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Characterization of Lipopolysaccharide Heterogeneity in *Salmonella enteritidis* by an Improved Gel Electrophoresis Method

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***Salmonella enteritidis* field isolates of different phage types and pathogenicities were assessed for changes in lipopolysaccharide (LPS) structure, using an improved method of polyacrylamide gel electrophoresis (PAGE) that revealed the same degree of structural detail as mass spectroscopy. The method allowed characterization of an LPS chemotype that may be associated, regardless of phage type, with increased virulence of *S. enteritidis*. The virulent variant SE6-E21, which efficiently contaminates eggs and yields high numbers of organisms from chick spleens, had an O-antigen/core ratio of 2.8, as determined from gels by densitometry, and 1.67 μg of mannose per μg of 2-keto-3-deoxy-octulosonic acid (KDO), while the avirulent variant SE6-E5 had O-antigen/core ratios of 1.2 and 1.00. The association between O antigen and virulence was also seen on analysis of five new field isolates. One of the new field isolates generated a mixed population of smooth and semismooth variants in agreement with its mixed virulence in chicks. When LPS was purified from large-volume cultures, only the most virulent isolate yielded high amounts of O antigen (1.6 μg of mannose per μg of KDO), while the other isolates had ratios characteristic of semismooth variants (≤1.0 μg of mannose per μg of KDO), including the isolate of mixed virulence. These results indicate that the improved PAGE method might provide a rapid, sensitive, in vitro assessment of field isolate virulence prior to the performance of definitive infectivity trials.**

Salmonella enteritidis is a persistent human pathogen contracted primarily through consumption of contaminated eggs (16, 24). Because current techniques for classifying *S. enteritidis* isolates have not resulted in a reliable method for correlating virulence and egg contamination, progress in defining the molecular events leading to outbreaks in people has been slow. One investigation of *S. enteritidis* virulence showed that variants isolated from a single parent culture had different abilities to contaminate eggs and to be recovered from the spleens of chicks, and these variants were determined to have different amounts of lipopolysaccharide (LPS) O antigen linked to core (18). Since a central factor in determining the virulence of gram-negative organisms is the structure of LPS (1, 8, 14, 19, 20), we investigated whether a simple method to detect quantitative changes in O-antigen/core linkage ratios could be devised. Since many investigators use polyacrylamide gel electrophoresis (PAGE) analysis to study the LPS characteristics of gram-negative bacteria by interpreting differences in the migration patterns of discrete sugar moieties (3, 4, 6, 11–13, 17, 25), we investigated whether PAGE techniques would show differences in LPS structure as isolated from a group of isolates at the time infectivity trials were performed. Results show that if improvements to existing techniques are made, LPS PAGE detects virulent isolates of *S. enteritidis* that are characterized by the production of increased amounts of O antigen relative to core.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and virulence assay. A previously described field isolate of *S. enterica* serovar enteritidis (*S. enteritidis*) (SE6) was used as the source for the different chemotypes shown in Fig. 4 and 5 and in infectivity trials (Table 1) (18). Virulence characteristics for three variants obtained from SE6 were as follows for equivalent infective doses: SE6-E21 yielded 10^6 CFU from spleens of chicks infected intraperitoneally at 5 days of age and collected at 8 days of age (CFU per spleen) and resulted in 42% contaminated eggs in intravenously infected hens; SE6-E5 yielded 10^4 CFU per spleen and less than 1% contaminated eggs; SE6-R yielded 10^3 CFU per spleen and less than 1% contaminated eggs. Cultures were grown at 42°C for 16 h on brilliant green agar medium (BBL) to obtain isolated colonies from cultures stored in deep stabbed nutrient agar. Single colonies were used to inoculate 10 ml of brain heart infusion (BHI) broth, and cultures were grown for 16 h at 42°C in tubes (20 by 150 mm). SE6-E5 was found to have an optical density and cell concentration yield similar to those of the parent isolate SE6, as determined by growth curve analysis. Comparisons of SE6-E21, SE6-E5, and SE6-R optical density and aggregation properties were performed by inoculating 1,000 cells into 1 ml of BHI broth in cuvettes. Readings were obtained from unmixed and mixed cells every 20 min. Incubation was at 42°C, and trials were performed in triplicate for each isolate from isolated colonies. Additionally, log-phase and early stationary growth phase, washed cells were assayed for total protein (milligrams per milliliter) (Bio-Rad DC protein assay). Bovine serum albumin was used to establish limits for regression analysis of protein concentrations, and samples with optical densities of greater than 0.5 (2.5×10^8 CFU) were diluted twofold to maintain readings within standard limits.

Five field isolates (SE5, SE8, SE9, SE15, and SE23) (Table 1), which were examined at the fourth passage after isolation, plus the characterized semismooth variant SE6 (passage 7) were compared for virulence attributes, using a quantitative chick spleen assay as previously described (18). Specifically, six groups of nine or ten chicks each were housed in separate Horsfall isolator cages, and each group was infected by the intraperitoneal route at an average dose of 10^6 organisms per chick. An uninfected chick was included in each cage as a control to show that contact infections occurred with these isolates. Data are expressed as number of CFU at a 10^2 dilution of spleen contents.

