JOURNAL OF BACTERIOLOGY, Feb. 1990, p. 1145–1147
0021-9193/90/021145-03$02.00/0

Vol. 172, No. 2

Detection of Lipopolysaccharides by Ethidium Bromide Staining after Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

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A rapid and easy method for staining lipopolysaccharides with ethidium bromide is described. Lipopolysaccharides could be visualized by ethidium bromide with almost the same sensitivity as found with the silver-staining method in less than 30 min. The ethidium bromide-staining method was particularly suitable for staining lipopolysaccharides possessing acidic O-specific polysaccharides, which were poorly visualized by silver staining.

A very sensitive method for detecting lipopolysaccharides (LPS) in polyacrylamide gels by silver staining (6, 18) was developed after trials involving electrophoresis of the dyed LPS (7), periodic acid-Schiff staining (7, 14, 17), and fluorographic analysis of ^{3}H-, ^{14}C-, or ^{33}P-labeled LPS after sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis (PAGE) (4, 14, 15). The silver-staining method shows good sensitivity for detecting LPS after SDS-PAGE. However, this method includes a time-consuming process until LPS bands appear in the gel, and preparation of the staining solution requires delicate handling of alkaline and silver nitrate solutions. Recently, we found that ethidium bromide (EtBr), a fluorescent dye used to stain nucleic acids in gels, stained the LPS in agarose or polyacrylamide gels during analysis of the *rfb* gene of *Escherichia coli* O9 (10). We describe an easy, rapid, and sensitive method of staining LPS with EtBr.

LPS was prepared by the following rapid method. A 1.5-ml overnight culture of bacterial cells was centrifuged in a model KM15200 microcentrifuge (Kubota, Tokyo, Japan) at 16,000 × *g* for 30 s. The bacterial pellet was suspended in 100 μl of TAE buffer (40 mM Tris acetate [pH 8.5]–2 mM EDTA) and mixed with 200 μl of alkaline solution containing 3 g of SDS, 0.6 g of Trizma base (Sigma Chemical Co., St. Louis, Mo.), and 6.4 ml of 2 N NaOH in 100 ml of H_2O. The mixture was heated at 55 to 60°C for 70 min and then mixed with phenol-chloroform (1:1, vol/vol). The supernatant of the mixture after centrifugation at 16,000 × *g* for 10 min was mixed with 200 μl of H_2O and 50 μl of 3 M sodium acetate (pH 5.2). LPS was precipitated by adding 2 volumes of ethanol. The precipitate was dissolved in 200 μl of 50 mM Tris hydrochloride (pH 8.0)–100 mM sodium acetate and precipitated with 2 volumes of ethanol. The final precipitation of LPS was dissolved in 50 μl of H_2O. The concentration of LPS was approximately 2 mg/ml. LPS samples were mixed with an equal volume of 0.1 M Tris hydrochloride (pH 7.0) buffer containing 4% SDS, 40% (wt/vol) sucrose, 2% (vol/vol) 2-mercaptoethanol, and 0.02% bromophenol blue. SDS-PAGE was performed as described by Tsai and Frasch (18). After electrophoresis, gels were removed from glass plates and immersed in a 30-μg/ml EtBr solution for 10 s. The destaining step was carried out in water with gentle shaking for 10 to 30 min. LPS bands were observed by using a transilluminator (wave length of 302 nm), and gels were photographed with a sensitive film, Polaroid type 667 (ASA 3000). Silver staining was performed by the method of Hitchcock and Brown (6).

First, the sensitivities of silver and EtBr stains were compared by using *E. coli* O111:B4 LPS (Difco Laboratories, Detroit, Mich.), the O-specific polysaccharide portion of which was chemically characterized and found to be a neutral polysaccharide (K. Eklund, P. J. Garegg, L. Kenne, A. A. Lindberg, and B. Lindberg, Abstr. IXth Int. Symp. Carbohydr. Chem., abstr. no. 493, 1978). Various amounts of *E. coli* O111:B4 LPS were applied to SDS-polyacrylamide gels, and LPS bands were visualized by silver or EtBr stain. At all concentrations of LPS tested, LPS bands could be visualized by both stains, although silver staining produced more distinct bands (Fig. 1). Even with 0.5-μg samples (Fig. 1, lanes 1), the major bands of *E. coli* O111:B4 LPS were detected by both stains. This appeared to be the minimum amount of LPS detectable by both staining methods. With 8-μg samples (Fig. 1, lanes 5), the LPS bands of low molecular weight, which corresponded to LPS molecules possessing short O-specific polysaccharide chains (fewer repeating units), were stained rather faintly by the EtBr stain. However, LPS molecules having long O-specific polysaccharide chains (many repeating units) were stained by both methods with nearly equal intensities. The fact that high-molecular-weight LPS bands were more strongly stained than the low-molecular-weight bands by EtBr suggests that EtBr dye tends to combine more readily with LPS molecules of high carbohydrate content than with those of low carbohydrate content.

