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A novel relationship between O-antigen variation, matrix formation, and invasiveness of *Salmonella enteritidis*

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SUMMARY

Salmonella enterica Enteritidis in chickens serves as a reservoir for salmonellosis in humans and the structure of its lipopolysaccharide (LPS) has been used to assess invasiveness. Culture from chick spleens generated colonies with an unusual wrinkled morphology, and it is designated the lacy phenotype. To characterize the nature of the morphological change, three isogenic variants were compared. Only the lacy phenotype produced a temperature-dependent cell surface matrix composed of several proteins in association with LPS high molecular weight O-antigen. Flagellin and a 35 kDa protein were identified as specific proteinaceous components of matrix. Both proteins cross-reacted with a monoclonal antibody previously determined to specifically detect the g-epitope of the Enteritidis monophasic flagella (H-antigen). These results suggest that O-antigen in association with protein contributes to cross-reactivity between molecules. The lacy phenotype was more organ invasive in 5-day-old chicks than isogenic variants producing low molecular weight O-antigen. However, it was no more efficient at contaminating eggs after oral inoculation of hens than a variant that completely lacked O-antigen, thus the lacy phenotype is classified as an intermediately invasive organism. The distinctive colonial phenotype of SE6-E21^{lacy} was used to investigate environmental factors that decreased O/C ratios and contributed to attenuation. In so doing, it was found that growth in complement at 46 °C caused matrix producing cells to hyperflagellate and migrate across agar surfaces. These results suggest that the structure of O-antigen might influence the secretion and/or the function of Enteritidis cell-surface proteins. The data also reveal a greater heterogeneity than has been assumed in the phenotype, and possibly the infectious behaviour, of Enteritidis.

INTRODUCTION

Salmonella enterica serovar Enteritidis is a food-borne pathogen of humans associated most often with consumption of contaminated eggs in the United States [1–4]. Enteritidis isolates have been subdivided by a variety of methods in attempts to identify molecular markers useful for associating human outbreaks with chicken flocks producing contaminated eggs [5]. Phage-typing is frequently used to subdivide Enteritidis [6]. However, phage are unable to distinguish between isolates with differing concentrations of surface receptor except when the amount of phage receptor becomes rate-limiting for attach-

ment [7]. This limitation is significant for determining the relationship between phage-type and pathogenic potential of strains since receptors can be virulence factors that decrease in amount and function before a change in phage-type is detected [8]. Indeed, the Enteritidis scheme has shown that there is no absolute correlation between phage-type and virulence in chickens [9], and genetic analyses have indicated that few distinct genetic lineages of Enteritidis exist [5, 10–13].

An important receptor in phage-typing is lipopolysaccharide (LPS). LPS is associated with efficient contamination of eggs and recovery of high numbers of organisms from chick spleens regardless of phage-type [9, 14]. Heterogeneity and non-stoichiometric modifications to the complex structure of LPS [15], correlate with differences in invasiveness of Enteritidis. An O-antigen to core (O/C) ratio of 1·6 μg rhamnose/μg KDO or greater distinguished invasive from attenuated strains [9, 14]. Enteritidis O-antigen exists as high molecular weight (HMW) and low molecular weight (LMW), and it is HMW O-antigen that identifies virulent Enteritidis [unpublished data]. While investigating how isolates could be subdivided according to invasiveness and O/C ratios, it was found that stored cultures of virulent Enteritidis had converted to a new wrinkled colonial phenotype without loss of O-antigen. This is a phenotype associated with survival of organisms under extreme conditions such as excess acid and peroxidation [16]. This paper reports colony immunoblotting to detect HMW O-antigen, and further evidence of it as a method to subtype O-antigen positive Enteritidis is presented. Here we suggest that colony morphology can be used as an indicator of HMW O-antigen, and population shifts were used to determine environmental factors that result in O-antigen variation.

MATERIALS AND METHODS

Enteritidis variants and growth conditions

Three Enteritidis isogenic LPS variants that were rough (SE6-R), semi-smooth (SE6-E5), and smooth (SE6-E21^{lacy}) were grown for 16 h at 42 °C to stationary phase in 20 ml brain heart infusion (BHI) broth (Difco, Detroit, Michigan) without aeration, or on brilliant green (BG) agar (BBL, Cockeysville, Maryland). SE6-E21^{lacy}, SE6-E5, SE6-R are isogenic strains, and were isolated from a single parent isolate after conducting infectivity studies [14]. Storage of the previously described virulent strain of Enteritidis in nutrient agar stabs for over 6 months generated SE6-E21^{lacy} [9, 14]. The O/C characteristics of SE6-R and SE6-E5, with respective O/C ratios of 0 and 1 μg rhamnose/μg KDO, have remained the same for over 3 years, and because they are stable, they were designated as control variants for conducting infectivity studies and *in vitro* comparisons. Biochemical profiles and serotyping were performed according to standard techniques using commercially available profiling (Enterotube; BBL) and serotyping (Difco) reagents. Phage-typing was performed by National Veterinary Services Laboratory, Ames, Iowa. Plasmid DNA was isolated from 100 ml of cells grown in BHI broth for 16 h at 37 °C in a shaking water bath using the method of Portnoy and White [17].

To detect the unusual colony morphology associated with the SE6-E21^{lacy} phenotype, stationary phase cells were diluted to obtain isolated colonies on BG agar, which were grown for 16 h at 42 °C. The lacy phenotype was induced by growth of colonies for an additional 24 h at 25 °C, and matrix was collected from these colonies after a total of 72 h growth at 25 °C as described below.

Virulence characterization

Five-day-old Leghorn chicks, hatched and raised in a forced-air positive pressure (FAPP) biocontainment facility, were inoculated orally with isogenic LPS variants SE6-R, SE6-E5, and SE6-E21^{lacy} in three separate experiments. Chicks were housed in Horsefall isolator units 2 days before infection, and cultures of cage papers were negative for the presence of *Salmonella* spp. prior to infection. The dose of Enteritidis was determined directly from the inoculum by enumerating colonies on BG agar. Spleens were collected and cultured 3 days after inoculation as previously described [9, 14]. In addition to chick experiments 2 groups of 12, 30-week-old hens were inoculated orally with $2{\cdot}7 \times 10^9$ cells. Isolation of bacteria from eggs was described elsewhere [18].