Isolation of LPS for PAGE and gas chromatographic analyses. LPS was extracted from the five field isolates and parent isolate SE6 at the time of infectivity assays. LPS was isolated by a procedure that combined aspects of several established techniques (2, 5, 10, 26). Specifically, 5×10^9 cells were suspended in 200 μl of TAE buffer (40 mM Tris acetate [pH 8.5], 2 mM EDTA) and mixed with 400 μl of lysis buffer (100 mM sodium dodecyl sulfate [SDS], 50 mM Trizma base, 0.128 M NaOH). Phenol-chloroform, 600 μl, was added to the cellular suspension, and the mixture was vortexed vigorously for 1 min and then heated at 65°C for 15 min. Phenol-chloroform stocks were prepared by mixing 500 g of phenol (Gibco/BRL, New York, N.Y.) with 100 ml of 1 M Tris (pH 8.0), 12.5 μl of *m*-cresol, 0.5 ml of β-mercaptoethanol, and 0.25 g of 8-OH-quinolone. To this solution, an equal volume of chloroform-isoamyl alcohol (24:1, vol/vol) was

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TABLE 1. Sources of *S. enteritidis* isolates

Isolate	Supplier	Source	Supplier's accession no.	Yr isolated
SE5	C. E. Benson, University of Pennsylvania	Ovary	81332B	1988
SE6	C. E. Benson, University of Pennsylvania	Egg	19299-52-1	1988
SE8	C. E. Benson, University of Pennsylvania	Spleen	575	1988
SE9	H. M. Opitz, University of Maine	Ovary	Me #18	1989
SE15	H. M. Opitz, University of Maine	Not known	ME #1	1989
SE23	J. Salisbury, Maryland Department of Agriculture	Farm isolate	51-0072-2	1990
SE6-E21	J. G. Petter, USDA,[a] Athens, Ga.	Egg	4.22.B7	1991
SE6-E5	J. G. Petter, USDA,[a] Athens, Ga.	Egg	4.20.B7	1991
SE6-R	J. G. Petter, USDA,[a] Athens, Ga.	Spleen	4.15.B7	1991
SE6-Rc	J. G. Petter, USDA,[a] Athens, Ga.	Aged culture	B.A3	1992

[a] USDA, U.S. Department of Agriculture.

added, and the stock solution was stored at 4°C for no longer than 1 month. After being heated, samples were centrifuged in an SS34 rotor for 15 min at 4°C. The supernatant was precipitated with 200 μl of H_2O, 50 μl of 3 M NaOAc (pH 5.2), and 1.0 ml of ice-cold 100% ethanol. Samples were resuspended and incubated first with DNase and RNase (Boehringer Mannheim, Indianapolis, Ind.) and then with proteinase K (Amresco, Solon, Ohio) (22). After another phenol-chloroform extraction without heating, samples were precipitated by mixing pelleted material with 200 μl of a solution containing 50 mM Tris HCl (pH 8.0) and 100 mM sodium acetate, followed by the addition of 2 volumes of ethanol. The yield of LPS from approximately 5.0×10^9 cells was 500 μg. The final precipitate was dissolved in 50 μl of H_2O. Generally, 2 μl (approximately 20 μg) was enough for band detection depending on the conditions discussed herein. LPS from *S. enterica* serovar Minnesota (*S. minnesota*) was purchased as phenol extracts (Sigma).

For quantitative analysis of O antigen, approximately 1 mg of LPS was extracted from three different cultures of variants SE6-E5 and SE6-E21 grown in 1 liter of BHI at 42°C for 16 h. After delipidation, LPS was analyzed for neutral sugars by alditol acid derivatization and gas chromatography as previously described (18), with mannose, glucose, and galactose included as internal controls. Colorimetric analysis of the core component 2-keto-3-deoxyoctulosonic acid (KDO) was performed by using standard techniques (27). Quantitative analysis of O-antigen/core ratios was similarly determined for the five field isolates and parent isolate SE6. In a separate experiment, LPS was extracted from 5 liters of SE6-E21, SE6-E5, and SE6-R. After enzymatic treatment with RNase and proteinase K, LPS samples from SE6-E21 and SE6-E5 were dialyzed, lyophilized, and then suspended in distilled H_2O to concentrations of 5 mg/ml for determination of optical densities. SE6-R LPS could not be analyzed because it formed an insoluble aggregate under these conditions.

PAGE of LPS. PAGE was performed with one of two buffer systems containing either urea or deoxycholate (DOC) as denaturing agent (12, 25). Gels were 1.0 mm thick, 15 by 15 cm, and 12.5% acrylamide and were prepared according to Tsai and Frasch except that urea gels were poured with a 4% stack and both stack and resolving gels were brought to 4 M urea by the addition of crystalline urea to solutions which were degassed for 20 min before pouring (25). Running buffer consisted of 0.025 M Tris, 0.192 M glycine, and 0.1% SDS. For DOC gels, a 5% stack was used. No degassing was necessary, but DOC gels were prerun. Running buffer was composed of the following: sodium DOC, 2.5 g/liter; glycine, 14.4 g/liter; and Tris, 3.0 g/liter. LPS samples were boiled in buffers containing loading dye for 3 min immediately prior to loading (7). After the run, the gels were placed in 250 ml of fixing solution (40% ethanol, 5% acetic acid) overnight.

Staining methods. The gels were visualized as described by Tsai and Frasch (25), with modifications according to Krauss et al. (13) or with modifications with a commercially available silver stain kit (Bio-Rad) as presented in Results. The conditions used were as follows.

