The resolution of bacteroides lipopolysaccharides by polyacrylamide gel electrophoresis

J. P. MASKELL

Department of Medical Microbiology, The London Hospital Medical College, London E1 2AD

Summary. The lipopolysaccharides (LPS) of the 10 species of the genus *Bacteroides* (*sensu stricto*) were extracted by the proteinase K method and their resolution compared by several methods of polyacrylamide gel electrophoresis (PAGE). These included sodium dodecyl sulphate (SDS)-PAGE with and without urea, polyacrylamide gradient gels and Tricine [N-Tris (hydroxymethyl) methyl glycine]-SDS-PAGE. The original discontinuous system showed good resolution of LPS from *B. thetaiotaomicron*, *B. caccae* and *B. ovatus* and this was enhanced by urea; *B. vulgatus* showed a typical ladder pattern associated with repeating polysaccharide units of the O side chains. The LPS profiles of the other species, including *B. fragilis*, were poorly resolved; the majority of components migrated with the leading edge of the wave front. The resolution of the LPS of these species was marginally improved with gradient gels but the majority of components were separated only within the regions of high polyacrylamide concentration. The Tricine-SDS system was consistently superior to the other methods, with excellent resolution of the LPS profiles of all *Bacteroides* species.

Introduction

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) has been used extensively for the study of lipopolysaccharide (LPS) and other bacterial surface structures. The original discontinuous system¹ has proved useful for the separation of the LPS components of organisms such as *Salmonella* spp.² and *Pseudomonas aeruginosa*.³ The LPS of some organisms, especially those with rough LPS, has proved more difficult to resolve with this method and various modifications have been proposed to improve its performance, such as the addition of urea.⁴ Improved resolution of rapidly migrating low molecular weight components may be accomplished with gradient gels. *Bacteroides fragilis* LPS has proved notoriously difficult to resolve because of its lack of classical structure and several reports^{5–7} suggest that only a few heavily stained bands may be visualised, the majority of components migrating with the dye front. The poor resolution of *B. fragilis* LPS has led to differences of opinion as to whether or not this organism produces LPS with a classical ladder pattern in gels,⁸ or whether they have only rough LPS.⁵ However, the LPSs of some of the species closely related to *B. fragilis* are less difficult to resolve,⁷ because they appear to possess a structure similar to that found in aerobic gram-negative organisms. The LPS of some *B. vulgatus* strains have been shown⁹ to produce a classical ladder pattern of repeating poly-

saccharide units. The LPS structure of the other species within the genus *Bacteroides sensu stricto* (i.e., the fragilis group of gram-negative anaerobic bacilli) is not well documented.

A new method of PAGE has been described¹⁰ which is discontinuous, like the original system,¹ but utilises a cathode buffer containing Tricine which serves as the trailing ion and allows stacking and destacking of low molecular weight proteins resulting in enhanced resolution. This method has been used to increase the resolution of lipopolysaccharides and lipo-oligosaccharides of gram-negative bacteria.¹¹ A much improved separation of LPS was reported with Tricine compared with the glycine buffer system.¹ Unlike previous methods, it was shown that the rate of migration of LPS components was generally directly related to their molecular weight.¹¹

These methods were compared for the resolution of LPS of the reclassified genus *Bacteroides*¹² which now consists of *B. fragilis* and nine closely related species.

Materials and methods

Bacterial strains

These were *B. fragilis* NCTC nos 9343 and 10584, *B. thetaiotaomicron* NCTC 10582, *B. vulgatus* NCTC 11154, *B. ovatus* NCTC 11153, *B. distasonis* NCTC 11152, *B. uniformis* ATCC 8492, *B. eggerthii* NCTC 11155, *B. caccae* ATCC 43185, *B. merdae* ATCC 43184, *B. stercoris* ATCC 43183 and one clinical isolate of *B. fragilis* isolated at the London Hospital.

Strains were identified with the ATB 32A (API Laboratory Products, Basingstoke) system.

Preparation of LPS

A modified version of the proteinase K method,² was used. Cultures were inoculated on to Wilkins Chalgren Anaerobe Agar (Oxoid) and incubated anaerobically for 48 h. Growth was suspended in phosphate-buffered saline (PBS, pH 7) and washed twice by centrifugation for 10 min at 1000 *g* in a bench centrifuge. The pellet was resuspended in PBS to an extinction at 525 nm (E_{525}) of 0.5–0.6 and 1.5-ml volumes were centrifuged at approximately 10 000 *g* for 1 min in an Eppendorf 5414 microfuge. The pellet was blotted dry with tissue paper and resuspended in 75 μ l of solubilisation buffer, which consisted of SDS 2% w/v, 2-mercaptoethanol 4% v/v, glycerol 10% v/v and bromophenol blue 0.01% w/v in 1.0 M Tris-HCl buffer, pH 6.8. The suspensions were heated at 100°C for 10 min in a heating block (Dri Block-Techne DB1) followed by the addition of 50 μ g of proteinase K (Sigma Protease X1) in 15 μ l of solubilisation buffer and held overnight at 55°C, then stored at –20°C.

