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Morphological Heterogeneity Among *Salmonella* Lipopolysaccharide Chemotypes in Silver-Stained Polyacrylamide Gels

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The morphological heterogeneity of lipopolysaccharides (LPSs) among salmonella mutants with different LPS chemotypes was analyzed in silver-stained polyacrylamide gels. The biochemical differences in the LPS chemotypes were reflected in the unique profiles of the purified LPSs. The LPS profiles in the whole-cell lysates were also unique for each chemotype. (Whole-cell lysates were assessed by a method which preferentially silver stains LPS and by a proteinase K digest of whole-cell lysates. The silver-stained LPS profiles of proteinase K-digested lysates were similar to the homologous purified LPS and could be used to preliminarily characterize the LPS chemotype before purification.) In summary, biochemical variation in LPS composition can be detected in silver-stained polyacrylamide gels.

The surface of the gram-negative bacterium mediates its interactions with the host. Lipopolysaccharide (LPS), a major component of the surface, has received much attention; in *Salmonella* species, the chemical composition, biosynthesis, genetic regulation, and immunochemistry of LPS have been well characterized.

Rigorous analyses of LPS by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) have been hampered by the fact that LPS does not stain well with carbohydrate stains (periodic acid-Schiff [16] or alcian blue methods). In the past, intrinsic radiolabels (5, 12, 13), carbohydrate dye (Procion red [1, 7]), and periodic acid-Schiff stain (12, 15) have been used to visualize LPS in polyacrylamide gels. Analysis of biochemically characterized LPS by SDS-PAGE has revealed that the O-specific polysaccharides, core oligosaccharides, and lipid A content contribute to the morphological heterogeneity of SDS-PAGE LPS profiles (5, 7, 12, 13).

To determine whether or not SDS-PAGE might be utilized to fingerprint bacterial LPS as it has been used to fingerprint bacterial proteins, we examined the LPS of biochemically characterized salmonella chemotypes. Incorporating the important discovery of Tsai and Frasch (periodate oxidation [17]), we used two silver stains, one which preferentially stains the LPS in bacterial whole-cell (WC) lysates (denoted Ag-LPS) and one which stains LPS and proteins (denoted Ag-LPS and proteins) to visualize purified LPSs. We also evaluated the LPS in WC lysates and in protein-digested lysates and compared the staining patterns with that of the purified LPS.

MATERIALS AND METHODS

Bacteria and culture conditions. *Salmonella minnesota* S218 and rough (R) mutants R60, R345, R5, R7, R3, and R595 (provided by Mark Peppler, Laboratory of Microbial Structure and Function, Hamilton, Mont.) were used. (The proposed LPS structures of these strains are shown in Fig. 1C [3, 9].) Organisms were grown at 36°C for 18 h under a moist atmosphere containing 5% CO_2 on gonococcal clear typing medium as described previously by James and Swanson (6).

^{32}P-intrinsic labeling of strain S218. Organisms were inoculated into 250 ml of gonococcal HEPES broth, described previously by Mayer and coworkers (11) (Proteose Peptone no. 3 [Difco Laboratories, Detroit, Mich.], 15 g; soluble starch [BBL Microbiology Systems, Cockeysville, Md.], 0.5 g; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES] sodium salt [Sigma Chemical Co., St. Louis, Mo.], 2.15 g; HEPES acid, 5.0 g; NaCl, 5.0 g; 1% IsoVitaleX [BBL]; and 0.5% sodium bicarbonate [per liter of distilled water]). The peptone solution was dephosphorylated by the addition of $MgCl_2$ at pH 10, the resulting precipitate was removed by centrifugation, the pH was readjusted to 7.2, and 100 μCi of ^{32}P (orthophosphoric acid, carrier free; New England Nuclear Corp., Boston, Mass.) was added. Cultures were incubated in a shaking water bath (37°C). ^{32}P-labeled organisms were harvested after 5 h and washed twice. Organisms were solubilized in SDS as described below.

WC lysates and proteinase K digestion. Organisms grown on solid medium were harvested with a sterile Dacron swab and suspended in 10 ml of cold Dulbecco

phosphate-buffered saline, pH 7.2 (2), to a turbidity of 200 Klett units (blue filter, Klett-Summerson colorimeter). A portion (1.5 ml) of this suspension was centrifuged for 1.5 min (Microfuge B; Beckman Instruments, Inc., Fullerton, Calif.). The pellets were solubilized in 50 μl of lysing buffer containing 2% SDS (BDH, Poole, England), 4% 2-mercaptoethanol, 10% glycerol, 1 M Tris (pH 6.8), and bromphenol blue. Lysates were heated at 100°C for 10 min. For protein digestion, 25 μg of proteinase K (PK) (Boehringer Mannheim GmBH, West Germany) solubilized in 10 μl of lysing buffer was added to each boiled lysate and incubated at 60°C for 60 min. Lysing buffer without PK (10 μl) was added to paired controls before incubation at 60°C for 60 min. PK (25 μg) was also added to WC lysates in the sample well during electrophoresis (at 0, 30, 60, and 120 min). In this experiment (see Fig. 3), PK migrated either concurrently or behind the WC lysates; proteolysis occurred during electrophoresis.