(i) Method I (Tsai and Frasch). Oxidation was performed by placing gels for 5 min in 1.4 g of periodic acid plus 200 ml of fixing solution. Gels were then washed three times every 15 min with 400 ml of H_2O. Staining was achieved by immersing gels for 10 min in a solution containing 2.0 ml of concentrated NH_4OH, 28.0 ml of 0.1 N NaOH, 5.0 ml of 20% $AgNO_3$, and 115.0 ml of H_2O for 10 min. The 20% $AgNO_3$ was added dropwise to the rest of the solution while the solution was stirred to prevent stabilization of a brown precipitate. NH_4OH was kept tightly sealed in aliquots at 4°C. DOC gels were washed three times in H_2O as before, while urea gels were washed six times in H_2O. Gels were developed in a solution containing 12.5 mg of citric acid, 125 μl of 37% formaldehyde, and 250 ml of H_2O. Developing was stopped by rinsing the gel in water.

(ii) Method II (Krauss modification). Oxidation and staining were performed as in method I. Developing was done in a solution 10 times more concentrated than that used in method I.

(iii) Method III (present modification). Oxidation and staining were performed as in method I. Developing was performed with a commercially available kit prepared according to the manufacturer's directions.

Densitometric analysis of gels. A scanning densitometer (Hoefer model GS300) was used in the transmitting mode to obtain densitometric data. Data were compiled and analyzed by using the integration parameters available with GS365W software (Hoefer).

MS analysis of LPS by electrospray propulsion. Briefly, a putative Rc chemotype variant of parent isolate SE6 was grown in 50 ml of BHI broth for 48 h at 37°C, which yielded approximately 100 mg (wet weight) of cells when collected by centrifugation (SS34 rotor; $10{,}000 \times g$ for 10 min). LPS was extracted as described above, and lipid A was hydrolyzed by heating at 100°C in 1% acetic acid for 90 min (21). Lipid A precipitate was removed by centrifugation, and the supernatant was dried by evaporation. Inositol (20 μg) was added as an internal standard, and the sample was dried and prepared for mass spectrometric (MS) electrospray propulsion by Hakomori methylation (27). Briefly, 0.5 ml of dry dimethyl sulfoxide was added to a dry sample and stirred until dissolved, followed by the addition of 0.5 ml of 2 M potassium dimethylsulphynil anion with a dry syringe under nitrogen. Sample was stirred for 2 h and then cooled in an ice bath until frozen, and 1.0 ml of iodomethane was added slowly. After the sample was stirred for 16 h at room temperature, 0.5 ml of water was added, and the sample was vortexed gently and then evaporated under N_2. Sep-Pak C_{18} reverse-phase cartridges were preconditioned with 8 ml of acetonitrile and 8 ml of water, and the methylated sample was added slowly to the top of the cartridge and washed with 8 ml of water. The sample was eluted from the cartridge with 2 to 3 ml of acetonitrile and dried. For electrospray propulsion, the sample was dissolved in 50% acetonitrile–0.1% formic acid and coated onto the sample platform. The flow rate was set at 2 μl/min as delivered by a Harvard infusion pump. The API Biomolecular Mass Analyzer (PE Science, Thornhill, Calif.) was set in the positive ion mode, and ion spray voltage was 5,000 V with an orifice potential setting of 35 V.

Immunoblot analysis of LPS. After LPS was extracted from the six field isolates shown in Fig. 7, twin urea-PAGE gels were prepared. One gel was stained and developed according to method III, while the other gel was transferred to a nitrocellulose membrane, using a semidry apparatus according to directions (Hoefer). Blocking solution, antibodies, and washing solutions were prepared and used according to standard techniques (18, 22). Primary antibody was rabbit anti-group D1 (factors 1, 9, and 12; Difco), and secondary antibody was alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (Pierce).

RESULTS

Improving detection of LPS components. When *S. minnesota* LPS was separated through DOC gels and stained according to the three methods given above, the best results were obtained with the Tsai and Frasch method with developing conditions as described in method III (Fig. 1). This represented a fivefold increase in sensitivity over method II. DOC gel conditions were best at visualizing core LPS versus the O-antigen region, with 1 μg being the detectable limit. For urea-PAGE shown in Fig. 2, modifications to oxidation and developing conditions were included to establish what step might account for the difference in results as shown in Fig. 1. Method IV included an increase in oxidation and fixing to 20 min as suggested by Fomsgaard et al. (5), but was otherwise similar to method III. Method V replaced the developing so-

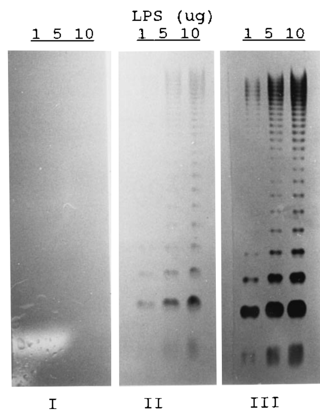

FIG. 1. DOC PAGE analysis of *S. minnesota* LPS. Three identical gels were prepared and loaded with 1, 5, and 10 μg of LPS. Each gel was silver stained and processed according to method I, II, or III as noted.

lution used in method III with that used in methods I and II, and oxidation in fixative also lasted for 20 min. A summary of method differences is given in Table 2. When *S. minnesota* LPS was stained under the five different conditions, it was found that method IV was less sensitive than method III, while method V did not detect any sugar moieties. To determine whether differences in amount loaded on DOC gels could be seen, especially as it related to O antigen, different amounts of *S. minnesota* LPS were electrophoresed in duplicate sets (Fig. 3). Method III or I was used to develop the gels, and once again it was found that LPS was detected by method III (Fig. 3A) but not method I. When the blank gel was reprocessed by method III, it was found that concentration-dependent differences in LPS O antigen could be seen in the previously negative gel (Fig. 3B) and that reprocessing of the gel resulted in some additional improvement in detection.