To compare EtBr and silver stains with respect to detection of LPS samples possessing neutral or acidic O-specific polysaccharide portions, both kinds of LPS were used. As LPS samples possessing neutral O-specific polysaccharide portions, LPS from *Salmonella typhimurium* (5) and *E. coli* O8 (16) were used. As LPS samples possessing acidic O-specific polysaccharide portions, LPS from *Shigella dysenteriae* 3 (1), *Shigella dysenteriae* 9 (3), *Shigella flexneri* 6 (2), *Shigella boydii* 4 (13), and *Shigella sonnei* phase I (9) were used. EtBr-stained LPS samples from *Salmonella typhimurium* and *E. coli* O8 produced bands almost as distinct as those produced with the silver stain (Fig. 2, lanes 1 and 2, respectively). However, a remarkable advantage of the EtBr stain was demonstrated when LPS samples having acidic O-specific polysaccharides were stained. By using EtBr on LPS samples from *S. dysenteriae* 3 strains 60H-44,

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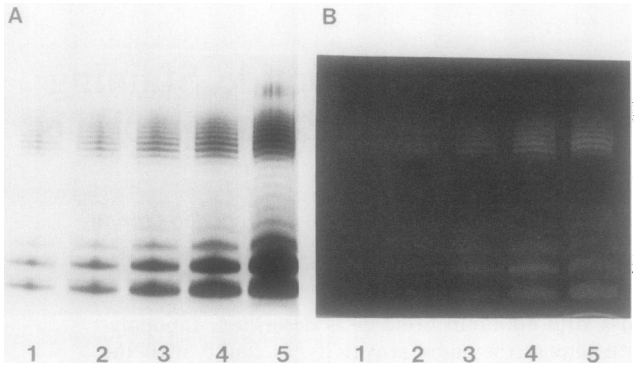

FIG 1. Sensitivity of silver (A) and EtBr (B) stains for LPS after separation by SDS-PAGE. Various amounts of *E. coli* O111:B4 LPS were analyzed. Lanes: 1, 0.5 μg; 2, 1 μg; 3, 2 μg; 4, 4 μg; 5, 8 μg.

61H-229, 62H-289, and 60H-201, *S. dysenteriae* 9 strain 62H-52, *S. flexneri* 6 strain 87-54, *S. boydii* 4 strain 83-2, and *S. sonnei* phase I strains 9 and 1196, LPS bands were discerned as clearly as when LPS from *Salmonella typhimurium* and *E. coli* O8 consisting of neutral sugars were used (Fig. 2B, lanes 3 to 11). These EtBr-stained samples showed clear bands except for LPS from *S. sonnei* phase I. In contrast, when LPS samples from these *Shigella* strains were silver stained, two to three bands of low molecular weight, corresponding to LPS molecules possessing fewer repeating units of the O-specific polysaccharide portions, were clearly visible, but the bands corresponding to LPS molecules with many repeating units of the polysaccharide portions were either not stained or only faintly stained (Fig. 2A, lanes 3 to 11).

Because of the limited utility of silver staining for LPS possessing acidic O-specific polysaccharide portions, there have been no reports describing the heterogeneity of LPS molecules from *Shigella* spp. with respect to the lengths of O-specific polysaccharide portions. We have revealed this feature for the first time by using an EtBr stain. However, in contrast to the other LPS samples, LPS from *S. sonnei* phase I, also having an acidic O-specific polysaccharides, did not show clear banding patterns. One reason for this poor separation of *S. sonnei* LPS bands may be that fewer sugars constitute one repeating unit of LPS from *S. sonnei* phase I than of other LPS samples from *Shigella* spp. The numbers of sugars that constitute one repeating unit of LPS are as follows; two for *S. sonnei* phase I, four for *S. dysenteriae* 9 and *S. flexneri* 6, and five for *S. dysenteriae* 3 and *S. boydii* 4 (1–3, 9, 13). For this reason, the molecular weights of individual molecules having variable lengths of O-specific polysaccharides of *S. sonnei* phase I LPS are too low to make distinct bands under this SDS-PAGE condition. The repeating unit of the O-specific polysaccharide of LPS from *S. sonnei* phase I consists of unusual components such as 2-amino-2-deoxy-L-altruronic acid (11) and 2-acetoamido-4-amino-2,4,6-trideoxy-D-galactose (9). It has not been ascertained whether the structural peculiarity of the repeating unit of the O-specific polysaccharide interferes with separation of LPS bands.

