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Reduction of *Salmonella* Enteritidis in the Spleens of Hens by Bacterins That Vary in Fimbrial Protein SefD

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Abstract

The objective of this research was to determine whether variation in the presence of fimbrial protein SefD would impact efficacy of bacterins as measured by recovery of *Salmonella enterica* serovar Enteritidis (*Salmonella* Enteritidis) from the spleens of hens. Two bacterins were prepared that varied in SefD content. Also, two adjuvants were tested, namely, water-in-oil and aluminum hydroxide gel (alum). Control groups for both adjuvant preparations included infected nonvaccinated hens and uninfected nonvaccinated hens. At 21 days postinfection, *Salmonella* Enteritidis was recovered from 69.7%, 53.1%, and 86.0% from the spleens of all hens vaccinated with bacterins lacking SefD, bacterins that included SefD, and infected nonvaccinated control hens, respectively. No *Salmonella* was recovered from uninfected nonvaccinates. Results from individual trials showed that both bacterins reduced positive spleens, but that the one with SefD was more efficacious. Alum adjuvant had fewer side effects on hens and egg production as compared to water-in-oil. However, adjuvant did not change the relative recovery of *Salmonella* Enteritidis from spleens. These results suggest that SefD is a promising target antigen for improving the efficacy of immunotherapy in hens, and is intended to reduce *Salmonella* Enteritidis in the food supply.

Introduction

Salmonella enterica serovar Enteritidis (*Salmonella* Enteritidis) accounts for nearly 20% of laboratory-confirmed illnesses of salmonellosis in recent years (CDC, 2014). Consumption of shell eggs and egg products is associated with risk of infection from *Salmonella* Enteritidis in humans (CDC, 2003; Cowden *et al*., 1989; Elson *et al*., 2005). A large multistate outbreak of *Salmonella* Enteritidis led to a recall of approximately 500 million eggs. The experience reinforced the necessity of coordinating quality assurance programs with federal regulatory measures (FDA, 2009; CDC, 2011). Management practices that incorporate vaccination to impede introduction and re-emergence of pathogenic serotypes such as *Salmonella* Enteritidis on-farm can be important additions to quality assurance programs for reducing foodborne illness (Gast, 2007; FDA, 2014).

Vaccination of chickens to reduce *Salmonella* Enteritidis on-farm is not a mandatory requirement in the United States (FDA, 2014). Vaccination programs appear to partially protect chickens from infection or transmission of this pathogen through eggs (Vielitz *et al*., 1992; Gast *et al*., 1993; Nassar *et al*., 1994; Hassan and Curtiss, 1996, 1997; Lopes *et al*., 2006; Adriaensen *et al*., 2007). With a goal of improving vaccine efficacy in mind, whole genome comparisons were performed between phenotypic variants of *Salmonella* Enteritidis to find promising antigenic targets (Guard *et al.*, 2011). The gene *sefD*, which is a critical component of the fimbria SEF14, was disrupted by deletion of a single nucleotide in the genome of a non-egg-contaminating strain. Fimbrial proteins are often promising vaccine candidates (Rajashekara *et al*., 2000).

Fimbria SEF14 is encoded by the *sef*ABCD operon, and it is transcribed under regulation of the *sef*R gene (Clouthier *et al*., 1993; Edwards *et al*., 2000, 2001). The operon is restricted to *Salmonella* Enteritidis and other closely related O-antigen group D *Salmonella.* The production of SefD protein was shown to be temperature dependent (Morales *et al*., 2012). Others have shown SefD to be required for cell binding and macrophage internalization, which contributes to the survival of *Salmonella* Enteritidis *in vivo* (Edwards *et al*., 2000). Thus, expression of the fimbria may contribute to

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transfer of the pathogen from the environment to the oral mucosa of the chicken, after which it would be repressed at the body temperature (Morales *et al*., 2012). Because differences in phenotype and virulence associated with expression of SefD were evident, experiments were conducted to explore whether bacterins that varied in SefD content also varied in efficacy against *Salmonella* Enteritidis in laying hens. Two strains, namely, *Salmonella* Enteritidis *sef*D mutant 29108 (ΔSefD 29108) and complemented mutant 100713 (SefD+ 100713) (Morales *et al*., 2012), were used to prepare bacterins. In addition, two adjuvants were used in formulating the vaccine to minimize side effects of vaccination.