LPS heterogeneity within a single *S. enteritidis* isolate. The methodology developed with *S. minnesota* was applied to the analysis of LPS from pathogenic *S. enteritidis*, which is known to undergo chemotype variation (18). LPS samples extracted

TABLE 2. Summary of method differences

Method	Oxidation (min)	Staining	Developing
I	5	Tsai and Frasch (25)	1×[a]
II	5	Tsai and Frasch (25)	10×[b]
III	5	Tsai and Frasch (25)	C[c]
IV	20	Tsai and Frasch (25)	C
V	20	Tsai and Frasch (25)	1×

[a] Tsai and Frasch (25).
[b] Krauss et al. (13).
[c] This paper. C, commercially available developer.

from three phenotypically different colonies of *S. enteritidis* isolated from a single parent were electrophoresed in DOC gels. Phenotype differences were seen as significantly different optical densities (difference of >0.05 optical density unit at a wavelength of 600 nm; Student's *t* test; $\alpha = 0.05$) at stationary phase as grown in 20 ml of BHI broth for 16 h incubation at 42°C, with no significant difference in cell concentration present between cultures (18). A fourth phenotype, presented here, is characterized by a stationary-phase optical density of 0.7 in comparison to 0.9 for the avirulent rough phenotype SE6-R, 1.0 for the avirulent semismooth variant SE6-E5, and 1.1 for the virulent smooth variant SE6-E21, as determined without aeration of cultures (18). LPS samples from isolates SE6-R, SE6-E5, and SE6-E21 had been previously analyzed by gas chromatography (18), while the LPS obtained from the fourth variant with an optical density of 0.7 was determined to be a deeper core chemotype, most likely an Rc LPS chemotype (SE6-Rc), as indicated by comparative electrophoresis with characterized core chemotypes of *S. typhimurium* (data not shown). MS analysis of delipidated LPS from SE6-Rc by electrospray propulsion identified peaks at 2,165 (peak 1) and 2,217 (peak 2) atomic mass units (AMU). Additional peaks following peak 1 and 2 are variably methylated products of derivatized molecules (Fig. 4). The two peaks were also detected by DOC PAGE (Fig. 5), with no more detail in structure detected by MS than by DOC PAGE. In addition, DOC PAGE detected additional bands that were not detected by MS. While these results do not definitively characterize the structure of

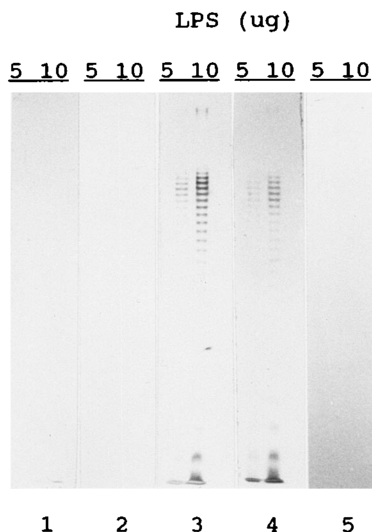

FIG. 2. SDS-urea-PAGE analysis of *S. minnesota* LPS. A single gel was prepared, and lanes were loaded with 5 and 10 μg of LPS in repeated pairs of lanes. See Table 2 for a summary of method differences.

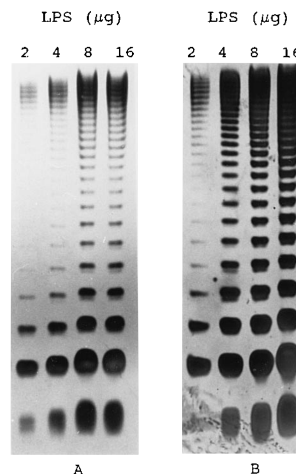

FIG. 3. DOC PAGE analysis of *S. minnesota* LPS. Two sets of lanes were prepared in a single gel. Each set was loaded with 2, 4, 8, and 16 μg of LPS. (A) Stained according to method III. (B) Originally stained according to method I, which resulted in a blank gel. Gel B was restained according to method III to obtain the gel shown.

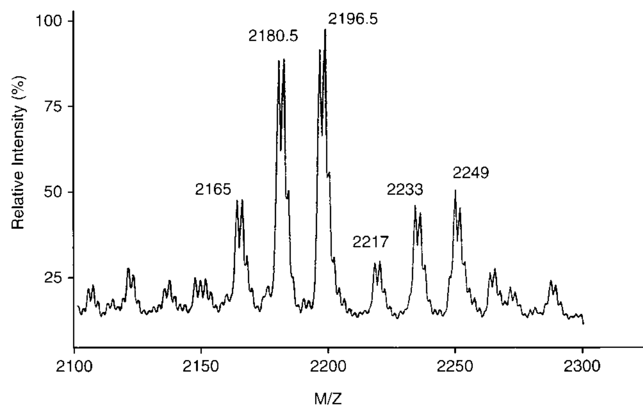

FIG. 4. Molecular weight in AMU of a putative Rc chemotype isolated from *S. enteritidis*. By using whole-number approximations, calculations for core AMU were made with LPS core structure as depicted by Rick (20). Phosphoethanolamine, $C_2H_8N_1O_4P_1$, 142 AMU; PPEtn, $C_2H_9N_1O_7P_2$, 222 AMU; KDO, $C_8H_{14}O_8$, 238 AMU; heptose, $C_7H_{14}O_7$, 210 AMU; glucose and galactose, $C_6H_{12}O_6$, 180 AMU; phosphate, $H_2P_1O_4$, 97 AMU (subtract 1 AMU per bond). The Rc chemotype, without lipid A, had a molecular mass of 2,165 AMU (peak 1), while the two additional peaks immediately following are due to variations in methylation. Another peak at 2,217 AMU (peak 2), also followed immediately by two variably methylated products, is separated from peak 1 by 52 AMU.