The polysaccharide portion of LPS has been suggested to be the reactive component in silver staining (18), and recently Kropinski et al. suggested that the fatty acids of the lipid A moiety might be the binding site of silver ions through the formation of π complexes (12). The results reported here suggest that the presence of acidic components in the O-specific polysaccharides, such as hexuronic acids (*S. boydii* 4, *S. flexneri* 6, and *S. sonnei*), glucolactic acid (*S. dysenteriae* 3), and pyruvate in ketal linkage (*S. dysenteriae* 9) (8), decreased the sensitivity of the silver stain. The reason for the finding that only the low-molecular-weight LPS bands that correspond to LPS molecules possessing short O-specific polysaccharide chains can be visualized by silver staining may be that absolute amounts of acidic components which may act to decrease the sensitivity of the silver stain are small in these LPS molecules. Whether the precise mechanism of the preferential silver staining of the low-molecular-weight LPS is due to the lack of acidic components that might interfere with the binding of silver ions to LPS or to the lack of steric or chemical interference

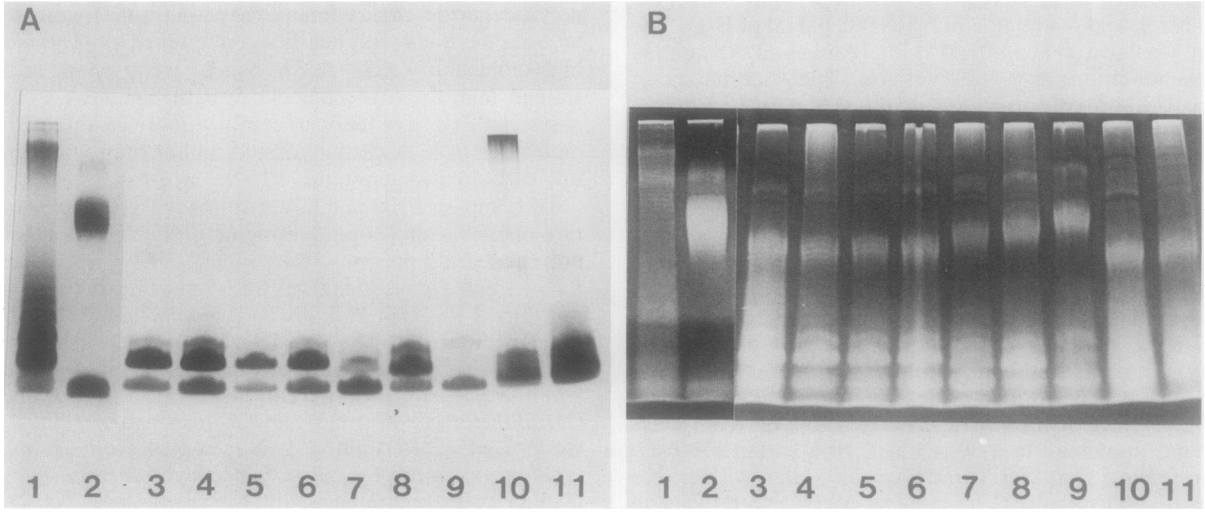

FIG. 2. Detection by silver (A) and EtBr (B) stains of LPS possessing neutral and acidic O-specific polysaccharide portions after separation by SDS-PAGE. Samples (10 μg) were analyzed on SDS-polyacrylamide gels. LPS was derived from *Salmonella typhimurium* (lane 1), *E. coli* 08 (lane 2), *S. dysenteriae* 3 (lanes 3 to 6), *S. dysenteriae* 9 (lane 7), *S. flexneri* 6 (lane 8), *S. boydii* 4 (lane 9), and *S. sonnei* phase I (lanes 10 and 11).

of oxidation of the O-specific polysaccharides in the longer side chains is unclear. There has been another report that sugar composition affects the results of silver staining of LPS. Hitchcock and Brown (6) reported the presence of doublets stained in gray and red in silver-stained gels on which LPS from *Salmonella typhimurium* had been separated. They discussed the possible involvement of the constituents common to the sugars in the outer core, because the doublets bands were present in the LPS from S, SR, Ra and Rb_2 but absent in the rougher chemotypes (RcP^- to Re). Our results provide evidence that an extreme of the influence of sugar composition of LPS on silver staining is failure to stain. In the silver-staining procedure, there is a step in which sugar constituents are oxidized by periodic acid. After this step, LPS is stained by silver nitrate in an alkaline solution. LPS from *E. coli* O8, *S. dysenteriae* 9, and *S. flexneri* 6 contain two, LPS from *S. typhimurium* contains three, and LPS from *S. dysenteriae* 3 and *S. boydii* 4 contain four oxidized sites in one repeating unit. There is no oxidized site in the repeating unit of *S. sonnei* phase I. It can therefore be concluded that there is no correlation between the number of oxidized sites in O-specific polysaccharide portions and sensitivity to silver stain. The lack of such a correlation seems reasonable, given the previous result that the LPS preparations without both the side chain and the core region react with the silver stain in polyacrylamide gels (12). At present, the exact mechanism of the EtBr stain is not clear. However, this new method is not affected by the acidic components in the O-specific polysaccharides. The tendency of the EtBr dye to combine more easily with LPS possessing long O-specific polysaccharides than with LPS possessing short O-specific polysaccharides may suggest the presence of tertiary structures of the O-specific polysaccharides that trap the EtBr molecules.

We acknowledge M. Saito, Aichi Prefectural Institute of Public Health, for his kind gift of bacterial strains.

This study was supported by grant-in-aid for scientific research 01480177 from the Ministry of Education, Science and Culture of Japan to M.O.

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