Isolation, electrophoresis, and immunoblotting of matrix

Matrix was isolated from 3-day-old colonies by lysing cells scraped from BG agar in an alkaline lysis buffer (100 mM sodium dodecyl sulfate [SDS], 50 mM Trizma base, and 0·128 M NaOH) [9, 14]. Lysis was for 10 min at 25 °C, after which the insoluble matrix was pelleted

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at 10000 g for 10 min, washed three times with 20 mM Tris–HCl, pH 7·5, and finally suspended in the same buffer at 100 μl per 50 μg (wet weight) cell pellet. Polyacrylamide gel electrophoresis (PAGE) was done by standard protocol [19, 20]. Prior to loading, matrix in buffer was passed through a 20 gauge needle to facilitate solubilization. An equal volume of loading buffer was added to sample, and 40 μl of this suspension was boiled for 10 min prior to electrophoresis. From top to bottom in gels, molecular weight markers were phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase and β-lactoglobulin.

For immunoanalysis of electrophoresed matrix, proteins were transferred from gels to nitrocellulose membranes using a semi-dry transfer apparatus (Hoefer, San Francisco, California) and following the manufacturer's recommended procedures [19]. Transfer of proteins to nitrocellulose membranes was confirmed by Ponceau S staining [19]. Monoclonal antibodies (MAbs) used for western blot characterization of matrix were obtained by immunization of mice with formalin killed Enteritidis whole cell preparation as described elsewhere [21], and MAbs were diluted 1:5. Secondary conjugated antibody was goat anti-mouse alkaline phosphatase labelled antibody (Pierce, Rockford, Illinois). A commercially available developer was used to produce signal (BCIP/NBT; Kirkegaard and Perry, Gaithersburg, Maryland).

Analysis of cell density, complement resistance, and cell surface proteins

To determine the maximum cell density (CFU/ml) to which the smooth lacy phenotype of Enteritidis could grow, SE6-E21 was serially passaged 10 times with selection of 1 culture out of 10 that produced the highest opacity in broth culture after 7 h incubation at 42 °C. This culture was diluted for counting on BG agar, and from which ten more cultures were grown from single colonies, and selected for highest opacity after 7 h incubation. Cultures were grown in 20 ml of BHI broth in 20 × 150 mm capped tubes without aeration. Opacity was determined at 600 nm using an LKBII spectrophotometer.

Complement resistance was determined by inoculating 10^3 cells from SE6-E21^{lacy}, SE6-E5, and SE6-R in 1 ml fresh 80% chicken plasma for 24 h at 42 °C. Plasma was prepared by the addition of 40 ml whole blood to 10 ml citrate buffer (Alsever's solution; Sigma, St Louis, Missouri). and removal of erythrocytes by centrifugation (20 min at 2500 g). Plasma was used within 3 days.

To recover cell-surface proteins after growth in complement, 1 ml stationary cells were vortexed vigorously for 1 min. Cells were harvested at 14000 g in a Beckman microcentrifuge for 2 min. Supernatant proteins were precipitated by the addition of one-tenth volume of 100% tricarboxcylic acid and incubation for 1 h on ice in preweighed tubes. Pellets were collected by centrifugation for 10 min at 14000 g, weighed after removal of supernatant and resuspended in 50 μl Tris–HCl buffer, pH 7·5, per 100 μg protein, which is the amount of protein typically recovered from 1 ml vortexed cells.

Table 1. *Source of non-isogenic Enteritidis isolates*

H. M. Opitz, University of Maine, USA Supplier's accession numbers	
544-1	ME-12
544-2	ME-13
544-3	ME-14
612-1	ME-18
ME-1	ME-41

Colony immunoblot analyses

Colonies from the three isogenic LPS variants and ten non-isogenic Enteritidis isolates (Table 1) were grown for 3 days on BG agar at 42 °C, on inverted plates in sealed humidified containers. Intact colonies were transferred to nitrocellulose membranes (Schleicher and Schuell; Keene, New Hampshire) by placing membranes over colonies for 16 h at 4 °C. Membranes were lifted from colonies and washed three times with shaking in phosphate buffered saline [19] until no observable cells remained on the filters. Blocking reagent, incubation of filters with primary and secondary antibodies, and wash conditions have been described [14]. The polyclonal antiserum used for the detection of O-antigen was group D1, with factors 1, 9, 12 (Difco), diluted 1:100. Secondary conjugated antibody was goat anti-rabbit alkaline phosphatase labelled antibody (Pierce), diluted 1:500. Matrix-producing colonies could not be washed from filters once matrix formed, so the relationship between matrix production and O/C ratio was investigated by growing two sister cultures, which were incubated under the different conditions as described.

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Determination of LPS O/C ratios

Isolation of LPS from isolates SE6-E21^{lacy}, SE6-E5, and SE6-R, derivatization of neutral sugars, and gas chromatographic analysis of derivatized glycosyl residues were performed as previously described, and readers are referred to the original manuscripts for analytical detail [9, 14, 22, 23], KDO amounts (3-deoxy-D-mannooctulosonic acid) were determined using a colorimetric thiobarbituric acid assay [22].

Scanning electron microscopy (SEM)

Preparation of colonies for SEM was based on a procedure designed to examine fungal agar cultures [24]. Sections of colonies were excised from agar plates with a thin layer of agar left attached, and after fixation, dehydration, critical point drying, and sputter coating, sections were examined on a Philips 505 SEM. Stationary phase cell suspensions grown in BHI broth were spotted onto agar surfaces and processed similarly.