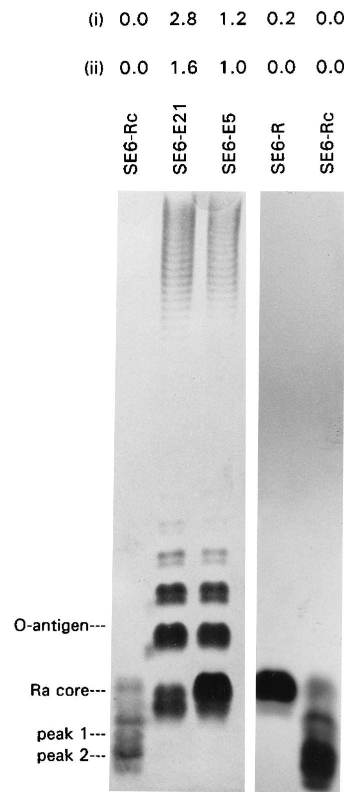

FIG. 5. DOC PAGE analysis of *S. enteritidis* LPS. Electrophoresis patterns for LPS as extracted from a putative Rc chemotype (lane 1), virulent *S. enteritidis* (lane 2), avirulent *S. enteritidis* (lane 3), avirulent Ra chemotype (lane 4), and Rc chemotype (lane 5). O-antigen/core ratios are listed as (i) integration analysis from densitometric readings or (ii) chemical analysis of three LPS samples from O-antigen-producing variants. (Sample values for SE6-E5 were 0.9, 1.0, and 1.1 μg of mannose per μg of KDO, while sample values for SE6-E21 were 1.6, 1.7, and 1.7 μg of mannose per μg of KDO; the ratio of O antigen/core was significantly different between SE6-E21 and SE6-E5 [Student's t test at $\alpha = 0.01$; pooled sample variance = 0.0067].)

the putative Rc chemotype, they do indicate that the improvements to PAGE technique result in detection of mixed LPS chemotypes corroborated by MS analysis and that structural changes of less than 180 AMU, the mass of glucose, can be detected by PAGE. Concentration differences in the higher-molecular-weight O-antigen region were not as evident on DOC gels as they were on urea gels unless densitometric data were obtained, and most of the signal difference was detected in the lower-molecular-weight region of the O-antigen molecule (Fig. 5). Specifically, ratios for the isolates in order of decreasing smoothness were 2.8 (SE6-E21), 1.2 (SE6-E5), 0.2 (SE6-R), and 0 (SE6-Rc chemotype) (Fig. 5). Chemical analyses showed that O-antigen/core ratios were significantly different between the virulent smooth variant SE6-E21 and the avirulent semismooth variant SE6-E5, while little variation between samples was shown (Fig. 5).

To further characterize the phenotypic differences among variants SE6-E21, SE6-E5, and SE6-R, cultures were grown with aeration in 1 ml of BHI in cuvettes, and optical densities were obtained every 25 min, before and after dispersal of cells (Fig. 6). Fig. 6A shows that cells of variant SE6-E21 remained in suspension during growth while cells of SE6-E5 and SE6-R aggregated, indicating that decreasing amounts of O antigen are associated with increasing aggregation of cells. After cells were dispersed (Fig. 6B), the stationary-phase optical densities were 1.40 for SE6-E21 and 1.25 for both SE6-E5 and SE6-R, and this small difference was reproducible. No significant difference in protein concentration was found for the three variants at equivalent optical densities (Table 3), indicating that the increased optical density was not a result of a difference in SE6-E21 biomass. To assess whether LPS determined the difference, concentrated and purified LPS from SE6-E21 and SE6-E5 was examined at wavelengths of 550, 575, 600, 625, and 650 nm. Differences were seen at all wavelengths, a maximum of 0.062 was observed at 600 nm, and a minimum difference of 0.033 was seen at 650 nm. The results suggest that an O-antigen structural difference in SE6-E21 LPS causes change in the light-scattering and/or light-absorbing properties of LPS. Initial work indicates that *N*-acetylglucosamine is significantly decreased in the core region of SE6-E21 LPS in comparison to SE6-E5 and SE6-R LPS (19a).

Analysis of field isolates for LPS heterogeneity. Marked differences existed between five field isolates and the semismooth avirulent variant SE6 in yield of cells per spleen obtained from infected chicks (Table 4). Essentially, chicks fell into two categories. Cages with even one chick that had greater than 2,000 CFU at the 10^2 dilution correlated with recovery of greater than 2,000 CFU from contact-infected birds. Conversely, if no chicks in a cage reached 2,000 CFU, then contact-infected birds had less than 100 CFU at the 10^2 dilution. These results suggest that isolates SE9 and SE15 were composed of mixed bacterial populations, and thus a wider variation in number of organisms recovered from the spleens of infected chicks resulted. Chicks came from flocks in their 25th generation of inbreeding (sister × brother crosses), and all experiments were performed at the same time in hatchmates randomly assigned to isolator cages. Thus, these results suggest that isolate differences account for variation in recovery from spleens more so than host immune factors.