LPSs. LPS from *Salmonella typhimurium* LT_2 (smooth [S]), *S. typhimurium* SF1512 (semirough [SR]), and *S. typhimurium* G30/C21 (deep rough chemotype Re) and LPS from *S. minnesota* R60 (chemotype Ra), R345 (chemotype Rb_2), R5 (chemotype RcP^-), R4 (chemotype Rd_2), and R595 (chemotype Re) were purified by either the Westphal and Jann procedure (18) or the Galanos et al. procedure (4) and were the kind gift of C. McLaughlin (Laboratory of Microbial Structure and Function). LPS from strains S218 (S) and R3 (Rd_2) was purified by the Westphal or the Galanos procedure in our laboratory.

SDS-PAGE. Preparations were subjected to SDS-PAGE with the Laemmli buffer system (8). The 4% stacking gel and the 12.5% separating gel did not contain SDS (Fig. 1B has a 15% separating gel). Electrophoresis was done at 35 mA of constant current with Tris-glycine (pH 8.3) plus 0.1% SDS buffer for approximately 2.5 h. Low-molecular-weight markers (Bio-Rad Laboratories, Richmond, Calif.) were used in several gels; the protein standards included phosphorylase (94 kilodaltons [Kd]), bovine serum albumin (68 Kd), ovalbumin (43 Kd), carbonic anhydrase (30 Kd), soybean trypsin inhibitor (21 Kd), and lysozyme (14.3 Kd). Several gels were counterstained with 0.2% (wt/vol) Coomassie brilliant blue (CBB) (R250; Fisher Scientific Co., Fairlawn, N.J.) in 25% (vol/vol) isopropanol–7% (vol/vol) acetic acid.

Silver stain. The sensitivity of the silver stain was sufficiently high so as to require careful cleaning of glass electrophoresis plates and glass staining dishes. These were washed with detergent and warm water and rinsed thoroughly, first with tap water and then with distilled water. A clean glass dish was used for each step. Plastic gloves, also rinsed with distilled water, were worn to handle the glass and the gels. In all steps, gels were agitated on an orbital shaker. Chemicals used were technical grade reagents and may be purchased from any scientific supply house.

Ag-LPS staining procedure. The following procedure was used for Ag-LPS staining: (i) overnight fixation in 200 ml of 25% (vol/vol) isopropanol in 7% (vol/vol) acetic acid; (ii) 5-min oxidation in 150 ml of distilled water with 1.05 g of periodic acid and 4 ml of 25% (vol/vol) isopropanol in 7% (vol/vol) acetic acid (solution made up just before use); (iii) eight 30-min washes, each time with 200 ml of distilled water; (iv) 10-min silver staining in a solution consisting of 0.1 N NaOH (28 ml), concentrated (29.4%) ammonium hydroxide (1 ml), 20% (wt/vol) silver nitrate (5 ml), and distilled water (115 ml) (make up solution just before use and stir constantly while making); (v) four 10-min washes, each time with 200 ml of distilled water; (vi) 10 to 20 min of developing in 250 ml of developer solution (citric acid [50 mg], 37% formaldehyde [0.5 ml], distilled water [amount sufficient to make 1 liter of solution]; made up just before use) at an optimal temperature of 25°C (if solution cools, staining of protein as well as LPS will occur); (vii) 1 h in a stop bath (200 ml of distilled water plus 10 ml of 7% [vol/vol] acetic acid); (viii) final wash with 200 ml of distilled water and then storage (gel may be stored in a zip-lock plastic bag with a small amount of water to prevent desiccation).

Use of concentrated (29.4%) ammonium hydroxide is essential for the preferential staining of LPS. To maintain the quality of this reagent, we place small amounts of ammonium hydroxide from a freshly opened bottle into small (25- to 50-ml) bottles with caps that can be tightly sealed. Loss of "strength" of the reagent results in persistence of the brown precipitate in the staining solution. Addition of more ammonium hydroxide will dissolve the precipitate; however, preferential staining of LPS will not result.

Ag-LPS and protein staining procedure. The Ag-LPS and protein staining procedure is identical to the Ag-LPS staining procedure, except for the following. In step ii (5-min oxidation), 10 ml of 40% (vol/vol) ethanol in 5% (vol/vol) acetic acid was added to the water-periodic acid solution. In step iv (silver staining), 3 ml of ammonium hydroxide was used (concentrated ammonium hydroxide was diluted with distilled water to 75% [vol/vol] of the original concentration [final concentration, 22%]).