Electrophoresis of LPS

A Protean Cell (Bio-Rad) vertical electrophoresis tank was used for all methods, with separating gels of approximately 140 × 120 × 1.5 mm. LPS samples were loaded into the slots under buffer in volumes ranging from 10 μ l to 30 μ l, depending on the method. Four methods of electrophoresis were used for the separation of LPS components.

1. *The discontinuous gel system.*¹ Acrylamide and NN-methylene bisacrylamide concentrations were adjusted to 13.5% and 0.25% w/v, respectively, in the separating gel, and to 6% and 0.6% w/v, respectively, in the stacking gel. SDS was omitted from the running and stacking gel mixtures. The separating gel was overlaid with SDS 0.1% in water immediately after pouring, and discarded before adding the stacking gel mixture. Electrophoresis was performed at a constant current of 35mA for approximately 6 h or until the dye front had reached the lower edge of the gel.

2. *A urea-based method.*⁴ The stacking gel was identical to that used above. The separating gel contained 13.5% and 0.25% acrylamide and bisacrylamide respectively and 4 M urea in 1.5 M Tris-HCl buffer, pH 8.8. Polymerisation was initiated by the addition of 70 μ l of TEMED and 100 μ l of ammonium persulphate 10%. Electrophoresis was performed at a constant 20mA through the stacking gel and 25mA through the separating gel. The running buffer used was as for method 1 but with half the concentrations of glycine and Tris (pH 8.3). Electrophoresis was stopped when the dye front had reached the bottom of the gel.

3. *Gradient gel.* This system was identical to method 1 but a gradient was formed so that the acrylamide

concentration increased from approximately 5% at the top of the separating gel to approximately 20% at the bottom. A BRL (Bethesda Research Labs, Inc.) gradient former was used for preparing the gradient and for filling the apparatus. The stacking gel and running buffer were identical to those used in method 1. Electrophoresis was performed at a constant 60V overnight and stopped just before the dye front had reached the bottom of the gel.

4. *A modification¹¹ of the Tricine SDS system.*¹⁰ The separating gel was prepared at a final concentration of T 16.5%, C 6%, where T represents the percentage of bisacrylamide to the total concentration (C) of acrylamide. The gel was polymerised by the addition of 100 μ l of ammonium persulphate 10% and 10 μ l of TEMED and layered with SDS 0.1%. The stacking gel (about 4 cm depth including slots) was prepared at a final concentration T 4%, C 3% and polymerised by the addition of 150 μ l of ammonium persulphate 10% and 15 μ l of TEMED within 30 min of pouring the separating gel. The cathode buffer was 0.1 M Tris-HCl, 0.1 M N-Tris(hydroxymethyl)methyl-glycine (Tricine) and SDS 0.1%, pH 8.25, the anode buffer 0.2 M Tris-HCl, pH 8.9, and the gel buffer 3.0 M Tris, SDS 0.3%, pH 8.45.¹⁰ Electrophoresis was performed at a constant 30V until the samples had entered the stacking gel then a constant 100V for approximately 30 h.

With all methods, after the stacking gel had polymerised, the comb was removed and the slots were washed three times with SDS 0.1% then filled with running buffer; for the Tricine method the cathode buffer was used.

Silver staining

LPS components were stained with silver.¹³ The stain was developed at 30–35°C to eliminate the staining of residual protein remaining after proteinase digestion of extracts.

Results

The LPS profiles of 11 reference strains representing the species of the genus *Bacteroides* obtained with the original Laemmli system are shown in fig. 1. The strain of *B. vulgatus* (lane 3) differed from the others in that it showed a classical smooth LPS ladder profile reflecting the multiple repeating units of the O side chain. The LPS profiles of *B. thetaiotaomicron*, *B. ovatus* and *B. caccae* were similar. *B. caccae* and, to a lesser extent, *B. thetaiotaomicron* and *B. ovatus* showed a series of double bands; with *B. caccae* these were a distinct brown colour and a series of low molecular weight grey bands were seen concentrated at the bottom of the gel below the lowest molecular weight doublet. The LPS of other species, including *B. fragilis*, tended to show relatively few diffuse bands and it was apparent that the majority of the components were concentrated behind the leading wave.

Fig. 1. LPS profiles of 11 reference strains and one clinical isolate of *Bacteroides* spp. separated by method 1. Lane 1, *B. fragilis* NCTC 9343; 2, *B. thetaiotaomicron* NCTC 10582; 3, *B. vulgatus* NCTC 11154; 4, *B. ovatus* NCTC 11153; 5, *B. distasonis* NCTC 11152; 6, *B. uniformis* ATCC 8492; 7, *B. eggerthii* NCTC 11155; 8, *B. caccae* ATCC 43185; 9, *B. merdae* ATCC 43184; 10, *B. stercoris* ATCC 43183; 11, *B. fragilis* NCTC 10584; 12, *B. fragilis*—clinical isolate.

The LPS profiles of *B. thetaiotaomicron*, *B. ovatus* and *B. caccae* are shown in fig. 2, separated with a gel containing 4 M urea. The resolution of the LPS of these species was enhanced, with many bands evident between the doublets seen with method 1. However, this method gave relatively poor resolution of the LPS of other species.

