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# Detection of Two Smooth Colony Phenotypes in a *Salmonella enteritidis* Isolate Which Vary in Their Ability To Contaminate Eggs

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***Salmonella enteritidis* isolates were obtained from eggs after infection of Leghorn hens with a parent isolate (SE6) known to only infrequently contaminate eggs. Isolates from eggs exhibited two phenotypes that were subtly different. One phenotype was typically smooth, while the other was transiently rough. Both sets of isolates were phage type 13A and positive for D1 epitopes. Immunoblot analysis of entire colonies and gas chromatographic analysis of purified lipopolysaccharide revealed that the phenotypic difference between isolates was due to a quantitative difference in O antigen and possibly a qualitative difference in the lipid A core region. In addition, the two isolates had different opacity properties when examined at 600 nm. When the two isolates were used to inject egg-laying hens, a significant difference in the ability to contaminate eggs was detected.**

*Salmonella enteritidis* is a motile group D member of the salmonellae capable of infecting a broad range of animal hosts. During the past 10 years, the incidence of *S. enteritidis* outbreaks in humans has increased markedly (20). Infection correlates strongly with ingestion of one type of food. Grade A shell eggs, with no evidence of shell cracks, are repeatedly implicated as the food source responsible for the current increase in human outbreaks (20, 21). In contrast, illness from other *Salmonella* spp. can be traced to a multitude of foods except eggs (2). The association of *S. enteritidis* with eggs indicates that vertical transmission occurs through the reproductive organs of the chicken (16, 20). Since *S. enteritidis* is capable of infecting humans by ingestion via contaminated eggs, factors which appeared to increase the efficiency of egg contamination by *S. enteritidis* were explored.

Recent research in my laboratory revealed that *S. enteritidis* field isolates might differ in their ability to produce or link O antigen (region III) to the core (region II) on the lipopolysaccharide (LPS) molecule in vitro (17). O antigen linked to the core (O-LPS) of *Salmonella* species is important in pathogenesis because it is recognized as being associated with organisms which avoid complement inactivation and phagocytosis in vivo (10, 12). In addition, coordinate regulation of other virulence properties appears to occur with biosynthesis of O-LPS in *Escherichia coli* (1). O-LPS is thus a proven virulence factor for many gram-negative pathogens. The subject addressed here is how heterogeneity in the LPS structure of naturally occurring *S. enteritidis* isolates relates to the process of egg contamination.

## MATERIALS AND METHODS

**Birds.** Mature hens and 5-day-old chicks were obtained from the facility's specific-pathogen-free, closed Leghorn flock. Chicks were housed in modified Horsfall isolation units maintained in a negative-pressure containment building, with 9 or 10 chicks in each unit (prior to losses due to mortality). Hens were housed in layer cages, one bird per cage, in environmentally controlled disease containment buildings.

The *S. enteritidis* phenotype used for each study is explained in the figure legends. Hens were moved into cages 1 week before the study was initiated. The hen house and cages were decontaminated between each infection study, and only one isolate at a time was used in the room.

**Isolates.** The *S. enteritidis* isolates used in these experiments came from an isolate (19299-52-1) originally obtained from C. Benson, University of Pennsylvania, and is referred to in this paper as parent isolate SE6. The isolate which appeared to have a low level of O antigen (SE6-E5) originated from an infected egg recovered on day 5 postinfection; the other isolate originated from an egg obtained on day 21 postinfection (SE6-E21). In addition, an isolate with the Ra chemotype and no group D1 reactivity was isolated from the spleen of a chick infected with SE6.