When LPS was examined by urea-PAGE gel, as extracted from an equal weight of packed cells, SE8 had the most O antigen, while SE9 had an intermediate amount (Fig. 7A). The other three isolates, SE5, SE15, and SE23, had considerably less O antigen, and these amounts were similar to O-antigen

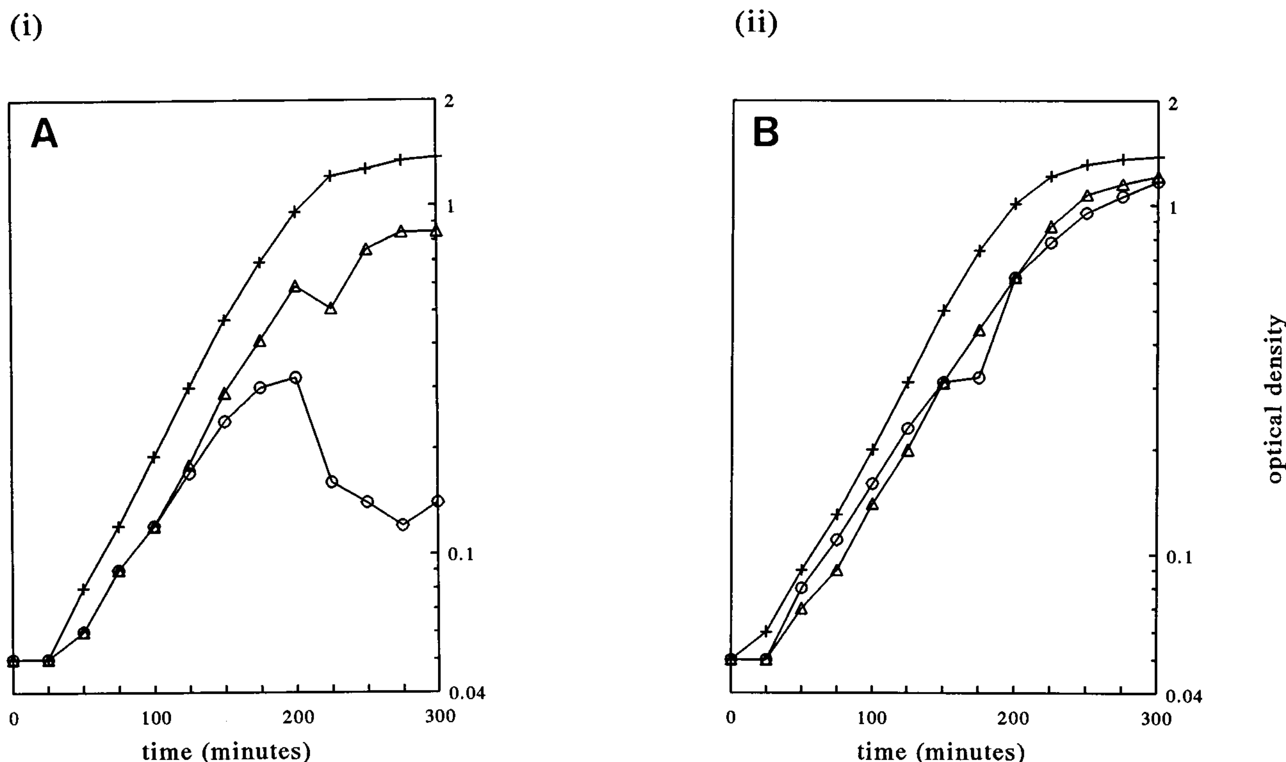

FIG. 6. Aggregation and optical density characteristics of *S. enteritidis* variants during growth. (A) Optical densities of cells of SE6-E21 (+), SE6-E5 (△), and SE6-R (○), allowed to aggregate and precipitate for 20 min between readings. SE6-R begins to aggregate about 30 s after dispersal. (B) Optical densities of dispersed cells. The small, discernible difference between stationary-phase readings of SE6-E21 and those of the other variants was significant (Student's *t* test at α = 0.05; average standard deviation = 0.0296 optical density unit). Symbols as in panel A.

amounts detected in the LPS of the semismooth variant SE6 (Fig. 7A). Relative amounts of O antigen for isolates shown in the silver-stained gel (Fig. 7A) were confirmed by immunoblot analysis of LPS with an O-antigen-specific polyclonal antiserum (Fig. 7B), and these data also indicate that a contaminating molecule does not contribute to signal. Quantitative analysis by gas chromatography confirmed that SE8 had the highest O-antigen/core ratio and that at a ratio of 1.6 it had amounts similar to those of virulent variant SE6-E21. However, LPS extracted for gas chromatographic analysis from isolate SE9, which had evidence of mixed populations, did not show a difference in O-antigen/core ratios compared with those of the semismooth variants SE6, SE15, and SE23 (SE5 sample was not processed successfully). This result was possibly due to outgrowth of a semismooth population in the larger-volume cultures used for LPS extraction, and this possibility was substantiated by detecting that growth characteristics for isolates SE9, SE15, and SE23 were similar to those of parent isolate SE6 (optical density of 1.0 in 20 ml of BHI, 20- by 150-mm tubes, with no statistical difference in cell concentration; mean = 5×10^8 CFU/ml) and by confirming loss of O-antigen signal for isolate SE9 in comparison to SE6 by reexamination of LPS by gel electrophoresis (data not shown).

TABLE 3. Total protein concentrations of *S. enteritidis* LPS variants during growth[a]

Optical density (600 nm)	Protein (mg/ml)		
	SE6-E21	SE6-E5	SE6-R
0.2	70	79	73
0.4	103	114	110
0.6	133	147	145
0.8	166	183	183
1.0	195	215	213
1.2	231	253	255

[a] Means of three assays were analyzed. There was no significant difference between variants (Student's *t* test at α = 0.05; average standard deviation = 17.73 mg/ml).