Separation with the gradient gel system (fig. 3) shows the presence of multiple bands within the LPS of several of the LPS preparations which were not apparent with the previous methods. Nevertheless, most of the components appear to be concentrated within the higher acrylamide concentrations in the lower portion of the gel. The characteristic profiles of *B. caccae*, *B. thetaiotaomicron* and *B. ovatus* are less well defined than in previous methods and the ladder of *B. vulgatus* is concentrated within the higher acrylamide concentrations. The use of polyacrylamide 20% gels (not shown) showed poor resolution with much streaking of bands.

The Tricine-SDS gel method (fig. 4) produced a far superior separation compared with the other methods. The LPS components of all species were well resolved although this may be improved by adjusting the loading of preparations for each species. The three species showing closely related LPS structure when separated with the other methods appeared less similar

when separated by the Tricine-SDS method. The smooth high molecular weight ladders of *B. stercoris* and *B. eggerthii* were well defined in some of these gels. The LPS of several species which could not be separated by previous methods were resolved with this system. Between the two heavily stained, rapidly migrating bands of *B. fragilis* LPS resolved with method 1, a series of bands was observed in some gels, indicative of smooth LPS. Profiles of all species showed several bands within the central region of the gels which were not readily apparent with the other methods.

Discussion

The resolution of bacteroides LPS has proved difficult with previously available PAGE systems because many components remain associated with the SDS as it migrates through the gel. As a result, the majority of LPS components of species like *B. fragilis*, *B. distasonis* and *B. uniformis* are poorly resolved, whereas those of species such as *B. thetaiotaomicron*, *B. ovatus*, *B. caccae* and *B. vulgatus*, which presumably have different characteristics, are resolved by conventional methods.⁷ Therefore, although gradient gels may assist the separation of rapidly migrating components, resolution is hindered by 'attachment' to

Fig. 2. LPS profiles of (lane 1) *B. ovatus*, (2) *B. thetaiotaomicron*, and (3) *B. caccae* resolved with a separating gel containing 4 M urea as described in method 2.

Fig. 3. LPS profiles of 10 reference strains of *Bacteroides* spp. separated by gradient gel electrophoresis (method 3). Lanes 1–10 correspond to those in fig. 1.

SDS. Even the addition of urea, which is said to assist in separating components,⁴ did not significantly improve the resolution of the LPS of species not separated by the original system.¹ However, *B. thetaiotaomicron*, *B. ovatus* and *B. caccae* LPS were well resolved by this method; ladders of repeating polysaccharide units were seen between the series of doublets which were visualised in gels without urea. The Tricine-SDS system has the advantage of using Tricine instead of glycine as the trailing ion; this enables efficient stacking and destacking of components allowing them to separate,¹⁰ as far as can be ascertained, relative to their molecular weight. With this method it is also possible to load much larger amounts of extract without smearing, which is the usual result of doing so with other methods. It is interesting that the LPS profiles of *B. thetaiotaomicron*, *B. ovatus* and *B. caccae*, which appeared very similar with the Laemmli-based methods, were rather different when visualised with the Tricine method. Other species, the LPS profiles of which were poorly resolved with previous systems, showed much enhanced separation. The LPSs of *B. stercoris* and *B. eggerthii* contain a high molecular weight ladder when separated by this method, typical of smooth LPS. Within all LPS profiles obtained with this method, bands of unstained material are seen, the reason for this is unknown, but it is possible that these components are predominantly

lipid and do not allow silver binding. It has been suggested that silver staining does not stain lipid but only the polysaccharide components of LPS.¹⁴ It must also be considered whether the preparations used were impure LPS and may have contained capsular polysaccharide and glycan.¹⁵

The LPS of *B. fragilis* has been the most extensively studied of the genus and is usually described as consisting of two heavily stained bands constituting the core regions. According to one report,⁸ most strains have rough LPS which runs at the gel front followed by a common antigen and a series of closely spaced bands of smooth LPS. Other workers⁵ were unable to demonstrate smooth LPS in any of the 17 strains studied including the reference strain (NCTC 9343). In this study it was found that, using all methods, the major and minor core bands were seen; and in several clinical isolates and the reference strain a series of repeating units was seen between the core components with the Tricine system. Additional bands of higher molecular weight components were also seen. The smooth LPS of *B. fragilis* is probably difficult to demonstrate because it is masked by core bands in heavily loaded gels and too light to be seen when gels are loaded with less material.

In conclusion, it would appear that although the Tricine method generally gives by far the best separation of LPS of *Bacteroides* spp., the original Laemmli system, possibly with the addition of urea, provides an excellent, if rather variable, method for

Fig. 4. Resolution of *Bacteroides* LPS by Tricine-SDS-PAGE (method 4). Lanes 1–10 correspond to those in fig. 1.

visualising the profiles of *B. vulgatus*, *B. ovatus*, *B. thetaiotaomicron* and *B. caccae*. However, only the Tricine method may be useful as a guide to the

molecular weight of resolved components, although for typing purposes this is probably of little significance.

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