**Preparation of inoculum.** Cells from cultures stored at 4°C on brilliant green (BG) agar plates were characterized within 2 days before use in infection trials by growth curve characteristics and LPS density (by immunoblot analysis) as described above. Cells from these master plates were used to start cultures in brain heart infusion (BHI) broth at 42°C. When the culture reached an $A_{600}$ of 0.5 (LKB Ultraspec III), cells were pelleted at 4°C and 12,000 × *g* in a Sorvall SS34 rotor. After two washes with phosphate-buffered saline (PBS), cells were diluted in PBS according to a previously performed quantitative growth curve to obtain the desired concentration of cells. In this laboratory, an $A_{600}$ of 0.5 in BHI broth represents approximately $2.6 \times 10^8$ CFU/ml. After the final dilution in PBS, an aliquot of inoculum was plated on BG agar to obtain the exact number of CFU per milliliter and to check for contamination. Cells were delivered in 0.5 ml of PBS either intravenously for hens or intraperitoneally for chicks.

**Recovery of salmonellae from spleens and eggs.** Spleens were collected from chicks at day 3 postexposure. Immediately after the chicks were killed by cervical dislocation, the carcasses were packed in ice. Aseptically collected spleens were cultured by placing them in 200 μl of BHI broth in tubes containing 1.0-mm glass beads. Spleens were disrupted by agitation for 2 min in a mini-bead beater apparatus (Biospec). Fifty microliters of spleen suspensions was

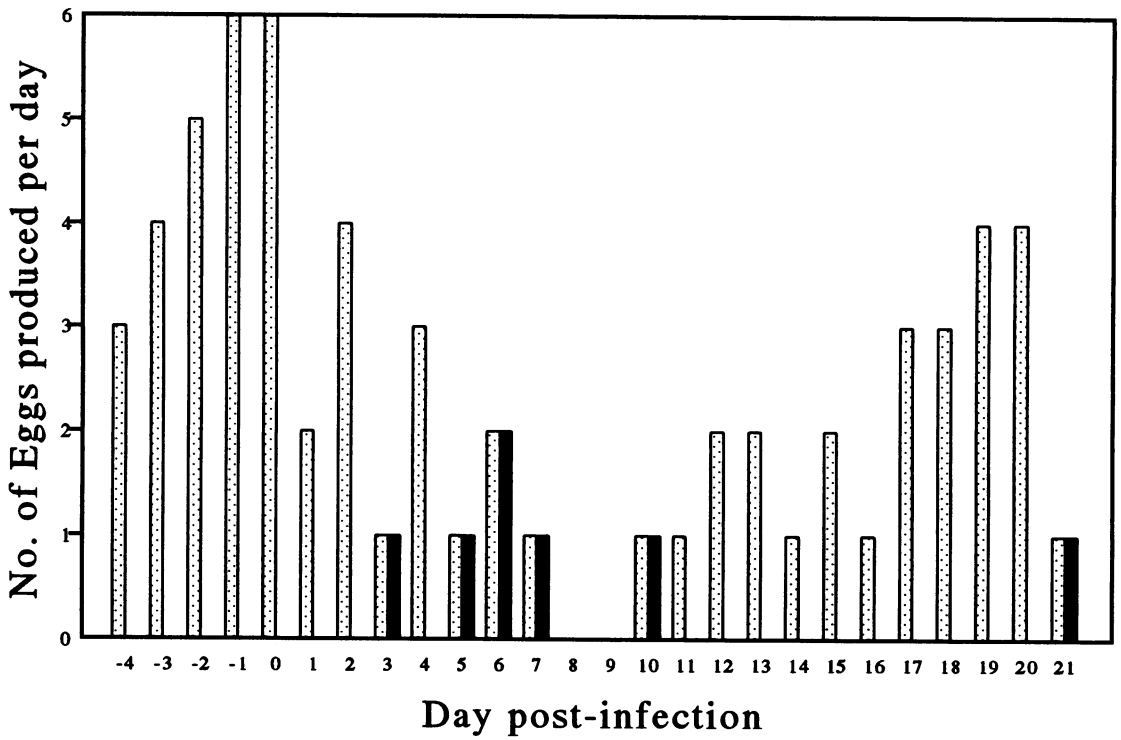


FIG. 1. Origin of SE6-E5 and SE6-E21. Stippled bars indicate total egg production per day, and solid bars indicate the number of those eggs found to be contaminated. E5 originated from an egg collected 5 days after infection; E21 originated from an egg collected on day 21 postinfection. The infective dose was $9 \times 10^7$ organisms. From days 1 through 21 postinfection, 39 eggs were produced and 7 were contaminated (18.0%). $N = 6$ birds.