RESULTS

Colony morphology and invasiveness of Enteritidis O-antigen variants

A morphological variant was isolated from SE6-E21 that formed a striking lacy colony, and it is referred to as the lacy phenotype (Fig. 1). The lacy phenotype investigated here, SE6-E21^{lacy}, was not found in cultures of the isogenic variants semi-smooth SE6-E5, which had an O/C ratio of 1·1 or less, or in rough SE6-R that lacked O-antigen. The lacy phenotype was found in 4 of 12 fresh Enteritidis isolates obtained from the spleens of naturally infected mice. The lacy phenotype was identified at 40 h grown on BG agar, because longer incubation detected colonies that produced matrix slowly. If the lacy appearance failed to extend to the edge of the colony, the colony was classified as weakly positive. Both smooth and semi-smooth SE6 variants were phage type (PT) 13a, harboured 2 plasmids (55 and 4·6 kbp), and produced identical biochemical profiles typical of salmonellae. Rough SE6-R differed only in that it was PT23 due to the loss of O-antigen.

To establish the virulence characteristics of smooth SE6-E21^{lacy}, infectivity assays were conducted in comparison to those of the minimally invasive isogenic LPS variants SE6-E5 and SE6-R. Results indicated

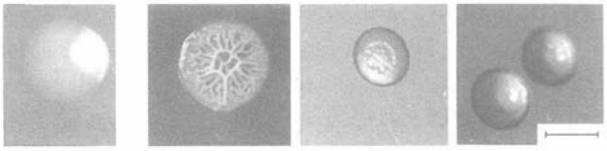

Fig. 1. Colonial phenotypic variants of Enteritidis. From left to right: smooth virulent SE6-E21, smooth SE6-E21^{lacy}, semi-smooth SE6-E5, and rough SE6-R, on BG agar. LPS O/C ratios are 1·6, 2·0, 1·0 and 0 (no O-antigen), respectively. Bar = 5 mm.

that smooth SE6-E21^{lacy} yielded significantly more organisms from chick spleens after oral inoculation than rough SE6-R at dosages of 7·2 and 2·5 × 10^8 CFU, respectively (Student's T test; alpha = 0·05) (Table 2, (i and iii)). There was some overlap in invasiveness of SE6-E21^{lacy} and SE6-E5 at dosages of $1{\cdot}20 \times 10^9$ CFU (Table 2, (i)). In experiment ii, SE6-E21^{lacy} was again compared to SE6-E5 at a dosage of $7{\cdot}0 \times 10^7$ CFU, and at this lower dosage, SE6-E5 was significantly less invasive.

Results from culturing eggs from hens infected with either SE6-E21^{lacy} or SE6-R indicated there was no significant difference in egg contamination between the 2 variants, with 1/175 and 1/199 eggs cultured positive respectively. Therefore, in spite of being more invasive in the chick assay, SE6-E21 was no better at contaminating eggs than avirulent SE6-R and SE6-E5, and all three variants contaminated no more than 1 % of eggs collected during a 21-day period following infection.

Matrix is O-antigen complexed with many proteins including flagellin and a protein of *ca.* 35 kDa

Analysis of LPS neutral sugars derived from O-antigen and measurements of core KDO indicated that the LPS O/C ratio for SE6-E21^{lacy} was 2 μg rhamnose/μg KDO. The ratios showed that SE6-E21^{lacy} produced significant amounts of HMW O-antigen compared to semi-smooth SE6-E5 and rough SE6-R, which had O/C ratios of 1 and 0 as previously determined. Thus SE6-E21^{lacy} produced HMW O-antigen similar to that of the SE6-E21 from which it originated. Since molecules other than O-antigen contribute to virulence, an attempt was made to identify other cell-surface changes that might contribute to the intermediate virulence of SE6-E21^{lacy}.

Electron micrographs of colonies from smooth SE6-E21^{lacy} showed that conversion to the lacy phenotype correlated with production of extracellular

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Table 2. *Invasiveness of three Enteritidis LPS variants for 5-day-old chicks*

LPS variant*	CFU × 10^9 oral dose	Number of chicks infected†	Number of positive spleens	Bacteria (range) per spleen
(i) SE6-E21^{lacy}	1·20	15	12	234 (5–1180)
SE6-E5	1·20	15	9	104 (5–370)
SE6-R	0·72	15	1	< 5
(ii) SE6-E21^{lacy}	0·07	9	7	270 (5–935)
SE6-E5	0·07	9	3	17 (5–35)
(iii) SE6-E21^{lacy}	0·24	40	17	154 (5–1170)
SE6-R	0·25	20	1	< 5

* O/C ratios are 2·0, 1·0 and none for SE6-E21^{lacy}, SE6-E5 and SE6-R, respectively.
† Three comparison experiments are indicated by roman numerals.

matrix that could encase cells (Fig. 2, panels 1–3, 5). The O-antigen deficient variants SE6-E5 and SE6-R, and uninduced SE6-E21, formed matrix weakly or not at all (Fig. 2, panels 4 and 6). Intact flagella were detected in SE6-E21^{lacy} matrix by EM (Fig. 2, panel 1, 3 and 5) but they were apparently not required for matrix formation since patches of cells were found without flagella (Fig. 2, panel 2). Flagella associated with the lacy phenotype frequently formed bundles (Fig. 2, panel 5). Pili were not seen to contribute to matrix formation. Matrix formation was associated with the ridges in colonies that formed the distinctive morphology seen on agar surfaces (Fig. 1), and cells in matrix-producing colonies formed a sharp leading edge in comparison to matrix-negative colonies (Fig. 2, panels 3 and 4). Results from SEM analysis indicated that observation of colonies is adequate for classification of colonies as positive, weakly positive, or negative for the lacy phenotype.

Matrix was easily recovered from cells scraped from agar. Approximately 50 3-day-old SE6-E21^{lacy} colonies yielded 200 μg of insoluble material, whereas tenfold more colonies from SE6-E5 and SE6-R yielded negligible matrix material (Fig. 3, left). Although matrix from the lacy phenotype included many proteins as seen on Coomassie stained gels, only two proteins of 40 and 45 kDa were conspicuous by SDS/PAGE in the small amount of precipitable material recovered from SE6-R and SE6-E5 (Fig. 3, left). Matrix-associated proteins less than 25 kDa were inconspicuous. Approximately 50% of the matrix disaggregated after repeated passage through a needle, while the use of detergents such as deoxycholate, triton X, and CHAPS did not.