RESULTS

The silver-stained profiles of purified salmonella LPSs in 12.5 and 15% SDS-polyacrylamide gels are shown in Fig. 1A and B, respectively (Fig. 1C is a schematic representation of the molecular structure of each LPS chemotype adapted from the reviews of Galanos and Luderitz [3, 9]). The S chemotype of *S. typhimurium* [S(st)] is comprised of approximately 40 regular doublets; the upper bands of the doublets are grey (G) and the lower bands are red (R). Although the LPS doublet ladder is distributed throughout the lane, several pairs are particularly prominent. The SR LPS of *S. typhimurium* [SR(st)] has three prominent doublets (also grey and red). Profiles of the Ra and Rb_2 chemotypes also have a prominent grey and red doublet, whereas the doublet is not seen in the rougher chemotypes (RcP^-, Rd_2, or either Re). The most distinguishing feature of the LPS patterns is the relative migration of the leading band; the core mutant chemotypes differ in that the mobility of the farthest migrating band increases as the core oligosaccharide shortens [Ra < Rb_2 < RcP^- < Rd_2; in the 12.5% gel, the Rd_2 and Re chemotypes of *S. minnesota* and *S. typhimurium* have

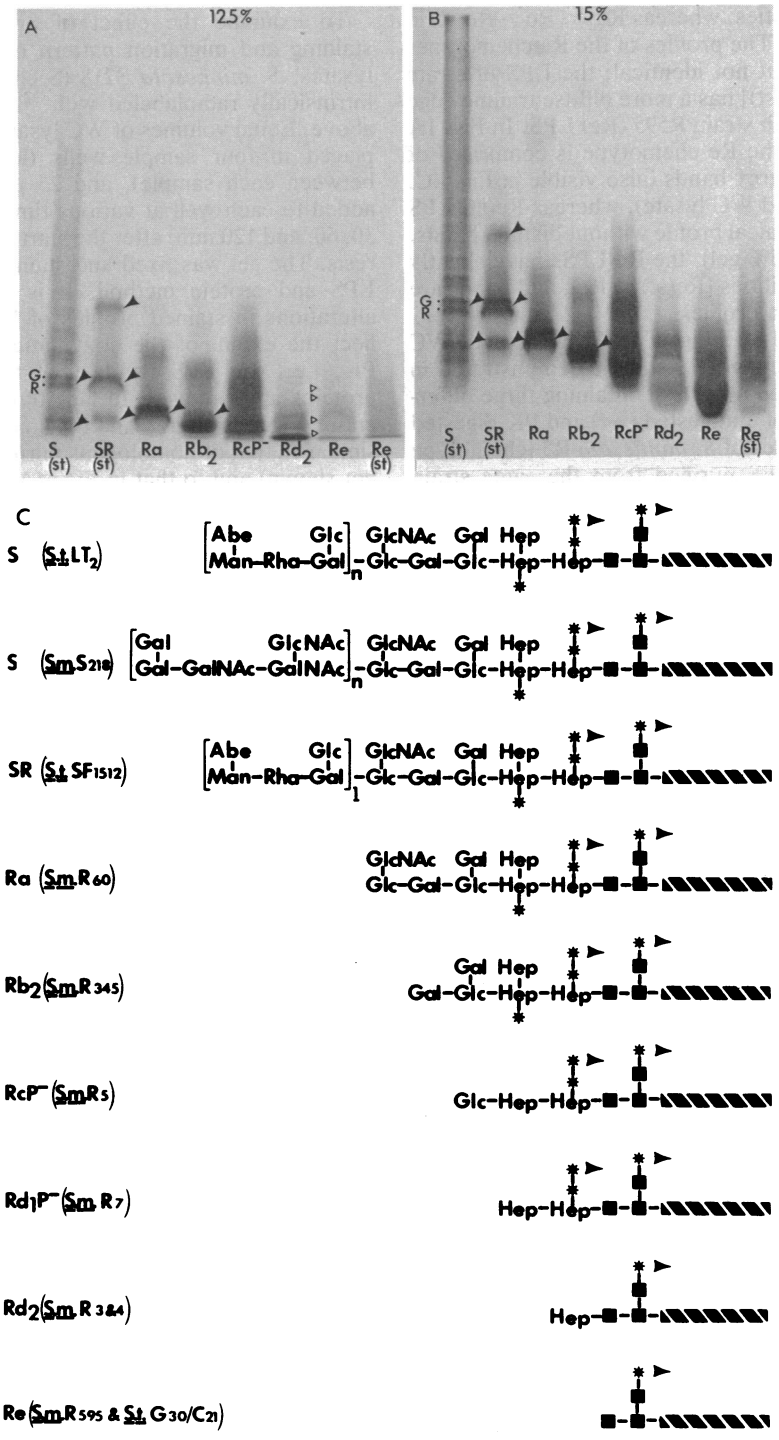


FIG. 1. Unique migration patterns of 10 μg of purified LPS in 12.5% (A) and 15% (B) polyacrylamide gels in silver-stained (Ag-LPS and protein) gels. The LPSs were extracted from the following strains (chemotypes): *S. typhimurium* (*S.t.*) LT_2 [S(st)], S1512 [SR(st)]; and G30/C21 [Re(st)]; *S. minnesota* (*S.m.*) R60 (Ra), R345 (Rb_2), R5 (RcP^-), R4 (Rd_2), and R595 (Re). The arrowhead (▲) indicates prominent grey (G) and red (R) doublets. The triangles (▷) are adjacent to four bands in the Re LPS (these bands stain yellow and are consequently difficult to photograph). (C) Schematic representation of the structures of the chemotypes shown in A and B (adapted from the reviews of Galanos and Luderitz [3, 9]). In addition, chemotypes S from *S. minnesota* S218 and Rd_1P^- from