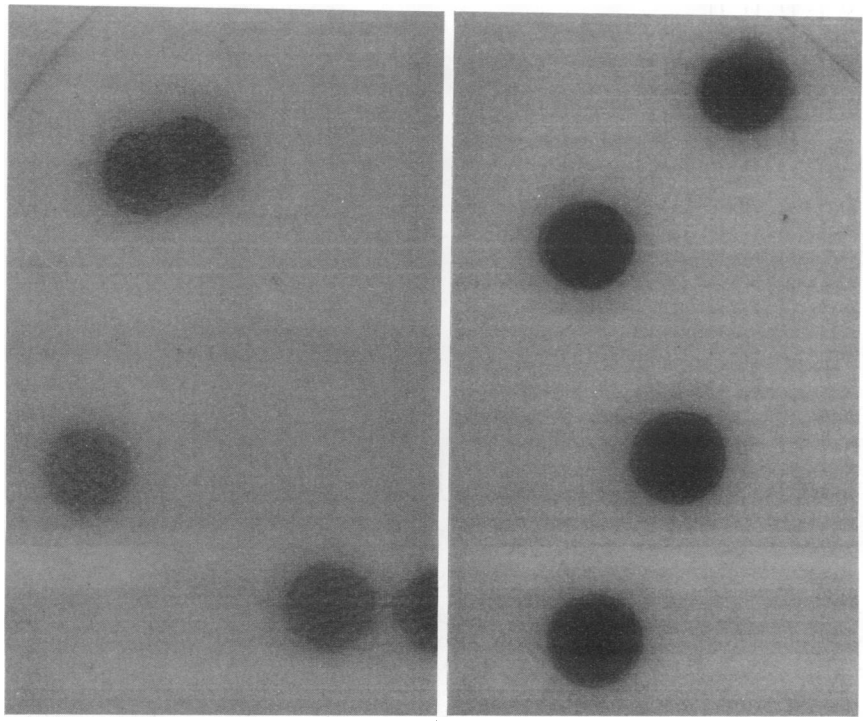

FIG. 2. LPS colony immunoblots of SE6-E5 (left) and SE6-E21 (right). Readers are referred to reference 18 for a complete discussion of the LPS molecule.