Materials and Methods

Bacterial strains

Salmonella Enteritidis PT4 (22079) was the parent strain used to derive all mutants, and it was isolated from a poultry farm environment in California (Kinde *et al*., 1996). The strain was previously characterized as a weak biofilm former and shown to be capable of egg contamination (Guard *et al*., 2011). Strains 29108 and 100713 were derived from strain 22079, and all steps in the construction of the mutant and its complement have been described (Morales *et al*., 2012). ΔSefD 29108 cannot produce SefD because it carries a nonpolar knockout mutation of *sef*D. SefD+ 100713 contains pCR2.1-TOPO with *F* (*lac*Za'-*sef*D+) and it was confirmed to have constitutive transcription of *sefD* (Morales *et al*., 2012). Biofilm formation, which is a marker of phenotype and virulence characteristics, was confirmed as described (Guard *et al*., 2011).

Description of hens and sample collection

A total of 309 specific-pathogen-free White Leghorn hens were obtained from the same laboratory. Each treatment group initially contained 20 birds, and the number of hens tested per group is stated in Table 1. Hens were kept in individual laying cages in a building with environmental monitors for light and temperature. Hens that showed signs of morbidity were euthanized and removed from the study without contributing data from culturing spleens. Water and feed were provided *ad libitum*. Hens were euthanized 21 days after challenge for sampling, which is a time associated postinfection with persistence in spleens but not oviducts (Guard *et al*., 2010). Spleens were aseptically removed and stored at 4°C until processed for isolation of *Salmonella* Enteritidis. Egg production was recorded from 14 days before the first immunization through 21 days after challenge. Mean daily egg production per treatment group per surviving hen was calculated for each group as described (Fialho *et al*., 2001). The length of time between vaccine doses varied between trials because flock egg production was allowed to rebound before potentially stressing hens with a second vaccination.

Preparation of cells for use in bacterins

ΔSefD 29108 and SefD+ 100713 were cultured from frozen stocks onto brilliant green (BG) agar (Acumedia, Neogen Corporation, Lansing, MI) at 37°C for 24 h. One colony-forming unit (CFU) was transferred to 10 mL of BHI broth and incubated for 24 h at 37°C with shaking at 150 rpm (Innova 4000; New Brunswick Scientific, Enfield, CT). Two (2) mL of cell culture was transferred to 500 mL BHI broth in 1-L flasks, and a total of up to 2 L was prepared per strain. Media for SefD + 100713 was supplemented with 100 μg/mL ampicillin to maintain the plasmid. Cultures were incubated in water baths at 37°C for 24 h with shaking and then placed at 4°C for 2 h. Cells were pelleted at 10,000 × *g* for 10 min at 5°C (Sorvall RC 4B Plus centrifuge). Pellets were washed twice in 300 mL sterile phosphate-buffered saline (PBS) (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl [pH 7.4]) and pelleted again. Pellet weights were recorded.

Batches of cultures were combined and suspended in 10 mL PBS, and viable cell counts were determined. Bacterins are vaccines prepared from inactivated cells prepared by adding a kill step. To kill cells, preparations contained in 50-mL capped vials were submerged in a water bath at 60°C for 60 min. Thimerosal was added to a final concentration of 0.01%. To confirm that cells were killed, a 0.5-mL volume of the final preparation was tested by cultivation onto BG and in BHI. Inactivated cells were kept at −80°C until use. Methods describing other details of characterization, namely, determination of relative amounts of lipopolysaccharide (LPS) O-antigen, transmission electron microscopy (TEM), protein extraction and immunoblot analysis for presence of SefD, and tests for stability are described in Supplementary Data S1 (Supplementary Data are available online at www.liebertpub.com/fpd).