Immunoblot analysis of matrix transferred to nitrocellulose membranes showed that of 10 Enteritidis MAbs, 5 produced ladder-like patterns typically seen for O-antigen, with the exception that all 5 produced a dense band at 60 kDa (Fig. 3, right). Whereas HMW O-antigen and flagellin were shown to be components of matrix, other proteins were present in matrix as seen by SDS-PAGE (Fig. 3, left). One 35 kDa protein was detected by MAb 4H3, which also produced a similar immunoblot pattern as O-antigen reactive MAbs (Fig. 3, right). Two MAbs, (a) 2B10 and (b) 6F12, bound just the 35 kDa protein present in the Coomassie stained gel of smooth SE6-E21^{lacy} matrix without producing an O-antigen ladder, but only a weak signal could be detected for either MAb (Fig. 3, right). Thus these two anti-35 kDa MAbs were specific but of low affinity. Since both flagellin and the 35 kDa protein were recognized by MAb 4H3, this MAb appears to recognize an epitope formed in association between LPS with flagellin or the 35 kDa protein. These results indicate that LPS can alter the immunoreactivity of proteins and might be a source of cross-reactivity between proteins and carbohydrates. The inability to dissociate LPS from protein resulted in an unavoidable 'smeary' band pattern on immunoblots since carbohydrates in association with proteins are known to decrease definition of banding patterns in SDS/PAGE.

Heterogeneity of Enteritidis O-antigen to core ratios as detected by colony immunoblots

Since quantitation was required to characterize changes in O/C ratios, colony immunoblotting using group D1 antiserum was tested as a simple method for

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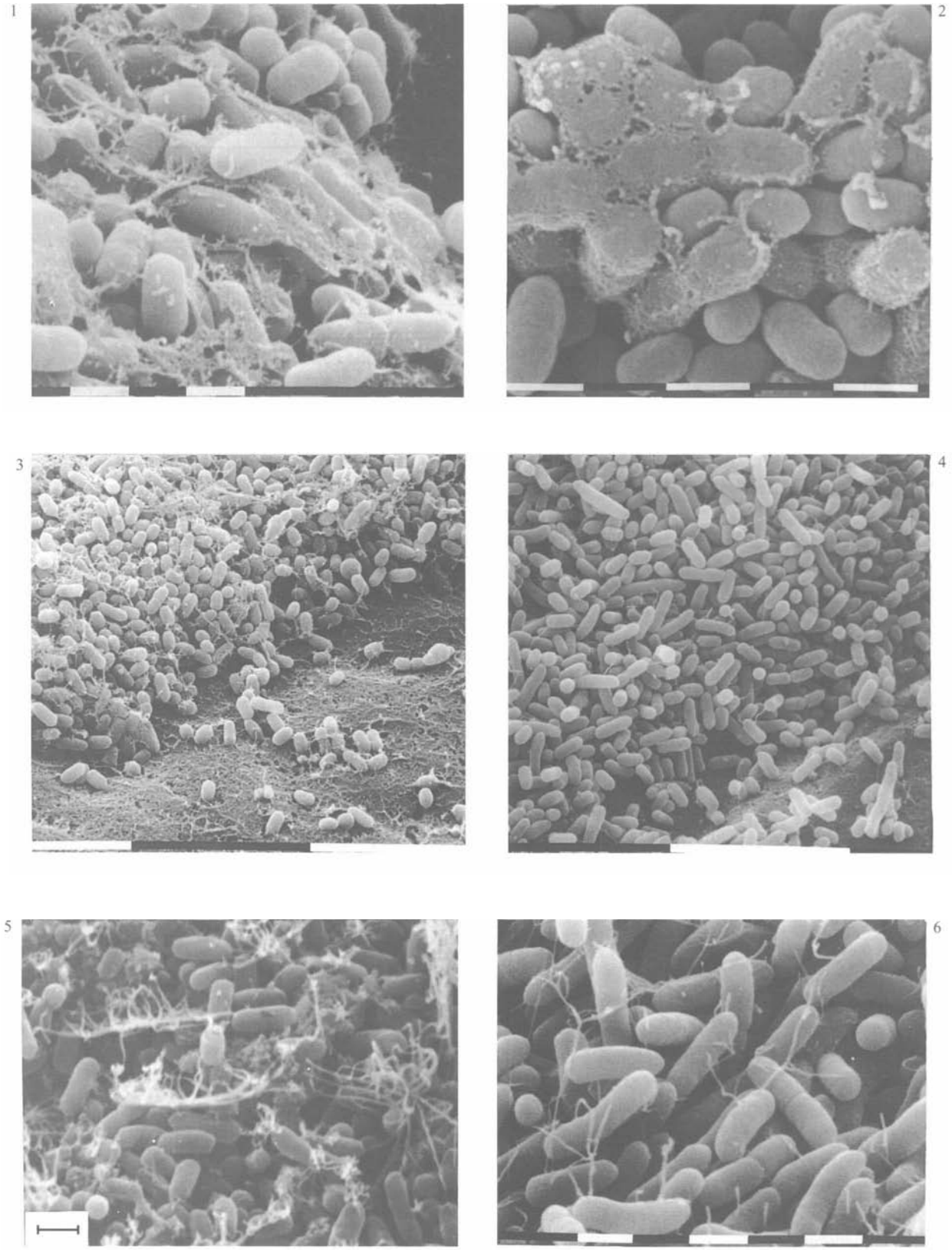