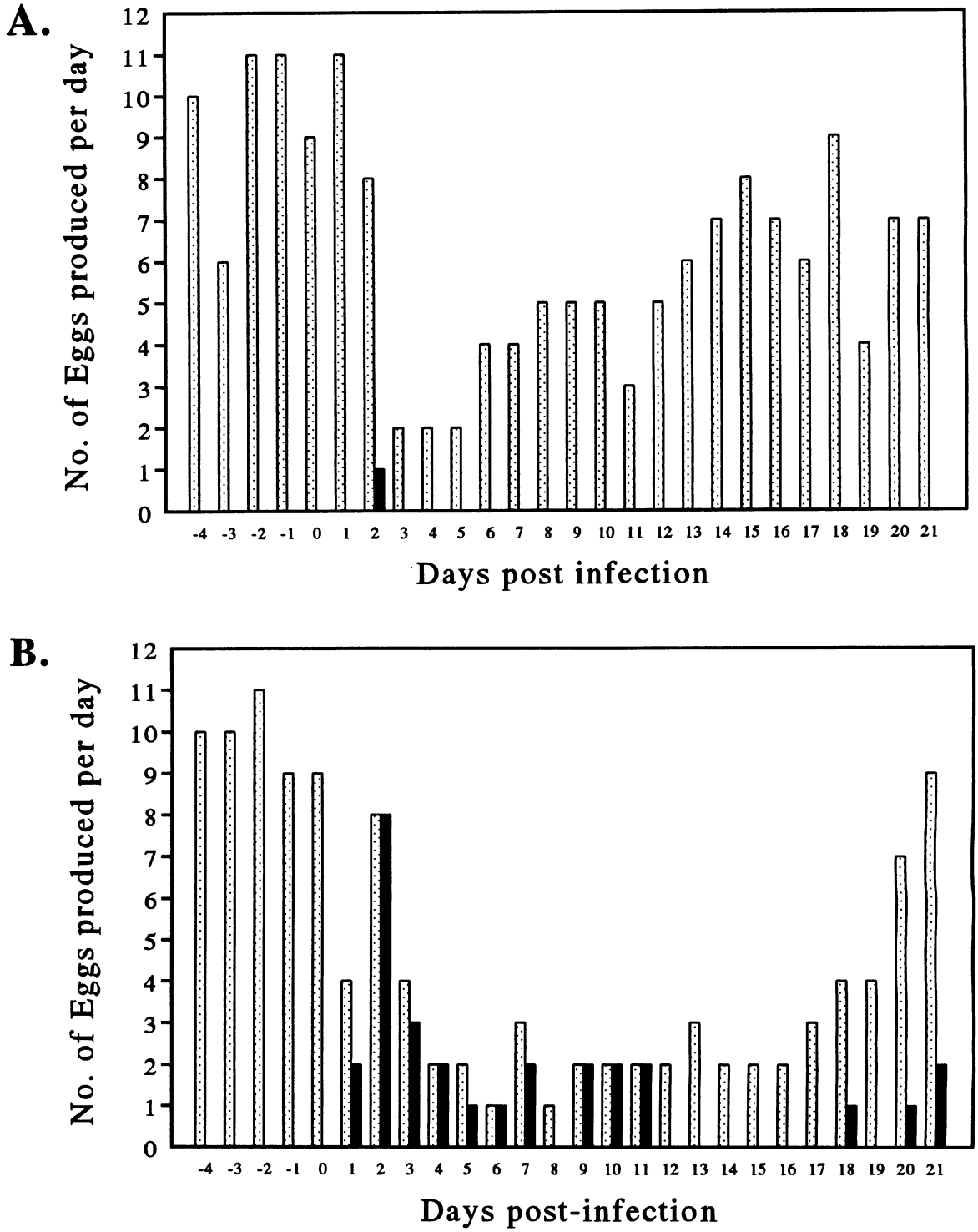


FIG. 3. Results from culturing eggs obtained from chickens infected with either SE6-E5 (A) or SE6-E21 (B). Stippled bars indicate total egg production per day, and solid bars indicate the number of those found to be contaminated. (A) Infective dose, $5.4 \times 10^7$ CFU. Of 117 eggs produced after infection, 1 was positive for *S. enteritidis* (0.8%). Another trial at $2.6 \times 10^7$ CFU/ml produced no contaminated eggs. (B) Infective dose, $3.0 \times 10^7$ CFU. Of 69 eggs produced after infection, 29 of those were positive (42%). $N$ = 12 birds.

TABLE 1. Growth and opacity properties of SE6-R, SE6-E21, and SE6-E21 grown under different conditions of $O_2$ diffusion

| BHI[a] (ml) | Growth ($10^9$ CFU/ml) | | | Opacity ($A_{600}$) | | |
|---|---|---|---|---|---|---|
| | SE6-R | SE6-E5 | SE6-E21 | SE6-R | SE6-E5 | SE6-E21 |
| 1.0 | 2.3 | 2.8 | 3.9 | 1.6 | 1.8 | 1.8 |
| 2.5 | 1.0 | 1.5 | 1.9 | 1.3 | 1.3 | 1.5 |
| 5.0 | 0.9 | 0.8 | 1.1 | 1.1 | 1.0 | 1.2 |
| 10.0 | 0.8 | 0.5 | 0.7 | 1.0 | 0.9 | 1.1 |
| 15.0 | 0.5 | 0.5 | 0.9 | 0.9 | 1.0 | 1.1 |
| 20.0 | 0.5 | 0.5 | 0.7 | 0.9 | 1.0 | 1.1 |
| 25.0 | 0.4 | 0.5 | 1.2 | 0.9 | 1.0 | 1.1 |
| 30.0 | 0.5 | 0.5 | 1.0 | 1.0 | 1.0 | 1.1 |