Preparation of water-in-oil emulsified bacterins

Cell suspensions, 4 mL, were mixed in a PRO400 homogenizer (ProScientific Inc., Oxford, CT) at 8000 rpm for 2.5 min with 16 mL of an oil mixture containing 80% (vol/vol) Hexadecane (Sigma-Aldrich, St. Louis, MO), 10% (vol/vol) Span 80 (Sigma-Aldrich), 7% (vol/vol) TWEEN 85 (Sigma-Aldrich), and 3% (vol/vol) Span 85 (Sigma-Aldrich) following a published protocol for producing oil-emulsion bacterins (Gast *et al*., 1993).

Adjustments to two batches of bacterins emulsified with a water-in-oil adjuvant

One liter of 16-h culture was used to produce a total of 5 mL of inactivated suspensions for the first vaccine dose. For the second round of dosing, cells were grown 8 h longer to increase cell density. One liter of a 24-h culture was concentrated to 5 mL by centrifugation as described previously, and an additional 20% (vol/vol) PBS was added. In the second set of hens used to evaluate the bacterins in water-in-oil adjuvant, 1 L of 24-h culture was concentrated to a final volume of 10 mL and an additional 20% (vol/vol) in PBS was added to the first and second vaccine doses. These adjustments were necessary to achieve the appropriate number of doses for the number of hens included in trials.

Details of vaccination of hens with bacterins in water-in-oil adjuvant

Hens receiving water-in-oil bacterins were vaccinated as two trials. Hens in the first trial began vaccination at 25.4 weeks of age with bacterins delivered in 0.2-mL volumes containing 3.3 or 3.7 mg total protein, respectively, for preparations made from ΔSefD 29108 and SefD+ 100713.

Table 1. Summary Description of Experimental Treatment Groups Vaccinated with *Salmonella enterica* Serovar Enteritidis Bacterins That Vary in SefD Content and Adjuvant

Experiment (adjuvant)	*Vaccine (adjuvant)*	*Initial number of hens*	*1st Vaccine dose[a] (mg/mL)*	*2nd Vaccine dose[a] (mg/mL)*	*Hen age at 1st and 2nd vaccination (wks)*	*Days between 1st and 2nd vaccination, and challenge*	*Challenge dose (CFU/0.5 mL)*	*% Mortality*	*% Positive spleens*
1 (Water-in-oil)	ΔSefD 29108[b]	21	3.3	0.6	25/32	45/28	6.9±0.7 exp7	4.8[c]	85.7
	SefD+ 100713[d]	20	3.7	0.6	25/32	45/28	6.9±0.7 exp7	15.0	58.8
	None	20	NA	NA	25/32	NA	6.9±0.7 exp7	5.0	100
	None	20	NA	NA	25/32	NA	NA	10.0	0
2 (Water-in-oil)	ΔSefD 29108	21	0.45	0.45	27/32	35/28	6.3±0.7 exp7	14.3	72.2
	SefD+100713	23	0.52	0.52	27/32	35/28	6.3±0.7 exp7	30.4	56.3
	None	24	NA	NA	27/32	NA	6.3±0.7 exp7	4.2	78.3
	None	20	NA	NA	27/32	NA	NA	15.0	0
3 (Alum)	ΔSefD 29108	24	0.37	0.43	25/29	28/25	6.0±0.4 exp7	0	58.3
	SefD+ 100713	24	0.4	0.36	25/29	28/25	6.0±0.4 exp7	0	50
	ΔSefD 29108	24	0.37	0.43	25/29	28/25	3.0±0.2 exp7	0	65.2
	SefD+ 100713	24	0.4	0.36	25/29	28/25	3.0±0.2 exp7	0	50
	None	24	NA	NA	25/29	NA	6.0±0.7 exp7	37.5	80
	None	20	NA	NA	25/29	NA	NA	0	0

[a]Protein concentration of total antigen in 0.2 mL of vaccine dose.
[b]ΔSefD 291082 is negative for SefD because of a knockout mutation in the gene *sefD*.
[c]One hen that died the last day of experiment was included for calculating mortality (20 survived), but the spleen was cultured and included in the 21 spleens cultured since it survived until the last day of the experiment.
[d]SefD+ 100713 is also a knockout mutant of *sefD*, but it is positive for protein SefD because it carries a complementing plasmid with constitutive expression of *sefD*.
CFU, colony-forming units; NA, not applicable.

