun.* **65**, 2747-2753 (1997).
9. Hitchcock, P.J., & Brown, T.M. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *J. Bacteriol.* **154**, 269-277 (1983).
10. Westphal, O. & Jann, K. Bacterial lipopolysaccharides. *Methods Carbohyr. Chem.* **5**, 83 (1965).
11. Lieberman, TD., *et al.* Parallel bacterial evolution within multiple patients ties novel genes to pathogenesis. *Nat Genet.* In Press, (2011).