similar mobilities, whereas Rd_2 < Re > Re(st) in the 15% gel]. The profiles of the Re chemotypes are similar but not identical; the LPS of strain G30/C21 [Re(st)] has a more diffuse trailing edge compared with strain R595 (Re) LPS. In Fig. 1A (12.5% gel), the Re chemotype is comprised of four distinct grey bands (also visible in Fig. 4C, the PK-treated WC lysate), whereas Re(st) LPS has a solid conical profile without distinct bands. In Fig. 1B (15% gel), the Re LPSs have slightly different mobilities [Re(st) < Re], and bands are visible in both profiles.

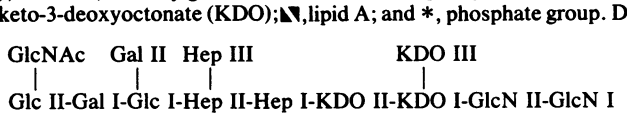
Preferential staining of LPS in bacterial WC lysates by the Ag-LPS stain is demonstrated in Fig. 2A. Paired samples containing three different volumes of undigested (wc) and PK-digested (pk) WC lysates of *S. minnesota* R5 (chemotype RcP^-) and LPS purified from the same strain were electrophoresed in a 12.5% polyacrylamide gel and stained as described above. The protein bands of the molecular weight markers and of the WC lysates are unstained except for a 32-Kd band and a 17-Kd band (apparent molecular weight) in the latter. The LPS of the WC lysate is visible as a sharply defined area comprised of narrow, well-defined bands compared with the diffuse broad bands of the PK-digested lysate and the purified LPS. The staining profile of 15 μl of PK-digested WC lysate is very similar to the profile of purified R5 LPS, including the diffuse upper edge. In both PK-digested material and purified LPS, additional upper bands are detected with increased amounts of material; with 20 μg of purified LPS, an additional band is visible. The leading edge of the purified LPS migrates farther than that of the WC or PK lysates; with smaller amounts of purified LPS (2 μg), the discrepancy in mobility (WC and PK versus LPS) is decreased (data not shown). The protein constituents of the undigested WC lysates are displayed by counterstaining with CBB (Fig. 2B). Several CBB-stained bands are present in the area adjacent to the silver-stained LPS in WC lysates. (Protein bands adjacent to LPS may contribute to the sharply defined staining pattern by compression of the LPS in the WC lysate.)

To examine the effect of proteins on the staining and migration pattern of LPS in WC lysates, *S. minnesota* S218 (S chemotype) was intrinsically radiolabeled with ^{32}P as described above. Equal volumes of WC lysate (10 μl) were placed in four sample wells (leaving a lane between each sample), and 25 μg of PK was added to each well at various time intervals (0, 30, 60, and 120 min) after the start of electrophoresis. The gel was fixed and stained by the Ag-LPS and protein method. It is assumed that alterations in stained profiles of WC lysate reflect the extent of the enzymatic digestion by PK, i.e., the LPS bands are stainable when proteins are removed. In lane 0′ (Fig. 3A), the entire lysate is altered and the staining profile is similar to that of homologous purified LPS (data not shown) and to that in Fig. 4A and 4C when PK digestion was completed before electrophoresis. The arrowheads indicate the demarcations between altered and unaltered staining pattern in the 30′, 60′, and 120′ lanes (the bands in the interceding lanes are artifacts; this material is not radioemitting [Fig. 3B]). Note the prominent dark band (*) which is stained in the time zero lysate but is not visible in the lanes in which PK alteration of the comigrating protein bands did not occur.

The autoradiogram of the dried gel (Fig. 3A) is shown in Fig. 3B. Several changes are apparent in lane 0′ (in which protein digestion appears to have taken place). Subtle changes are seen in the upper portion of the ladder where ^{32}P-labeled bands are more discrete and well defined compared with the smear of radiolabeled material in the same area in lane 120′. A large radiolabeled band which comigrated with the silver-stained band in Fig. 3A is present, as compared with several smaller bands in the same area in lanes 30′, 60′, and 120′. The lower radiolabeled band is concurrently diminished in size. These ^{32}P-labeled bands are insensitive to RNase digestion and are the same as in homologous ^{32}P-LPS (data not shown).