Vaccines were administered subcutaneously (SQ) in the neck. Six weeks later, hens at 31.7 weeks of age received a second immunization containing 0.60 mg total protein in both bacterins in 0.2 mL for hens. Birds were challenged intramuscularly (IM) 4 weeks after the second immunization with $6.9 \pm 0.7 \times 10^7$ CFU of parent strain 22079 in 0.5 mL of PBS. The second trial started vaccination at 26.7 weeks of age, again at 31.7 weeks of age, and then challenged was at 35.7 weeks of age. Bacterins ΔSefD 29108 and SefD+ 100713 had 0.47 and 0.52 mg per hen in the first and second vaccine dose, respectively. The infectious dose 4 weeks after the second vaccination in the second trial was $6.3 \pm 0.7 \times 10^7$ CFU of parent strain 22079.

Preparation of bacterins by emulsification in aluminium hydroxide gel (alum)

Cells were prepared as described for water-in-oil bacterin. To emulsify cells with alum, suspensions were first homogenized in a Bullet Blender® (NextAdvance, Averill Park, NY) as indicated in the protocol for *E. coli* cultures (NextAdvance, 2013). Four (4) mL of the homogenized cell suspensions were mixed in a PowerGen 125 homogenizer (Fisher Scientific) at wheel scale 4 for 2.5–3.0 min with 16 mL of Alhydrogel® (Sergeant, Clifton, NJ).

Details of vaccination of hens with alum

Hens from the same flock were split into two separate rooms. Each set of hens was vaccinated SQ at 25 and 29 wks with 0.2-mL doses containing 0.37 or 0.40 mg total protein from ΔSefD 29108 and SefD+ 100713 bacterins, respectively. Second vaccinations contained 0.43 or 0.36 mg in 0.22 or 0.18 mL, respectively. Four weeks after immunization, hens that were in one room were infected IM with $6.0 \pm 0.04 \times 10^7$ CFU of parent strain 22079 in 0.5 mL PBS. Hens in the second room received a challenge dose of $3.0 \pm 0.02 \times 10^7$ CFU. Both groups of hens, namely, those infected at 6.0×10^7 and 3.0×10^7 CFU, shared the same control group of hens to reduce the use of animals in research.

Cultivation of spleens for isolation of Salmonella *Enteritidis*

Spleens were held at 4°C after collection and processed 24 h after collection. Each spleen was placed in 100-mL sample bags with 10 mL of trypticase soy broth (Acumedia) and then pummeled for 1 min at high speed in a Stomacher® 80 Biomaster (Seward, Worthing, UK). Samples were incubated at 37°C for 48 h and a 100-μL aliquot was streaked onto BG agar. Plates were incubated for 24 h at 37°C and identities of suspected colonies were biochemically determined by the Enterotube II method (Difco BD, Detroit, MI).

Statistical analysis

The number of eggs produced daily by each group of hens and the average O-antigen LPS antibody titers against Group D *Salmonella* was compared using the Student *t*-test. Mean daily egg production per hen per experimental group, adjusted for any hens removed from the study due to morbidity or premature mortality, was evaluated using a multivariate analysis of variance (SYSTAT v13.1; Systat Software, Inc., San Jose, CA). Raw data, comprised of negative and positive spleens per group, were analyzed by Fisher exact test for determination of probability values (p-value).