Fig. 2. Characterization of Enteritidis cell-surface matrix by scanning electron microscopy (SEM). Vertical black and white alternating bars (bottom of frames) indicate size. Panel 1, matrix positive lacy phenotype with flagella. Bars = 1 μm; panel 2, cells of the matrix-producing lacy phenotype without flagella. Bars = 1 μm; panel 3, the leading edge of the lacy phenotype grown at 25 °C (permissive temperature). Cells aggregate and form ridges. Bars = 10 μm; panel 4, the leading edge of the lacy phenotype grown at 42 °C (unpermissive temperature). Cells do not aggregate or form ridges. Bars = 10 μm; panel 5,

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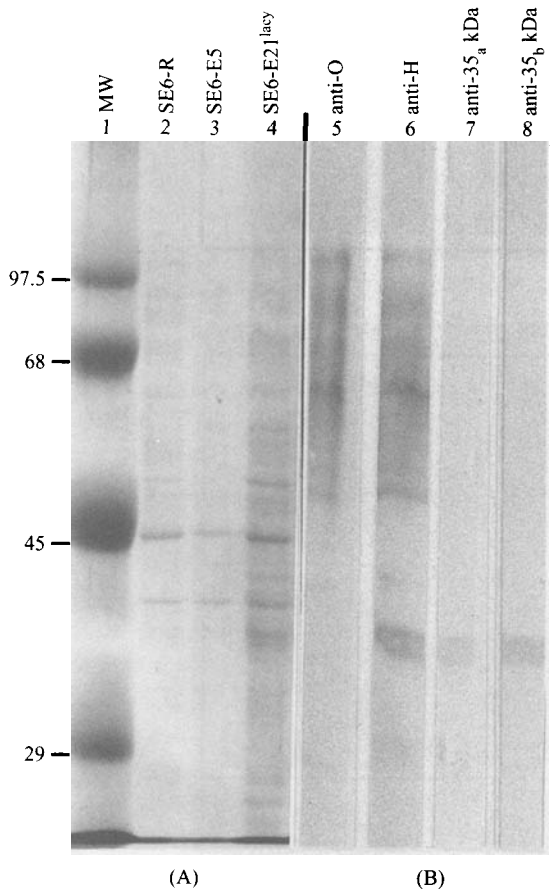

Fig. 3. Characterization of cell-surface matrix from the lacy phenotype of Enteritidis. (A) SDS–10% PAGE: Lane 1. MW markers as indicated in kDa. Lanes 2–4. Matrix from LPS variants SE6-R, SE6-E5 and SE6-E21^{lacy}; (B) Immunoblot: Lanes 5–8. Matrix from smooth SE6-E21^{lacy} transferred to nitrocellulose reacted with monoclonal antibodies raised against killed cells of Enteritidis. Lane 5, serovar group D1 O-antigen reactive MAb; lane 6, g-epitope flagella (H-antigen) specific MAb; lanes 7 and 8, two MAbs (*a*) and (*b*) specific for a 35 kDa protein.

detecting changes in O/C ratios. The lacy phenotype simplified interpretation of this method, because it was an easily recognizable correlate of HMW O-antigen. Thus the lacy phenotype together with a rough phenotype provided appropriate controls for ranking colony immunoblot signals on a scale from 0 to 2. Isolates with O/C ratios of 1 were used to further refine the ranking scale, and methods to generate this phenotype are described herein.

Using the isogenic LPS characterized variants as controls for the interpretation of O-antigen signal intensity (Fig. 4, top row), considerable O-antigen heterogeneity was detected in 2 of 10 field isolates examined as 15 mm colonies (Fig. 4, rows A and B; isolates ME1 and ME18). However, the most common pattern encountered (8 of 10 isolates) was that shown in Fig. 4, row C (isolate ME41), which is typical for colonies with O/C ratios of 1. These results indicate that O/C ratios of 1 are most common and associated with population homogeneity, whereas more intense signals indicate that either part or most of the population produced HMW O-antigen O/C ratios from 1·6 to 2·0. Thus finding O/C ratios that ranged from 1·6 to 2·0 indicated varying degrees of population heterogeneity that contributed to the final HMW O-antigen recovered. Colonies grew to 15 mm in 3 days as long as there were no more than 5 colonies on a plate. Heterogeneity was seen as changes in the amount of O-antigen signal between colonies from a single isolate (Fig. 4, row B), and as sectors within colonies radiating from the centre of the colony (Fig. 4, rows A and B). Minor sectoring occurred at the edge of colonies as colony growth slowed.

Nutrient limitation, O-antigen and loss of matrix

The effect of ageing on cultures was assessed by screening colonial phenotypes from cultures grown to stationary phase and longer. Results were that SE6-E21^{lacy} remained 100% matrix-positive at 16 and 24 h as examined by culture on BG agar. Upon ageing for 48 h, 30% of colonies were weakly positive, and at 72 h the population was composed of 13% lacy colonies, 86% weakly positive colonies, and 1% that did not form matrix. Slide agglutination with O-antigen specific antiserum showed that matrix-negative variants had no O-antigen. After 1 week, the original matrix-positive variant had been entirely lost; the population was now composed of 54% weak positives, 40% that did not form matrix, and 6% that were of a new LPS core variant, which could not be recovered from chick spleens at an i.p. dosage of 6×10^5 organisms.

Selection for maximum cell density, which is another method for placing cells under conditions of limiting nutrients, likewise produced a decrease in O/C ratios. As selection progressed, the CFU/ml increased from 1·07 to $3{\cdot}80 \times 10^9$, and A_{600} optical densities increased from 1·25 to 1·90 (Table 3). A marked shift in the composition of the population occurred at optical density 1·68 as SE6-R emerged to comprise 100% of the population. No change in cell

bundle formation by flagella in a lacy colony. Bar = 2 μm; panel 6, LPS variant SE6-R, which lacks O-antigen and does not form matrix at any temperature, produces typical flagella without detectable bundle formation. Bars = 1 μm.