DISCUSSION

Since contamination of eggs by *S. enteritidis* is sporadic (16), any prominent surface molecule which undergoes variation is

TABLE 4. Virulence characterization of *S. enteritidis* isolates

Chick no.	CFU of given isolate (phage type) from chick spleens					
	SE5 (8)	SE6 (13a)	SE8 (8)	SE9 (14b)	SE15 (8)	SE23 (14b)
1	61	74	>2,000	>2,000	147	440
2	70	58	>2,000	>2,000	166	476
3	41	75	>2,000	>2,000	107	324
4	38	86	>2,000	>2,000	770	274
5	103	95	>2,000	135	306	468
6	102	101	>2,000	167	>2,000	752
7	81	68	>2,000	672	312	386
8	66	77	>2,000	70	193	246
9	34	82	>2,000	299	412	518
10	ND[a]	153	104	ND	198	98
C[b]	78	79	>2,000	>2,000	>2,000	80

[a] ND, not done.
[b] C, contact-infected chick.

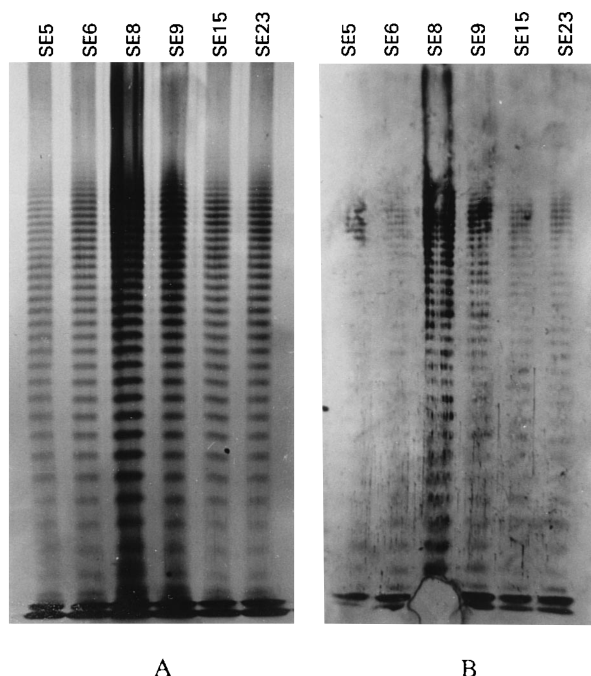

FIG. 7. Electrophoretic and immunoblot characteristics of LPS isolated from characterized *S. enteritidis* field isolates. LPS was isolated from equal weights of packed cells. (A) Analysis by urea-PAGE. O-antigen/core ratios, in micrograms of mannose per microgram of KDO, were 1.0 for SE6, 1.6 for SE8, 0.9 for SE9, 1.1 for SE15, and 0.6 for SE23. Sample from SE5 was not available. (B) Immunoblot of a twin gel transferred to nitrocellulose and reacted with group D1 O-antigen-specific polyclonal antiserum.

suspected of having a role in determining virulence. The change in methodology presented here was used to show that LPS heterogeneity occurs within *S. enteritidis* populations, that it can be detected by using a combination of readily available PAGE techniques and immunoblot analyses, and that large decreases in O antigen result in aggregation of cells during growth. While results show that increased ratios of O antigen/core are more likely than phage type to correlate with virulence in chicks, finding that O antigen decreases on passage indicates that continued investigation of heterogeneity is warranted before conclusions can be made concerning how LPS is associated with virulence outside of its established role in complement resistance. Comparison of the data presented here with published compositional data indicates that virulent *S. enteritidis* populations probably have peak integration ratios of O antigen/core of at least 2.8, while ratios of avirulent *S. enteritidis* variants are 1.2 or less as measured from DOC gels by densitometric analysis (18). Mechanisms by which O-antigen ratios change have been proposed by others (11, 15, 23), and they invoke alternative processing of core.

To analyze *S. enteritidis* with PAGE techniques, a change in developer is useful, and results obtained here are due to the inclusion of paraformaldehyde rather than formaldehyde in the commercial product. It is imperative to use an extraction technique that isolates LPS without bias for chemotype structure (2). In addition, the improvement in signal was seen in all gel systems, including a Tricine buffer system (courtesy of C. Schnaitman; data not shown), and a small-scale extraction procedure (7). Finally, increased fixing time while oxidizing (method IV) caused a decrease in signal, which is possibly due to leaching of LPS molecules with low fatty acid content, as seen by others (3). Since different organisms have different LPS and lipid A structures (1, 9, 19, 20), the conditions here are specific for *S. enteritidis*, *S. minnesota*, and possibly other salmonellae with O antigen composed of neutral sugars.