Results

Variation of SefD correlated with changes in cell shape, colony morphology, and lipopolysaccharide O-antigen

Colony morphology of SefD+ 100713 varied from that of ΔSefD 29108 and parental strain 22079, and the latter two were similar to each other. Parent 22079 and ΔSefD 29108 had colonies with a wrinkled appearance previously associated with biofilm formation, whereas those from SefD+ 100713 had a smooth appearance, when grown on selective agar media (BG agar, Acumedia) (Fig. 1) (Guard *et al.*, 2011). Growth characteristics also differed between strains. On average, the ΔSefD 29108 cell suspension had 3.93 ± 1.2 times more cells than that estimated for SefD+ 100713 per preparation, which was equivalent to CFU/mL of 3.27×10^{13} versus 0.83×10^{13}, respectively. However, pellet weights were very similar between strains (data not shown). Transmission electron microscopy showed that CFU counts differed because the length of SefD+ 100713 cells was up to 10 times greater than that of the ΔSefD 29108 cells (Fig. 2). Therefore, it appears that fewer but longer cells of SefD+ 100713 have similar pellet weights as compared to more numerous but shorter cells of ΔSefD 29108. Parental strain 22079 and mutant ΔSefD 29108 each produced similar amounts of LPS O-antigen binding sites, but more O-antigen than SefD+ 100713 ($p < 0.001$) (Supplementary Material S1). Overall, 22079 and 29108 had 2^3 more O-antigen binding sites than did SefD+ 100713.

SefD was detected in cell preparations made from SefD+ 100713

Expression of SefD was confirmed for SefD+ 100713 by the observation of a strong signal that was not detectable in parental 22079 or ΔSefD 29108 when the peptide-generated labeled antibody was used for immunoblots (Fig. 3). This result agreed with previous results that transcription of *sef*D was present for SefD+ 100713 strain and absent in ΔSefD

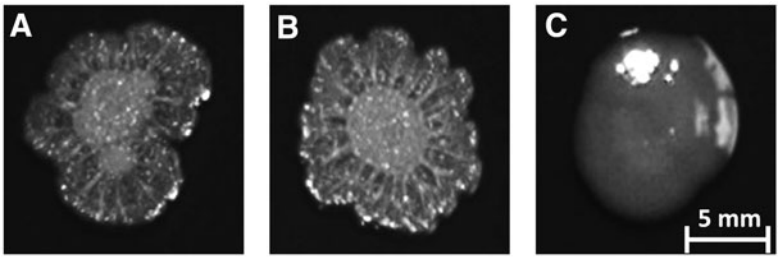

FIG. 1. Colony morphology of *Salmonella* Enteritidis that varies in SefD. Contrast and brightness of images were adjusted 30% to make image suitable for publication. **(A)** Parental strain 22079 PT4, which has a complete *sef*D gene but is negative for transcription. **(B)** ΔSefD 29108, which has a knockout mutation of *sefD*. **(C)** SefD+ 100713, which constitutively expresses SefD from a plasmid.

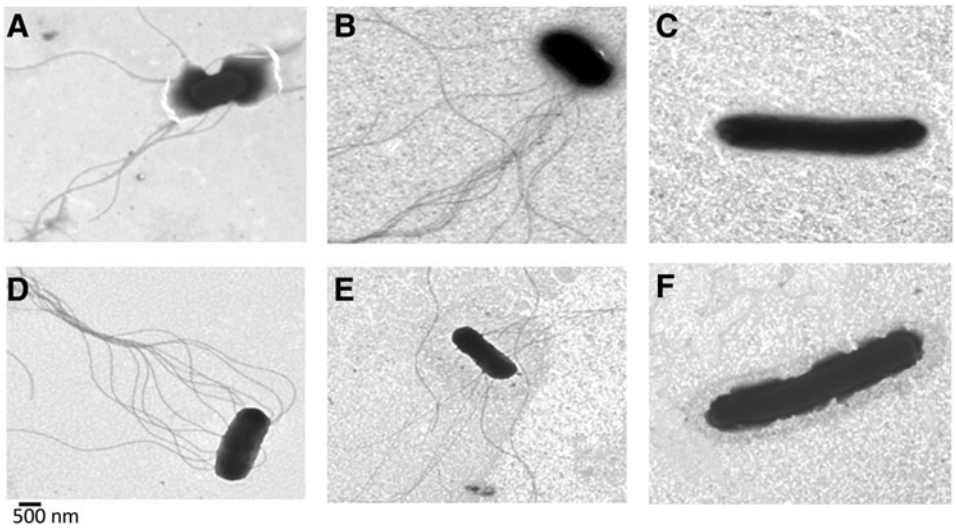

FIG. 2. Transmission electron microscopy micrographs of *Salmonella* Enteritidis. Bacteria were cultured in brain heart infusion broth for 24 h **(A, B, C)** or 16 h **(D, E, F)** at 37°C. (A, D), Parental strain 22079 PT4, which has a complete *sef*D but lacks transcription. (B, E), ΔSefD 29108, which has a knockout mutation of *sefD*. (C, F) SefD+ 100713, which constitutively expresses SefD from a plasmid.