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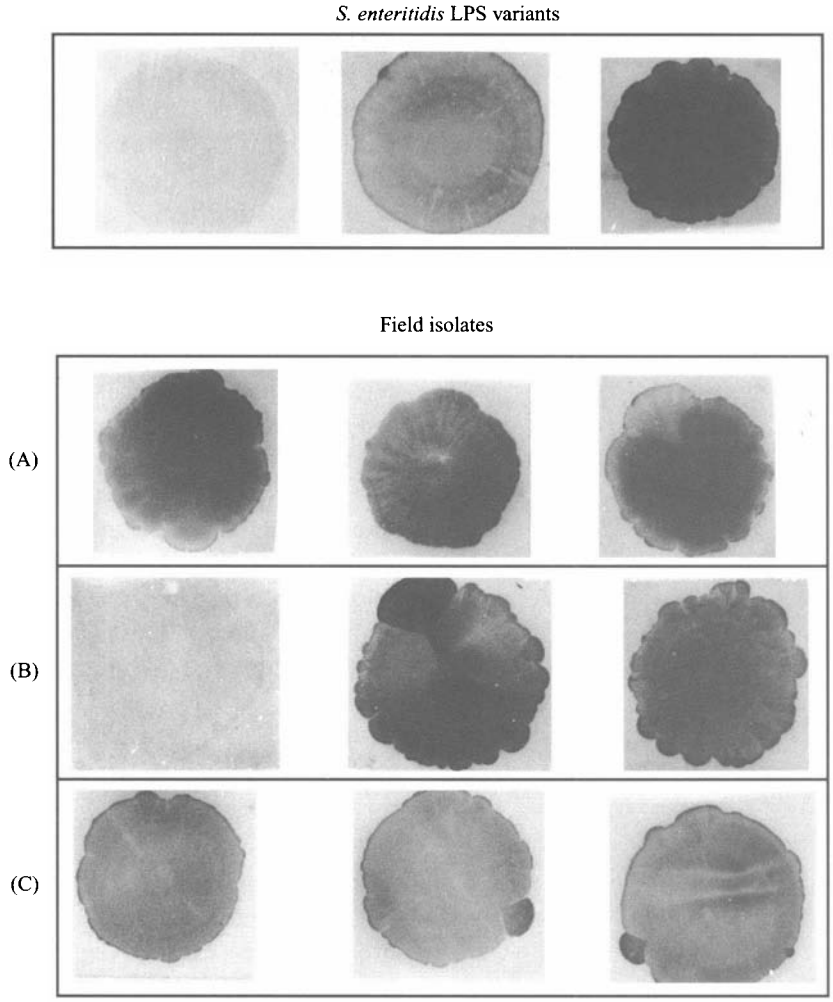

Fig. 4. O-antigen colony immunoblots of Enteritidis. At top, from left to right, characterized Enteritidis SE6 LPS variants with O/C linkage ratios of 0, 1 or 2. Rows A, B and C; immunoblots from three fresh field isolates. From left to right are different colonies picked randomly from each isolate. Primary antibody is commercially available polyclonal D1 antiserum.

Table 3. *Cell density and matrix formation by SE6-E21^{lacy}*

Serial passage	A_{600}	CFU/ml × 10^9	Matrix
1	1·25	1·07	+
2	1·43	2·35	+
3	1·55	2·29	+
4	1·63	2·23	+
5	1·65	2·89	+
6	1·68	2·92	+ and −
7	1·70	3·10	+ and −
8	1·75	3·01	+ and −
9	1·83	3·62	−
10	1·90	3·80	−

Cultures were grown at 42 °C in BHI for 6 h and counted on BG agar.

Table 4. *Growth of Enteritidis O-antigen variants in 80% chicken plasma**†*

	Incubation time (h)	
O-antigen variant	5	24
Smooth SE6-E21^{lacy}	1·30	0·14
Semi-smooth SE6-E5	0·67	0·57
Rough SE6-R	0·01	0·13

* Initial inoculum was 10^3 stationary phase cells.
† Data are CFU/ml × 10^9.

density or colony morphology was seen when smooth SE6-E21^{lacy} was passaged without selection for maximum growth. An additional selection experiment with SE6-E5 indicated that 1·10 was its maximal opacity, which correlated with 1·0 × 10^9 CFU/ml. These results

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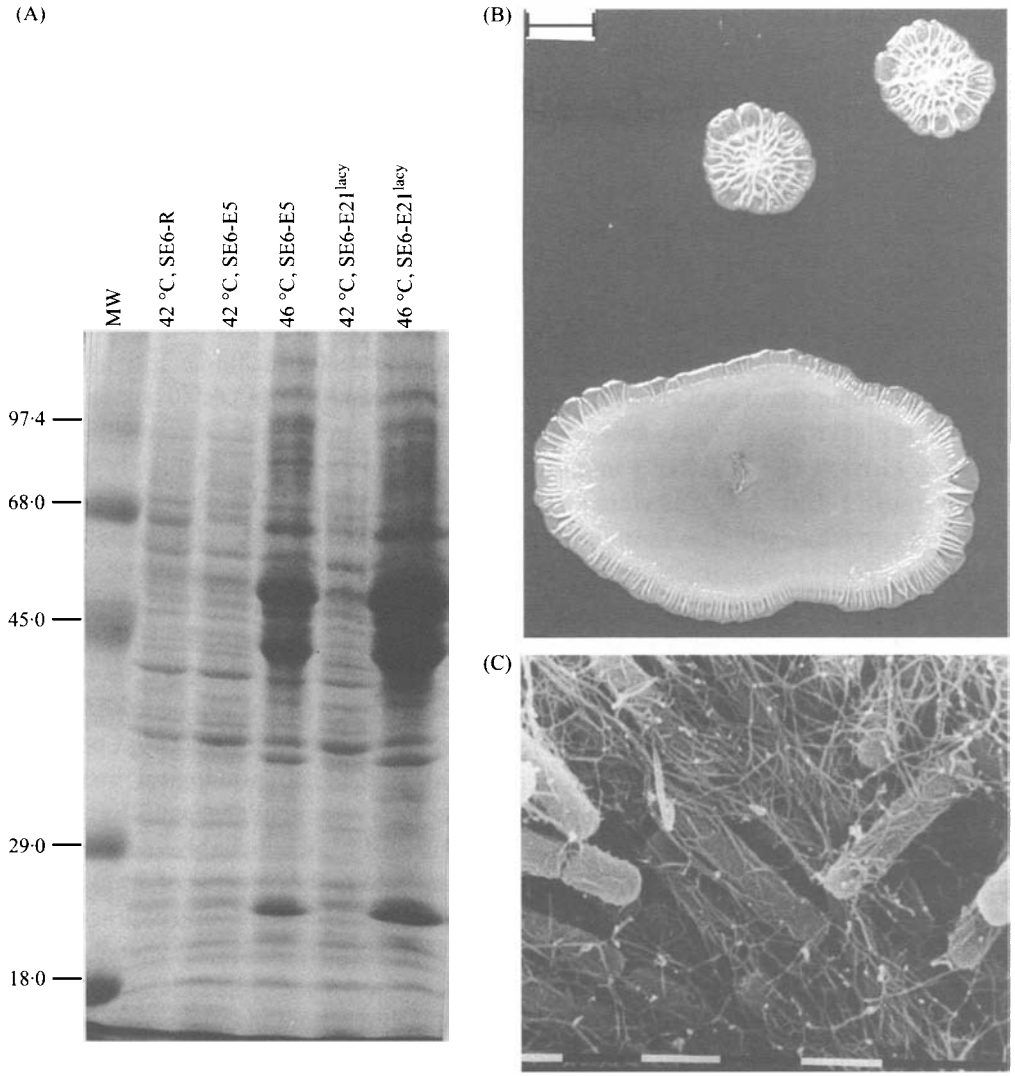