[a] In 20 by 150-mm tubes. Opacity differences of greater than 0.05 units are statistically significant (Student's $t$ test; $\alpha = 0.05$).

diluted 10-fold. In order to obtain plates with isolated colonies, 100 μl from each dilution, beginning at $10^{-2}$ and ending at $10^{-7}$, was plated on BG agar. Lower dilutions than this did not contribute to data analysis, and the $10^{-2}$ dilution yielded colony counts that were greater than the number of any residual bacteria that could be recovered from the capsule of flamed spleens by swabbing with medium.

Plates were incubated at 42°C overnight, and colonies were counted. Colonies were confirmed as *S. enteritidis* by biochemical analysis (Enterotube) and serology with commercially available group D1 antiserum. Phage types were determined by the National Veterinary Services Laboratories, Ames, Iowa. Whole contents of eggs were cultured as described previously (9). Eggs were collected from 4 days prior to infection through day 21 postexposure.

**Growth characterization.** Colonies of either SE6-E5 or SE6-E21 grown overnight at 42°C on BG agar plates were diluted in Luria-Bertani (LB) broth to an $A_{600}$ of 0.05. Cultures were grown statically for 280 min at 42°C to obtain growth curves in duplicate. For the information presented in Table 1, cultures were grown from single colonies for 24 h at 42°C in BHI broth.

**Colony immunoblots.** Cells were collected from the plates referred to above and diluted to obtain about 50 CFU per BG plate. These plates were incubated at 42°C until the colonies were about 4 mm in diameter. The colonies were overlaid with nitrocellulose, and the plates were held overnight at 4°C. Immunoblot analysis was performed with rabbit anti-group D1 antiserum (Difco) as the primary antibody. Handling of filters, washes, binding to secondary antibody (alkaline phosphatase-conjugated anti-rabbit immunoglobulin G [Pierce]), and signal development were performed according to standard techniques (19). Slide agglutination reactions with group D1, factor 9, factor 12, and factor 1 antisera (Difco) were performed according to the manufacturer's directions.

**GC analysis of LPS and lipid A-associated fatty acids.** LPS was isolated from SE6-E5 and SE6-E21 cultures grown in 1 liter of BHI broth incubated overnight at 42°C by the method of Darveau and Hancock (5) except that the cells were lysed in an alkaline lysis buffer (13). From these preparations, 500 μg of LPS was delipidated by heating at 100°C in 1% acetic acid for 90 min, and the precipitate was removed by centrifugation. The supernatant was dried by evaporation under $N_2$. Inositol (20 μg) was added as an internal standard. A standard sugar mixture (100 μg each of heptose, ribose, rhamnose, fucose, glucose, galactose, and mannose) with 20 μg of inositol added was prepared along with the sample during alditol acetate derivitization. Alditol acid derivitization was performed for neutral sugars by established techniques (23). The sample was run on a 30-m DB-1 column at an initial temperature of 190°C for 2 min and then at 240°C, increased at a rate of 10°C/min, and held at 240°C for 10 min or until the heptose peak was present.

For fatty acid analysis, 2.0 mg of LPS isolated from the different strains was used to prepare methyl esters by published techniques (3). Heptadecanoic acid ($C_{17}H_{34}O_2$), 200 μg, was included as an internal standard. Dried sample was diluted in 50 μl of chloroform and used for gas chromatography (GC) analysis. Columns and program conditions were used as described previously (3).