29108 and parent 22079 (Guard *et al*., 2012). Agreement between results from transcription studies and immunologic analysis of cell surface properties is important because it strongly suggests the fimbria was assembled on the outer membrane. It has been reported that isolation of SefD is difficult (Clouthier *et al*., 1994). Immunoblot assay was also done using a commercial SEF14 antibody to provide some evidence of the SEF14 fimbria content of bacterins (Edwards *et al*., 2000). The SEF14 antibody used under nonreducing conditions detected a band of ≈14 kDa in extracts from the parent, mutant, and complemented mutant (data not shown).

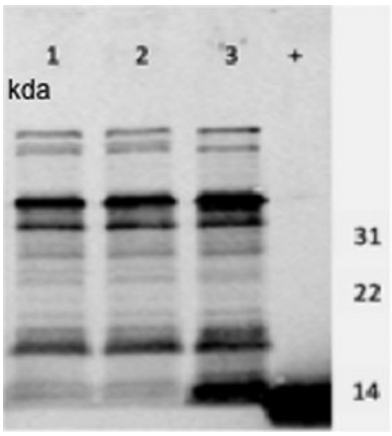

FIG. 3. Immunoblot assay of *Salmonella* Enteritidis strains used to produce bacterins that varied in SefD content. **Lane 1:** Parental 22079, no *sefD* transcription at 37°C but does have the intact gene. **Lane 2:** ΔSefD 29108, which has a knockout mutation of *sefD*. **Lane 3:** SefD+ 100713, producing SefD constitutively by a plasmid containing sefD. **Lane 4:** SefD neutralizing peptide, which was included as a positive control.

Salmonella *Enteritidis was reduced in spleens by vaccination, but more so by SefD+100713*

Overall, 49 of 57 spleens (86.0%) were positive at 21 days after challenge of unvaccinated hens (Table 1). For hens vaccinated with ΔSefD 29108 and then challenged, 60 of 86 spleens (69.8%) were positive (Table 1). For all hens vaccinated with SefD+ 100713 and challenged, 43 of 81 spleens (53.1%) were positive (Table 1). The range of positive spleens was between 50% and 60% for SefD+ 100713 and between 58.3% and 85.7% for ΔSefD 29108. These results were consistent across four different sets of birds divided as two independent trials for hens with water-in-oil bacterins and one trial of hens vaccinated with alum preparations as a paired set. Bacterin ΔSefD 29108 reduced the number of positive spleens as compared to unvaccinated controls that were also infected ($p = 0.02$ one tail). Bacterin SefD+ 100713 also reduced the number of positive spleens as compared to unvaccinated controls ($p < 0.0001$ one tail). In summary, results support that SefD+ 100713 performed better than did ΔSefD 29108 at reducing *Salmonella* Enteritidis in the spleens of hens under the experimental parameters described ($p = 0.02$ one tail). More details of statistical analyses presented in Table 1 are included in Supplementary Data S2.

Adjuvant-associated side effects on egg production and mortality

A 30-day moving average was applied to each group laying eggs daily (% daily egg production) to minimize daily fluctuations from variables such as when eggs were collected or minor differences between flocks due to weather and age (Fig. 4). Trends in egg production for hens infected with *Salmonella* Enteritidis are somewhat predictable. For example, no significant differences in egg production were observed between any of the groups before vaccination with either water-in-oil or alum