Fig. 5. Detection of Enteritidis hyperflagellation and swarming motility. (A) SDS–10% PAGE of cell-surface proteins collected from LPS variants grown at the indicated temperatures in 80% chicken plasma with complement; (B) Swarming motility (large colony) exhibited by some colonies of SE6-E21^{lacy} grown in complement at 46 °C and passaged to BG agar. Bar = 5 mm. (C) Hyperflagellation detected in the middle region of a swarm colony. Flagella towards the edge of the colony, where matrix formed again, were bundled (Fig. 2, panel 5). Bars = 1 μm.

again identified that variants with O/C ratios of 1 were stable upon passage and resistant to change from selection strategies imposed under laboratory conditions.

Avirulent variants with low O/C ratios grew and survived in complement

Enteritidis LPS variants differed in their ability to grow and survive in 80% chicken plasma. Surprisingly, the less virulent stable variant with an O/C ratio of 1 was the most complement resistant variant. Smooth SE6-E21^{lacy} attained a higher cell concentration in plasma after 5 h than did SE6-E5 (Table 4), but transmission electron microscopy showed that the lacy phenotype population was lysed upon prolonged incubation with complement (data not shown). The rough variant SE6-R grew poorly in plasma, but some growth took place since cell concentration at 24 h versus 5 h increased (Table 4). As initially isolated and tested at low passage (2 and 3), smooth virulent SE6-E21 was as complement resistant as was SE6-E5 at 24 h. Since the lacy phenotype had overgrown the ageing cultures of SE6-E5 on hand, it was not possible to reassess smooth virulent SE6-E21 for complement resistance in a direct comparison to the SE6-E21^{lacy} phenotype. These results again support the finding

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that the LPS variant SE6-E5 is stable over years, while highly virulent LPS variants are subject to change. Eventual loss of complement resistance also suggested that this could be a characteristic that differentiates organ invasive isolates that differ in their ability to contaminate laid eggs.

Smooth SE6-E21^{lacy} can be induced to hyperflagellate

An attempt was made to recover SE6-E21 from stocks overgrown with the lacy phenotype. In so doing, one growth condition was found that resulted in identification of yet another phenotype that may be associated with virulence. Growth of SE6-E21^{lacy} in complement at 46 °C versus 42 °C for 8 h resulted in a 30-fold enhancement of surface flagellation as examined by gel electrophoresis and EM (Fig. 5*a*, lanes 5 and 6 and Fig. 5*c*), and in the ability of cells to migrate across agar surfaces (Fig. 5*b*). Flagellin (54 kDa) was one of 4 proteins enhanced on the surface of these cells, as were unidentified proteins of ca. 45, 32, and 24 kDa (Fig. 5*a*). Tenfold more protein was recovered from semi-smooth SE6-E5 grown in complement at 46 versus 42 °C (Fig. 5*a*, lanes 3 and 4), but these cells did not migrate across agar surfaces when passaged to BG agar. Rough SE6-R did not survive growth at 46 °C. These results suggest that O-antigen is required to undergo this marked, albeit artificially induced, change in flagellation, and that HMW O-antigen improved the degree of hyperflagellation and enhanced motility.

DISCUSSION

To cause severe food-borne gastrointestinal disease in humans Enteritidis must survive quite different environments to those involved in the contamination of eggs. The data presented here showed that populations of pathogenic Enteritidis grown in laboratory cultures undergo radical cell surface variation, and provide an indication of how such a variation may be related to observed changes in invasiveness. Variations in cell surface were shown to be paralleled with substantial changes in the morphology of Enteritidis colonies growing on diagnostic laboratory media, a phenomenon reminiscent of other pathogenic bacteria, and of classical examples of bacterial surface variation, e.g. *Neisseria gonorrhoeae* and *Proteus mirabilis* [15, 26]. Central to surface and colonial variation appears to be the 'lacy' colonial phenotype that maintains a high ratio of O-antigen to core, which generates an associated matrix, and which under certain conditions hyperflagellates and swarms on agar surfaces. Recent investigations have suggested the importance of the lacy phenotype in the epidemiology of Enteritidis since it is enhanced in its resistance to exogenous acid and hydrogen peroxide [27]. Thus, matrix formation possibly provides protection to cells in hostile environments since time must be spent between hosts. Protection against environmental factors is a function sometimes attributed to biofilm formation [28].