The stained SDS-PAGE profiles of WC lysates (wc) and PK-digested WC lysates (pk) of seven *S. minnesota* LPS mutants are shown in

S. minnesota R7 are included (WC and PK profiles are shown in Fig. 4). LPSs of S, SR, and Ra chemotypes have complete cores. In addition, S and SR have O side chain polysaccharides (*n* or 1 repeating units). Strains which synthesize LPS chemotypes Rb_2, RcP^-, Rd_1P^-, Rd_2, and Re are defective in one or more enzymes necessary for synthesis of the complete core oligosaccharide. It is thought that the lipid A–2-keto-3-deoxyoctonate region of the LPS is conserved in all chemotypes; however, it should be noted that substituents may not be present in molar quantities. How variations in phosphates or amino groups might affect electrophoretic migration and silver staining is not known. Abbreviations and symbols: Abe, abequose; Gal, galactose; Glc, glucose; GlcN, glucosamine (in lipid A); GlcNAc, *N*-acetylglucosamine (in core); Hep, heptose; Man, mannose; Rha, rhamnose; ►, ethanolamine; ■, 2-keto-3-deoxyoctonate (KDO); ⋀⋁, lipid A; and *, phosphate group. Designation of sugars:



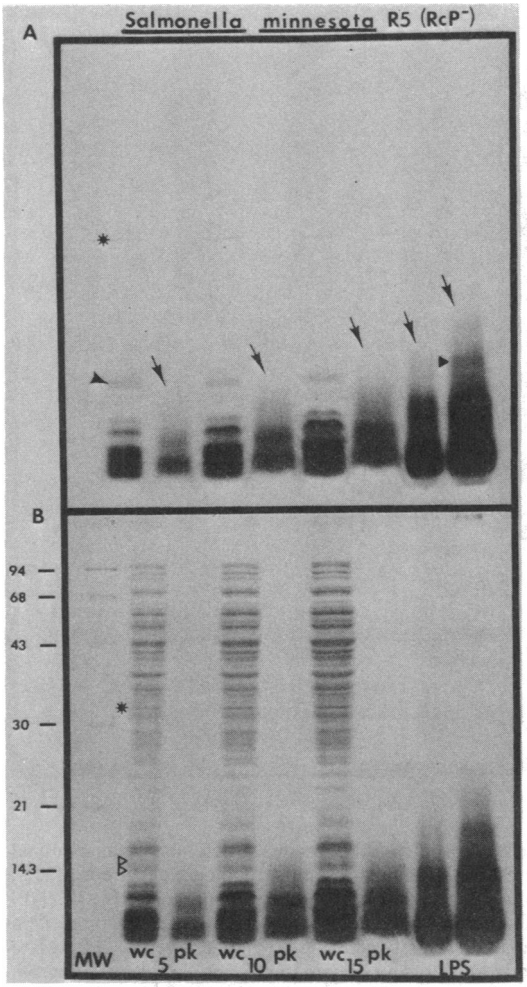


FIG. 2. Preferential staining of LPS with the Ag-LPS stain. Paired samples containing equal volumes (5, 10, or 15 μl) of WC (wc) and PK-digested (pk) lysates of *S. minnesota* R5 (RcP⁻) and 10 and 20 μg of homologous purified LPS were electrophoresed in a 12.5% SDS-polyacrylamide gel. Gel stained by the Ag-LPS method; molecular weight markers (MW; numbers on left) and the proteins of the WC lysate are unstained, except for the 32-Kd band (*) and a lower band of approximately 17Kd (▲). The bands of the purified LPS and the PK lysate have diffuse upper edges (→) compared with those of the WC lysates. Increased amounts of LPS (20 versus 10 μg) have an additional band (▶). (B) Same gel stained with CBB. In the WC lysate, two CBB bands (▷) are present in the area adjacent to the silver-stained LPS.

Fig. 4. In Fig. 4A, the gel was stained by the Ag-LPS method. The LPS staining pattern of each strain is unique; in all strains, more area stained in the PK-digested lysate than in the paired, undigested WC lysate. This is most apparent in the strain S218 profile; only a small portion of the LPS doublet ladder was stainable in the WC preparation, and PK digestion of proteins resulted in the staining of LPS comprised of 35 to 40 grey and red doublets (see Fig. 3A). In the WC lysates, a 32-Kd band stained in most strains as well as a band with a 17-Kd apparent molecular weight.

The CBB-stained gel is shown in Fig. 4B. In this gel, the stained profiles of the seven strains are very similar except for the portion of the lanes (bracket or arrowhead) which correspond to the areas which stain with Ag-LPS stain (Fig. 4A). In each strain, this area stains poorly or not at all with CBB. The 32-Kd protein, however, does stain with CBB.

In Fig. 4C, the same preparations are shown stained by the Ag-LPS and protein method. With this silver stain, the PK-digested lysates stain darker than the same areas in the gel when it is stained by the Ag-LPS method (Fig. 4A). The silver-stained WC lysate profiles of the seven strains are similar to each other except for the lower portions of the lanes corresponding to those areas (bracket or arrowhead) stained by the Ag-LPS method (Fig. 4A). The 32-Kd band is stained darkly by this method, as is the 17-Kd band.