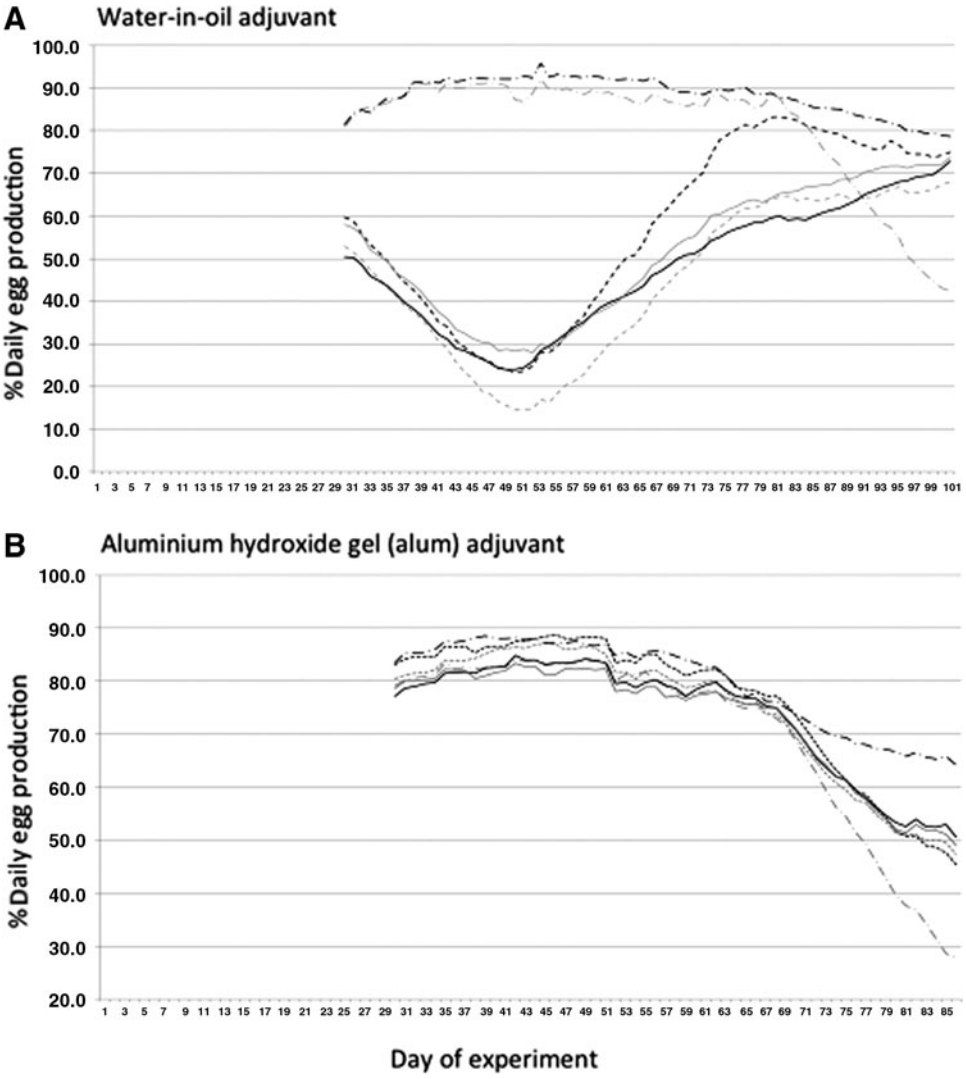

FIG. 4. Percent (%) daily egg production for hens that vary in vaccination and challenge status for *Salmonella* Enteritidis. A 30-day moving average was applied as discussed in text (**A** and **B**). Challenge was at day 80 for hens receiving bacterin in water-in-oil (A) and at day 65 for hens immunized with the alum adjuvant bacterin (B). Egg production was similar between the two groups receiving water-in-oil bacterin (data not shown), so only the data from the second group is presented. Lines associated with treatment groups are as follows:
a. (– – –) Vaccinated with bacterin ΔSefD 29108 then infected with wild type (WT).
b. (– – –) Vaccinated with bacterin SefD+ 100713 then infected with WT.
c. (–·–·–) Not vaccinated, but infected with WT.
d. (——) Vaccinated with bacterin ΔSefD 29108, but not infected.
e. (——) Vaccinated with bacterin SefD+ 100713, but not infected.
f. (–·–·–) Not vaccinated and not infected.

preparations ($p > 0.05$). An expected decline in egg production was observed for all treatment groups as hens passed peak production. It was also expected that egg production would drop ($p < 0.01$) in unvaccinated hens that were challenged (Fig. 4A and B, line c), and that it would remain highest for unvaccinated hens that were not challenged (Fig. 4A and B, line f).