**Titration of O-LPS on intact cells.** SE6-E5 and SE6-E21 cells were harvested from BG plates and vortexed until suspended. Ten microliters was removed from the heavy suspensions and diluted 10-fold. $A_{600}$ readings were used to estimate the number of cells present in each suspension by comparison with a previously performed scale; plate counts were performed to confirm estimates. Meanwhile, 100 μl of factor 9 antiserum (antityvelose) was diluted in a twofold series in 1× PBS, leaving 50 μl per well on a 96-well microtiter plate with rounded wells. To these wells, 50 μl of cells was added. Each sample was assayed three times. The plate was agitated for 15 min and then centrifuged at 1,000 rpm for 5 min in a Centricon MP6 unit. $A_{620}$ values were obtained on an automated plate scanner. Controls included an Ra chemotype SE6 isolate (rough phenotype) and whole cells from each isolate with no antiserum added.

## RESULTS

**Animal experiments.** Figure 1 shows the origins of SE6-E5 and SE6-E21, obtained when hens were infected with the SE6 parent. SE6-R was isolated from a chick spleen after exposure to the SE6 parent in a separate trial. When $6.5 \times 10^5$ organisms of SE6-R, the SE6 parent, SE6-E5, and SE6-E21 were injected intraperitoneally, it was found that the SE6-R, the SE6 parent, and SE6-E5 isolates failed to invade all of the chick spleens at the $10^{-2}$ dilution of spleen contents, while SE6-21 was recovered from 100% of the spleens at the $10^{-2}$ dilution (Fig. 2). In addition, SE6-E21 resulted in higher numbers of bacteria in spleens (Fig. 2). Wilcoxon rank sum analysis, using the number of CFU at the $10^{-2}$ dilution, indicated that SE6-E21 resulted in statistically

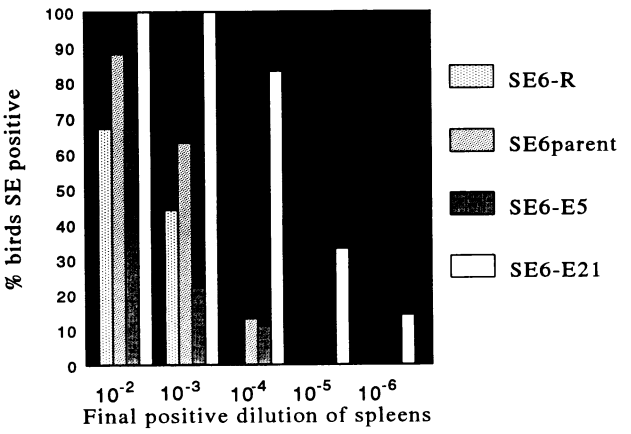


FIG. 4. *S. enteritidis* (SE) in spleens of 5-day-old chicks infected intraperitoneally with $6.5 \times 10^5$ organisms.

TABLE 2. Analysis of O antigen and fatty acids as isolated from SE6-R, SE6-E5, and SE6-E21[a]

| Analysis | O antigen content (μg of LPS/mg) | | |
|---|---|---|---|
| | SE6-E5 | SE6-E21 | SE6-R |
| Neutral sugar | | | |
| Rhamnose | 18.36 | 58.22 | —[b] |
| Mannose | 7.01 | 63.42 | — |
| Galactose | 4.28 | 81.22 | 1.56 |
| Glucose | — | 18.32 | 3.73 |
| Heptose | — | 10.94 | — |
| Fatty acid | | | |
| $C_{14}$:O (myristic acid) | 4.32 | 3.52 | 4.22 |
| 3-OH$C_{14}$:O (β-hydroxymyristic acid) | 26.98 | 26.20 | 28.03 |
| $C_{18}$:O (stearic acid) | 6.57 | 3.57 | 7.78 |

[a] Cells were grown under $O_2$ diffusion conditions equal to 20 ml in tubes (20 by 150 mm) (see Table 1).
[b] —, not detected.

significant different levels of infection in the chicks than the other two isolates ($P$ = <0.025 of type I error; rank summation was based on a rank scale of 100 CFU increments) (15). Mortality per group was as follows: SE6-R, 0 of 9; SE6 parent, 1 of 9; SE6-E5, 1 of 10; and SE6-E21, 3 of 10. Additional trials comparing the phenotypes had been performed previously at a dosage of $5 \times 10^6$ organisms intraperitoneally with similar results of a $10^2$- to $10^3$-fold difference in numbers of CFU recovered (17).