When results from previous infectivity studies were compared to results presented here, SE6-E21^{lacy} appears to be an intermediately virulent phenotype that retains a high O/C ratio and the ability to invade organs, but is no longer efficient at egg contamination. These results compare favourably with infection seen in other experiments with non-isogenic isolates, where some chicks yielded hundreds, but not thousands of CFU per spleen [9]. These results were also similar to those obtained earlier when SE6-E5 was used to infect egg-laying hens [14]. Keller and colleagues [29] used SE6-E21 in a histopathological investigation of tissues from hens after challenge with Enteritidis, and found that SE6-E21 was reliably detected in reproductive organs and in the contents of developing eggs, but not in laid eggs. Humphrey and colleagues confirmed that the lacy phenotype was associated with the ability of phage type 4 Enteritidis isolates to infect reproductive organs, but again these did not efficiently contaminate eggs [27]. Thus, the apparent niche for the lacy phenotype is internal organs, including the reproductive tract, but not eggs. Whereas these results suggest that invasiveness of SE6-E21^{lacy} might be less dose dependent than that for SE6-E5, additional experimentation is warranted with more non-isogenic strains before a correlation can be drawn between phenotype and dosage effect.

Since smooth SE6-E21^{lacy} can be induced to produce copious flagella and other proteins on the cell surface, it is probable that the physiological potential of Enteritidis is much greater than is observed in the laboratory. The 60 kDa flagellin protein associated with O-antigen had the amino acid sequence AQVINTNSLSLLTQNNLNKS as determined by Edman degradation N-terminal sequencing, and was exactly the same as that obtained for a 50 kDa band from *S. typhimurium* or a 54 kDa band from Enteritidis. This sequence is the consensus N-terminal sequence of the salmonellae gene product flagellin [30]. Therefore, flagellin is a specific component of matrix. The class of

MAb that produced this pattern yielded results identical to that of O-antigen typing sera in agglutination reactions [21]. The ability to hyperflagellate, which is a virulence trait of other invasive bacteria [31, 32], suggests that the intermediate virulence of smooth SE6-E21^{lacy} could be due to (1) decreased secretion of flagellin and possibly other virulence-associated proteins, (2) decreased production of a global regulator that is perhaps related to heat shock induced sigma factors, or (3) lack of exogenous growth factors, supplied either from the host or the environment, which improve production and secretion of flagellin and other proteins. In any of these cases, SE6-E21^{lacy} is impaired in its ability to undergo the processes that lead to efficient hyperflagellation and production of virulence factors on the cell surface. Since Enteritidis pilins are between 14 and 21 kDa [33, 34, 35], results again support the hypothesis that piliation does not contribute to matrix formation.

Finding that the semi-smooth variant SE6-E5 was well adapted to survival in plasma is another indication that HMW O-antigen influences virulence in more ways that by providing complement resistance and resistance to phagocytosis, which are functions generally attributed to O-antigen [36, 37]. A change in the positioning of a molecule such as the gene product of *tol*C [38], which is involved in secretory processes in bacteria and contributes to complement resistance, could result in highly virulent Enteritidis converting to complement sensitive SE6-E21^{lacy}. Of note is the finding that secretion of *tol*C by Enteritidis is difficult to obtain under laboratory conditions, which supports the concept that suppression of secretion of virulence factors occurs rapidly in cultures of Enteritidis [39]. Thus the role of HMW O-antigen could be to selectively facilitate the secretion of virulence factors such as flagella and *tol*C without loss of complement resistance. LPS is known to affect the secretion of proteins in other bacteria [40, 41].

While we have determined that O-antigen structure is associated with invasiveness, variation in other regions of LPS could also contribute to invasiveness and other measures of virulence such as mortality. Changes in both core and the lipid A region of LPS in the SE6 isogenic variants have been documented [14, unpublished data]. Whereas some changes in core appear to occur in concert with specific O-antigen structures, the degree of change that can occur in the lipid A region has not yet been exhaustively investigated. It is possible that associations between O-antigen and lipid A endotoxin structures will need characterization before the processes that optimize virulence are fully understood.

No laboratory condition has been found that preserves Enteritidis indefinitely, and passage through the host does not influence the incidence of O-antigen LPS variants recovered from organs, i.e. rough variants remain rough after passage through chicks. These findings suggest that *in vitro* factors are important for differentiation and development of virulent bacteria. This suggestion is somewhat contrary to the concept that the *in vivo* environment regulates virulence factors [42]. However, O-antigen could be a notable exception as a virulence factor because it is a complex carbohydrate with a malleable structure that seemingly reflects *post facto* the stresses encountered by bacterial populations during growth outside the host. Indeed, finding that smooth SE6-E21^{lacy} is complement sensitive after lengthy incubation suggests additional selection pressure supplied by the host could be required for development of optimal invasiveness and survival in the egg.

Identification of the lacy colonial phenotype and use of O-antigen colony immunoblot assays has particular application for monitoring bacterial populations during vaccination and challenge studies, especially since interest in the use of recombinant live vaccines and bacterins to protect against Enteritidis in poultry is increasing [43, 44]. Since previous research established that Enteritidis could produce mixed patterns of infection in chicks, the use of colony immunoblots provides additional support that the generation of population heterogeneity is part of the basic biology associated with Enteritidis [9, 14]. While one day old colonies had previously been used for this type of analysis [14], 15 mm colonies provided more information on O-antigen variation in populations of Enteritidis.

In view of current findings, it is probably imperative that a standard challenge isolate for evaluation of Enteritidis vaccines be used between laboratories. Chemical analyses of O/C ratios [9, 14] show differences between isogenic variants and provide objective assessment of LPS structure in comparison to crude methods for visualization of long chain O-antigen in gels [45–47]. Improved gel techniques have been described that can be used to detect virulent Enteritidis [9]. However, the simplest method for the characterization of an adequate, but not optimal, challenge isolate that is capable of infecting the reproductive organs of hens is to confirm that 100%

of Enteritidis cells in a culture produce the lacy colonial phenotype.

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