DISCUSSION

Differences in LPS profiles in polyacrylamide gels (Fig. 1A and B) appear to correlate positively with biochemical differences in LPS (Fig. 1C). Biochemical differences among the chemotypes include variations within the core oligosaccharides and the presence (or composition) of O side chains. The staining pattern of LPS in WC lysates also reflects the biochemical composition of the chemotype (Fig. 4A, B, and C). In the WC lysate, LPS comigrates with proteins and to some extent is affected by the proteins (Fig. 2A and B and Fig. 3A and B).

The silver-stained profile of *S. typhimurium* S LPS (Fig. 1A and B) is consistent with the findings of previous studies done by Jann and co-workers (7) (using Procion red-dyed LPS), by Goldman and Leive (5), by Palva and Makela (13), and by Munford and co-workers (12) (using radiolabeled LPS) in an SDS-PAGE system. The contribution of the composition of the sugars of the O side chains to LPS electrophoretic mobility is probably reflected in the differences in the LPS profiles of *S. typhimurium* S LPS (Fig. 1) and *S. minnesota* S LPS (Fig. 3A and B and Fig. 4A and C); these S LPSs are similar except for the sugars which comprise the O side chains (*S. typhimurium*, galactose, rhamnose, mannose, abequose, and glucose; *S. minnesota*, galactose, *N*-acetylglucosamine, and *N*-acetylgalactosamine [10]).

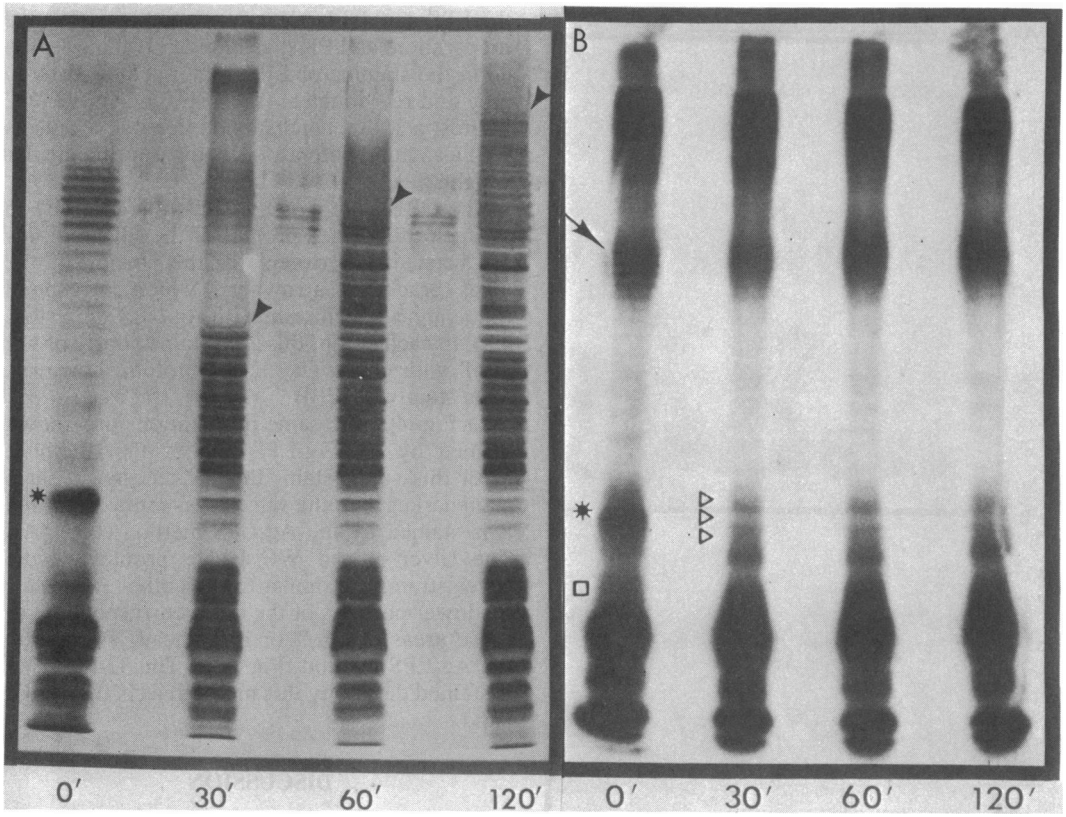


FIG. 3. (A) Effect of PK digestion on the silver-stained gel profile of WC lysates. Each lane contains equal volumes of SDS-solubilized WC lysates of *S. minnesota* S218 (the bands in the interceding lanes are artifacts; only SDS lysing buffer was added to these lanes). PK (25 μg) was added 0, 30, 60, or 120 min (lanes 0', 30', 60', and 120', respectively) after the start of electrophoresis. The gel was fixed and stained by the Ag-LPS and protein method. The arrowheads (➤) indicate the lines of demarcation between altered and unaltered WC lysate (compared with S218 of Fig. 4C). The prominent dark band (*) is present only in the 0' lane. (B) Autoradiogram of the same gel. ^{32}P radioemitting bands which correspond to the silver-stained ladder (→) are well defined compared with the smears in the 30', 60', and 120' lanes. A large radiolabeled band (which corresponds to the silver-stained band [*] in A) is present, compared with several smaller bands (▷) in lanes 30', 60', and 120' in the same area. A concurrent loss of radiolabeled material is seen in the large band below (□).