When hens were injected intravenously with either SE6-E5 or SE6-E21, a marked difference in egg contamination was observed. The lowest dose of SE6-E5 did not result in any contaminated eggs. An additional trial with SE6-E5 at twice the initial dose resulted in 0.8% positive eggs (Fig. 3A). Infection with SE6-E21 at a dose between those used in the aforementioned experiments resulted in contamination of 42% of the eggs through day 21 postexposure (Fig. 3B). A bimodal distribution of contaminated eggs and depressed total egg production was observed for isolate SE6-E21. When the SE6-E21 results are compared with the data obtained by infecting hens with the SE6 parent (Fig. 1), it can be seen that a higher dose of SE6 was required to depress egg production, to cause a bimodal pattern of contaminated eggs, and to recover more than 10% contaminated eggs.

**In vitro characterization of isolates by immunoblot and growth analysis.** On initial isolation from chicks or eggs, SE6-E5 colonies had the cobblestone appearance typical of rough phenotypes, even though they reacted readily with D1 antiserum in a slide agglutination assay. After passage or continued growth, this roughness changed to a smooth appearance. SE6-E21 colonies always appeared smooth. Single-colony growth curves for SE6-R, SE6-E5, and SE6-E21 showed that an opacity difference of 0.05 $A_{600}$ units was significant ($t$ test; $\alpha = 0.05$) for cultures grown to the stationary phase. Stationary-phase opacity differences were observed in other media, such as BHI. However, it was observed that SE6-E21 could obtain a higher cell number than SE6-E5 under different $O_2$ diffusion conditions in BHI (Table 1). SE6-E21 was the only isolate which exhibited increased growth under microaerophilic conditions.

Colony immunoblots also showed that the presence and expression of O-LPS differed between SE6-E5 and SE6-E21, as determined by signal intensity (Fig. 4). In contrast, both SE6-E5 and SE6-E21 reacted similarly with group D1 (factors 1, 9, and 12), factor 9, and factor 12 antisera in slide agglutination reactions.

**LPS structural analysis.** The results of GC analysis of neutral sugars and fatty acids from LPS isolated from each strain are shown in Table 2. The results indicated that only SE6-E21 had a neutral sugar profile typical of a D1 *Salmonella* strain. As expected, SE6-R was negative for neutral sugars typically found in O antigen. SE6-E5 yielded considerably lower neutral sugar quantities than SE6-E21, even though nearly equivalent amounts of β-hydroxymyristic acid were recovered from all three isolates. Since loss of heptose occurred for both SE6-E5 and SE6-R during alditol acetate preparation, the ratio of rhamnose to β-hydroxymyristic acid (micrograms of LPS per milliter) was found to be the most reliable method of expressing the ratio of O antigen to lipid