Both groups of hens that were vaccinated with water-in-oil preparations experienced a similar ($p < 0.01$) decline in egg production. Results of egg production for the second test of the water-in-oil bacterin are shown (Fig. 4A, lines a, b, d, and e). In contrast, hens vaccinated with bacterins emulsified with alum showed no significant decline in egg production after vaccination (Fig. 4B, lines a, b, d, and e). Thus, adjuvant had a major impact on the response of the hen to vaccination as assayed by egg production. Additional repetition would help to assess further how changing to the alum adjuvant impacted performance of bacterins.

Mortality also differed between groups, and it was highest for hens receiving SefD+ 100713 prepared in water-in-oil adjuvant (Table 1). Mortality was two- to three-fold higher for SefD+ 100713 than it was for ΔSefD 29108. Changing adjuvant to alum may have helped to decrease mortality and impact on egg production following vaccination. However, calculations suggest that up to 4 deaths per 24 hens over 21 days is within 1 SD of average loss (Supplementary Data S2). Again, further analysis would be necessary to determine whether a change of adjuvant lowered mortality or whether observed mortality was within parameters expected for hens in production.

Discussion

The presence of the fimbrial protein SefD appeared to improve the efficacy of a bacterin for reducing *Salmonella* Enteritidis in the spleens of laying hens under these experimental conditions ($p = 0.02$ Fisher exact test). Constitutive expression of SefD had consequences for the bacterial cell, and efficacy increased with loss of O-antigen, loss of biofilm, and presence of SefD. Expression of *sefD* impacted morphological transitions that somewhat resemble the sessile and planktonic states exhibited by other pathogens (Moreno-Paz *et al*., 2010). Bacterins that are currently marketed probably lack SefD, because wild-type strains appear to be functional *sefD* mutants (Morales *et al*., 2012). It is common to find natural mutants of *sefD*. For example, point mutations present

in *sefD* reference sequences of *Salmonella* Gallinarum, *Salmonella* Typhi, *Salmonella* Paratyphi A, and some field isolates of *Salmonella* Enteritidis occur at the same location within a penta-adenosine region of the open reading frame. *Salmonella* Enteritidis generates strain heterogeneity due to the accumulation of single nucleotide polymorphisms, and these experiments were conducted with a well-characterized and sequenced strain kept under selection pressure to grow to high cell density (Guard-Petter, 1998). It is possible that use of other strains could impact results.

Vaccination of egg-laying chickens is not mandated in the United States, but it facilitates comprehensive programs to reduce the risk of *Salmonella* Enteritidis entering the food supply (Gast, 2007; Pitesky *et al*., 2013). Products for vaccinating egg-laying chickens in the United States include genetically modified live vaccines of heterologous serotypes such as *Salmonella* Typhimurium (Hassan and Curtiss, 1997). These products would not contain *sefD* because the gene is known to be absent (McClelland *et al*., 2001). Vaccines made from killed cells of *Salmonella* Enteritidis could vary widely in SefD content because specific stimuli to initiate expression might be absent during production (Berghaus *et al*., 2011). In contrast, modified live vaccines that are made from related serovar group D1 serotypes would have the potential to express SefD (Matulova *et al*., 2012; Penha Filho *et al*., 2012; De Cort *et al*., 2013; Nandre *et al*., 2013).

Conclusions

Results presented here suggest that SefD is a specific antigen from *Salmonella* Enteritidis that should be explored for increasing efficacy of currently marketed vaccines. However, further experimentation is required to explore specifics of application, including addressing the most economical route of vaccination. Moreover, the adjuvant should be selected to avoid side effects in the hen.

Acknowledgments

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Disclosure Statement

No competing financial interests exist.

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