The nature of the LPS-silver ligands which results in the grey- and red-staining doublets is obscure. This staining pattern is present in S, SR, Ra, and Rb_2 LPSs but is absent in the rougher chemotypes (RcP^- to Re). This suggests that some constituents common to the sugars in the outer core may contribute to this silver-staining pattern. As can be deduced from Fig. 1C, the core sugars *N*-acetylglucosamine, glucose II, galactose I and II, and heptose III as well as the phosphate group on heptose II are deleted in the RcP^-, Rd_1P^-, Rd_2, and Re chemotypes. Goldman and Leive (5), using *S. typhimurium* LT_2, showed that each component of the doublets contained phosphorus (^{33}P) and galactose (^3H). Goldman and Leive also noted a shift of radiolabel from the lower band to the upper band in LPS which had been stored (−20°C) or in LPS extracted from outer membranes which had been stored at −80°C for 3 months. We did not observe a shift of grey and red staining in gels of LPSs which had been stored (4°C or −20°C). In a study of the S LPS of *Serratia marcescens* done in collaboration with M. Wilson and D. Morrison (manuscript in preparation), we found the ratio of grey and red staining of doublets varied depending upon the fatty acid constituents in lipid A. For the present, the biochemical basis of the grey-red staining pattern remains obscure.

The most consistent difference in the LPS patterns of the core-defective mutants (Ra to Re) is the relative mobility of the fastest migrating band. The mobility increases in positive correla-

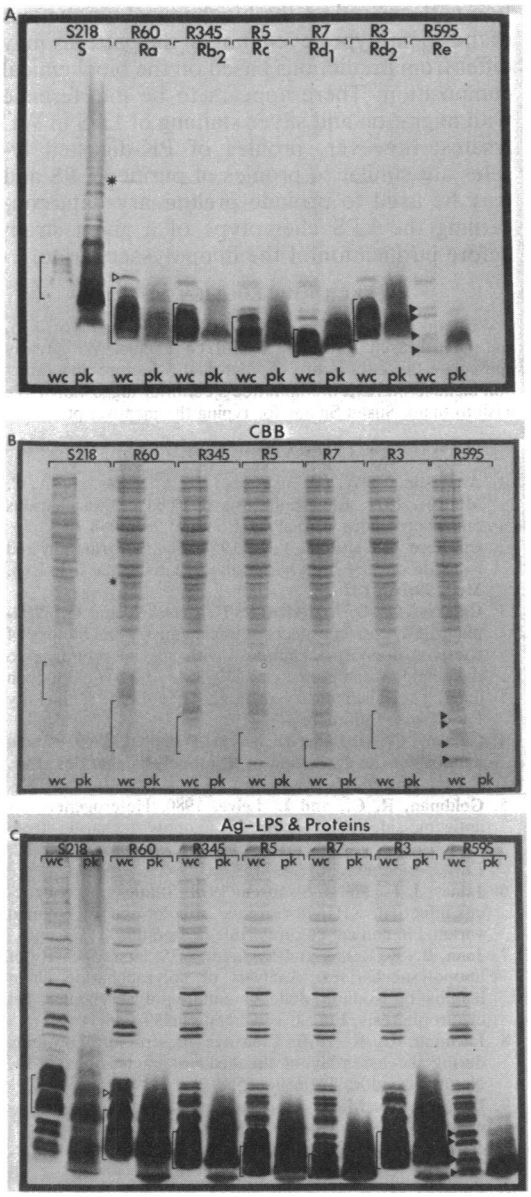


FIG. 4. WC lysates (wc) and PK-digested lysates (pk) of seven *S. minnesota* LPS mutants are displayed in 12.5% SDS-polyacrylamide gels. The gels were stained by the Ag-LPS method (A), with CBB (B), and by the Ag-LPS and protein method (C). In each gel, brackets ([) or arrowheads (▶) are used to identify the portion of the WC lysate profile which stains by the Ag-LPS method. Two bands (32 and 17 kd) stain by the Ag-LPS method in the WC but not in the PK preparation (*, ▷).

tion with the shortening of the oligosaccharide core, and although the absolute migration patterns (relative mobility) are affected by electrophoretic conditions (12.5 versus 15% gels), LPS chemotypes have unique migration patterns relative to one another in SDS-polyacrylamide gels (Fig. 1A and B).