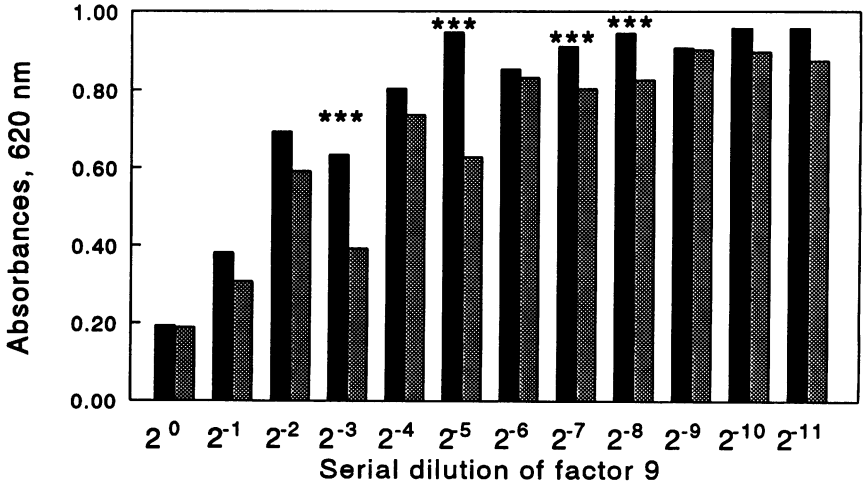


FIG. 5. Depletion of factor 9 (tyvelose)-specific antiserum by SE6-E5 (shaded bars) and SE6-E21 (solid bars). Bars indicate the means obtained by running samples in triplicate. Means which were statistically different between the two isolates (Student's $t$ test, $\alpha = 0.05$) are marked (***). Factor 9 was chosen because tyvelose undergoes no known form variation and is inadequately quantified by GC analysis because of acid lability.

A core. The ratios were 2.22, 0.68, and 0.00 for SE6-E21, SE6-E5, and SE6-R, respectively.

When whole cells were assayed for the quantity of O antigen by observing how they reacted with an antiserum which detected only tyvelose (factor 9), it was observed that isolate SE6-E21 depleted antiserum sooner than SE6-E5 (Fig. 5). O-LPS causes cells to remain in suspension, and cells remain suspended even when centrifuged if no antibodies are present to form complexes. Accordingly, the Ra chemotype strain lacking O antigen, SE6-R, pelleted throughout all dilutions in the assay, while cells from SE6-E5 and SE6-E21 remained in suspension when antiserum was not included.

## DISCUSSION

The results presented here show that *S. enteritidis* field isolates selected for investigation should be characterized according to LPS structure and growth characteristics. Currently, phage typing is used as part of the evidence for causality between human outbreaks and flock isolates (22). Here, phage typing appears to inadequately differentiate the biological behavior of SE6-E5 and SE6-E21, which are both phage type 13A. SE6-R is phage type 23, and review of the typing scheme indicates that at least 2, and possibly 3, of the 10 typing phages are O antigen specific (22). The phage type associated most frequently with human disease, phage type 8, is also the one most frequently isolated from randomly sampled spent hens (7). The frequency with which phage type 8 is isolated from both humans and flocks introduces the chance that an incidental rather than a causal relationship between isolates and flocks might be identified. Thus, different methods of isolate evaluation are needed to assess pathogenesis.

Characterization of isolates by the parameters presented here might prevent inadequate experimental design due to the emerging presence of a smooth phenotype that is avirulent, in part, because of a reduction in O-LPS. In view of the recently described role for coordinated regulation of LPS structure with other virulence factors, the necessity of excluding isolates such as SE6-E5 from animal studies becomes even more consequential than previously thought (1). It remains to be investigated whether the emergence of isolates SE6-E5 and SE6-R is due to stationary-phase induction of mutations (24).

The finding that heptose is selectively degraded during alditol acetate preparation for the two isolates with less O antigen, SE6-E5 and SE6-R, could indicate that microheterogeneity exists in this region between isolates with different ratios of O antigen to lipid A core. Such a change might render avirulent isolates such as SE6-E5 more susceptible to acyloxyacyl hydrolases or oxidation during the course of an infection (4, 6). It is not known if the decrease in recovery of secondary acyl groups for SE6-E21 is significant or not (Table 1), but it is known that pathogenic *E. coli* has an activated acyl carrier protein involved in transfer of acyl groups to prohemolysin (11). Perhaps pools of acyl groups are depleted on lipid A of isolate SE6-E21 because they are added to proteins undergoing double membrane translocation. Changes in the acylation of lipid A are known to affect endotoxic activity and could be why sharp drops in egg production were only encountered for isolate SE6-E21 (14). Investigation into the finer details of structure in this region continues.

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