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REFERENCES

1. **Brade, H., L. Brade, U. Schade, U. Zähringer, O. Holst, H.-M. Kuhn, A. Rozalski, E. Röhrscheidt, and E. T. Rietschel.** 1988. Structure, endotoxicity, immunogenicity and antigenicity of bacterial lipopolysaccharides (endotoxins, O-antigens), p. 17–45. *In* J. Levin, H. R. Büller, J. W. ten Cate, S. J. H. van Deventer, and A. Sturk (ed.), Bacterial endotoxins: pathophysiological effects, clinical significance, and pharmacological control. Alan R. Liss, Inc., New York.
2. **Darveau, R. P., and R. E. W. Hancock.** 1983. Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. J. Bacteriol. **155:**831–838.
3. **Dubray, G., and G. Bezard.** 1982. A highly sensitive periodic acid-silver stain for 1,2-diol groups of glycoproteins and polysaccharides in polyacrylamide gels. Anal. Biochem. **119:**325–329.
4. **Duchesne, L. G. M., J. S. Lam, L. A. MacDonald, C. Whitfield, and A. M. Kropinski.** 1988. Effect of pH and acrylamide concentration on the separation of lipopolysaccharides in polyacrylamide gels. Curr. Microbiol. **16:**191–194.
5. **Fomsgaard, A., M. A. Freudenberg, and C. Galanos.** 1990. Modification of the silver staining technique to detect lipopolysaccharide in polyacrylamide gels. J. Clin. Microbiol. **28:**2627–2631.
6. **Helander, I. M., A. P. Moran, and P. H. Mäkelä.** 1992. Separation of two lipopolysaccharide populations with different contents of O-antigen factor 12_2 in *Salmonella enterica* serovar *Typhimurium*. Mol. Microbiol. **6:**2857–2862.
7. **Hitchcock, P. J., and T. M. Brown.** 1983. Morphological heterogeneity among *Salmonella* chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. **154:**269–277.
8. **Jimenez-Lucho, V. E., and L. L. Leive.** 1990. Role of the O-antigen of lipopolysaccharide in *Salmonella* in protection against complement action, p. 339–354. *In* B. H. Iglewski and V. L. Clark (ed.), The bacteria, vol. 11. Academic Press, New York.
9. **Karibian, D., C. Deprun, and M. Caroff.** 1993. Comparison of lipids A of several *Salmonella* and *Escherichia* strains by ^{252}Cf plasma desorption mass spectrometry. J. Bacteriol. **175:**2988–2993.
10. **Kido, N., M. Ohta, and N. Kato.** 1990. Detection of lipopolysaccharides by ethidium bromide staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. J. Bacteriol. **172:**1145–1147.
11. **Klena, J. D., R. S. Ashford II, and C. A. Schnaitman.** 1992. Role of *Escherichia coli* K-12 *rfa* genes and the *rfp* gene of *Shigella dysenteriae* 1 in generation of lipopolysaccharide core heterogeneity and attachment of O-antigen. J. Bacteriol. **174:**7297–7307.
12. **Komuro, T., and C. Galanos.** 1988. Analysis of Salmonella lipopolysaccharides by sodium deoxycholate-polyacrylamide gel electrophoresis. J. Chromatogr. **450:**381–387.
13. **Krauss, J. H., J. Weckesser, and H. Mayer.** 1988. Electrophoretic analysis of lipopolysaccharides of purple non-sulphur bacteria. Int. J. Syst. Bacteriol. **38:**157–163.
14. **Kusumoto, S., N. Kusunose, M. Imoto, T. Shimamoto, T. Kamikawa, H. Takada, S. Kotani, E. T. Rietschel, and T. Shiba.** 1989. Synthesis and biological function of bacterial endotoxin. Pure Appl. Chem. **61:**461–464.
15. **MacLachlan, P. R., S. K. Kadam, and K. E. Sanderson.** 1991. Cloning, characterization, and DNA sequence of the *rfaLK* region for lipopolysaccharide synthesis in *Salmonella typhimurium* LT2. J. Bacteriol. **173:**7151–7163.
16. **Morris, G. K.** 1990. *Salmonella enteritidis* and eggs: assessment of risk. Fed. Vet. **47:**8–10.
17. **Palva, T., and P. H. Mäkelä.** 1980. Lipopolysaccharide heterogeneity in *Salmonella typhimurium* analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis. Eur. J. Biochem. **107:**137–143.
18. **Petter, J. G.** 1993. Detection of two smooth colony phenotypes in a *Salmonella enteritidis* isolate which vary in their ability to contaminate eggs. Appl. Environ. Microbiol. **59:**2884–2890.
19. **Raetz, C. R. H., R. J. Ulevitch, S. D. Wright, C. H. Sibley, A. Ding, and C. F. Nathan.** 1991. Gram-negative endotoxin: an extraordinary lipid with profound effects on eucaryotic signal transduction. FASEB J. **5:**2652–2660.

19a. **Rahman, M., and R. W. Carlson.** Personal communication.

20. **Rick, P. D.** 1987. Lipopolysaccharide biosynthesis, p. 648–662. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 1. American Society for Microbiology, Washington, D.C.
21. **Ryan, J. M., and H. E. Conrad.** 1974. Structural heterogeneity in the lipopolysaccharide of *Salmonella newington*. Arch. Biochem. Biophys. **162:**530–535.
22. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed., p. 16–23. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Schnaitman, C. A., and J. D. Klena.** 1993. Genetics of lipopolysaccharide biosynthesis in enteric bacteria. Microbiol. Rev. **57:**655–682.
24. **St. Louis, M. E., D. L. Morse, M. E. Potter, T. M. DeMelfi, J. J. Guzewuch, R. V. Tauxe, and P. A. Blake.** 1988. The emergence of grade A eggs as a major source of *Salmonella enteritidis* infections: new implications for the control of salmonellosis. JAMA **259:**2103–2107.
25. **Tsai, C. M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119:**115–119.
26. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides: extraction with phenol-water and further applications of this procedure. Methods Carbohydr. Chem. **5:**83–91.
27. **York, W. S., A. G. Darvill, M. McNeil, T. T. Stevenson, and P. Albersheim.** 1985. Isolation and characterization of plant cell walls and cell wall components. Methods Enzymol. **118:**3–40.