Interestingly, the SR LPS has a doublet with the same mobility as the Ra chemotype (Fig. 1); this doublet suggests the presence of a heterogeneous LPS population including Ra-like molecules. The doublet above the Ra-like one (most prominent) may represent molecules with complete core lipid A and one O side chain (SR). It would be expected, on the basis of the observations and conclusions of other workers (5, 7, 12, 13, 17), that the SR profile would be identical to the S ladder except much abbreviated (since the number of O side chains [*n*] equals 1 in the SR chemotype). Instead, the S LPS profile has faster-migrating material than the SR LPS; in addition, there are four doublets in the S ladder which are absent in the SR profile between upper and middle doublets, and the upper doublet does not correspond to a doublet in the S LPS of strain LT_2.

Of interest is the fact that the Re chemotype profiles of strains R595 (Re) and G30/C21 [Re(st)] are not identical. This was unexpected; even though these LPs are from two different species, the lipid A–2-keto-3-deoxyoctonate constituents are believed to be identical (3, 9). However, the R595 and G30/C21 Re LPSs can also be differentiated by thin-layer chromatography (C. McLaughlin, personal communication).

The silver-stained profiles of purified LPSs and the profiles of PK-digested WC lysates are similar with either staining method (Fig. 1, Fig. 2, and Fig. 4A and C). However, the silver-stained profiles of the WC lysate vary markedly depending upon which staining method is used. With the Ag-LPS method, only those portions of the LPS in the WC lysate which do not comigrate with proteins (see Fig. 4A and B) are stained. The area bracketed in the gel shown in Fig. 4A stains well with Ag-LPS method and corresponds to a CBB-refractory area in the gel shown in Fig. 4B. (This is most apparent for strain S218.) The possibility that proteins may interfere with the silver staining (and migration) of LPS is supported by analysis of the gels in Fig. 3A and B in which PK digestion of proteins occurred in the gel and resulted in various degrees of alteration of the lysate profile. In Fig. 3A, the silver-stained pattern (at time zero) is quite similar to that in Fig. 4A (for PK-digested material from strain S218), in which proteolysis occurred before electrophoresis. The autoradiogram of this gel (Fig. 3B) demonstrates the alteration in LPS migration pattern after proteolysis (these ^{32}P-labeled radioemitting bands are resistant to RNase digestion); the coelectrophoresis of proteins with LPS seems to influence the migration characteristics of the LPS. Compres-

sion of LPS by proteins may partially explain the well-defined banding of LPS seen in WC lysate compared with the diffuse upper edges of the PK-digested lysate and purified LPS (Fig. 2A).

In each of the R mutants, the relative mobilities of the fastest migrating LPS bands in WC and PK lysates are similar to each other (Fig. 4A) and are similar, albeit not identical, to those of purified LPS (Fig. 1A and B and Fig. 2A). A notable exception is the LPS of the Rd_2 chemotype. From the migration pattern of purified Rd_2 LPS (Fig. 1A and B) (extracted from strain R4), one would predict a faster migrating LPS in the WC and PK profiles of the mutant strain R3 (Rd_2 chemotype) (Fig. 4A). The discrepancy in the observed versus predicted mobility prompted us to purify the LPS from R3. The mobility of the purified LPS, the WC LPS, and the PK LPS of R3 were similar; the silver-stained profiles of R3 and R4 LPSs were not identical (data not shown). There are several possible explanations for the differences between the silver-stained LPS profiles of R3 and R4, both supposedly having the Rd_2 chemotype: (i) the strain (R3) is incorrectly labeled; (ii) R3 is a leaky mutant; and (iii) there is molecular microheterogeneity between the Rd_2 LPSs of R3 and R4. Experiments are under way to clarify this apparent discrepancy; however, the important conclusion and the reason we opted to include these data is that the LPSs of the WC, PK, and purified preparations in a given strain are similar enough to predict the migration characteristics of one based on the other.

The frequent staining of the 32- and 17-Kd bands with silver by the Ag-LPS method, best seen in Fig. 4A, may be due to LPS which comigrates with the protein, or it may be due to the marked affinity of this band for silver ions as evidenced by the dark staining with the Ag-LPS and protein method (Fig. 4C). The exact chemistry of the silver stain is unknown; however, if the chemistry of silver in the photographic process is applicable, preferential staining of LPS is most likely a reflection of the high-affinity ligands (aldehyde and pre-aldehyde groups) of periodate-oxidized LPS for silver compared with proteins rather than any specific interactions of silver with LPS. Hence, any ligands with high constants of association (K_a) for silver ions would also tend to stain. The Ag-LPS method depends upon limiting the number of silver ions in solution (with high concentrations of ammonium hydroxide) and thereby complexing silver to the most efficient ligands (LPS) in preference to weaker ones (protein) (14).

In summary, the profiles of LPS chemotypes in silver-stained polyacrylamide gels appear to be unique and reproducible. It seems likely, therefore, that the unique electrophoretic migration patterns reflect the biochemical uniqueness of the chemotypes even though the patterns may differ from predictions based on the biochemical composition. There appears to be interference with migration and silver staining of LPS in WC lysates; however, profiles of PK-digested lysates are similar to profiles of purified LPS and may be used to provide preliminary data concerning the LPS chemotype of a given strain before purification of the lipopolysaccharide.

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