Epidemiol. Infect. (2006), **134**, 967–976. © 2006 Cambridge University Press
doi:10.1017/S0950268806006054 Printed in the United Kingdom

Survival characteristics of *Salmonella enterica* serovar Enteritidis in chicken egg albumen

H. KANG, C. LOUI, R. I. CLAVIJO, L. W. RILEY AND S. LU*

Program in Infectious Diseases and Immunity, School of Public Health, University of California, Berkeley, CA, USA

(*Accepted 6 January 2006, first published online 28 February 2006*)

SUMMARY

Salmonella enterica serovar Enteritidis (SE) is a major foodborne pathogen primarily causing human infection through contaminated chicken eggs. To understand how SE survives in chicken egg albumen, we systematically and quantitatively analysed the survival properties of SE in egg albumen and identified factors affecting its survival. Survival assays of SE in egg indicate that egg albumen restricted the growth of SE. A major factor that controlled SE's growth in egg albumen was iron restriction, since egg albumen supplemented with iron allowed SE to grow, and iron acquisition mutants of SE showed decreased survival in egg albumen. In addition, low pH of albumen, high concentrations of bacteria and low incubation temperatures of bacteria with albumen facilitates the survival of SE. Our results suggest that egg albumen uses multiple mechanisms to control SE including iron limitation, surface interaction and possible enzymatic activities.

INTRODUCTION

Salmonella enterica serovar Enteritidis (SE) is is a major foodborne bacterial pathogen in the United States and around the world [1–6]. The incidence of SE has increased from 5% to nearly 20% in the United States, and is the second leading serovar of *Salmonella* after the serovar Typhimurium (*S. enterica* serovar Typhimurium) [2, 3]. In Europe, SE caused 85% of all cases of human infection by *Salmonella* in 1997 [5]. Its incidence has decreased since then due to the adoption of various control measures [5, 7–13]. Nevertheless, SE remains a foodborne pathogen that causes significant human disease and economic burden [2, 14, 15].

The main vehicle for the transmission of SE to human is the chicken egg [4, 8, 16–21]. In chickens, SE colonizes internal organs as well as the tissues of the reproductive tract including the ovaries, oviduct, cloacal and vaginal tissues [22–27]. SE from the ovaries can contaminate the yolk in some eggs, while the majority of *Salmonella* bacteria are deposited from the reproductive tract to the albumen (egg white) close to the yolk membrane or on the viteline membrane [28–30]. The contaminating bacteria survive in the albumen as the eggshell forms and persists after eggs are laid.

Although *S. enterica* serovar Typhimurium causes occasional human infection through cracked eggs, SE is the only bacterium that routinely causes human infection through intact eggs. Both serovars infect chickens and colonize internal organs including the tissues of the reproductive tract. Keller *et al.* reported that *S. enterica* serovar Typhimurium contaminated forming eggs (eggs recovered from reproductive tracts before they are laid) at higher frequencies than SE did in experimentally infected hens. Nevertheless, only SE

* Author for correspondence: S. Lu, Ph.D., 140 Warren Hall, University of California, Berkeley, CA 94720, USA.
(Email: sangwei@berkeley.edu)

could be isolated from laid eggs [24]. This indicates that forming eggs can eliminate most of the contaminating bacteria, and the association of SE with intact chicken eggs may be due to its enhanced survival ability in eggs, especially the egg albumen. Multiple studies have been carried out to investigate the colonization of chickens and contamination of eggs by SE [23, 24, 27, 31, 32]. However, the survival and persistence of SE in egg is poorly understood. It is generally believed that iron chelation by ovotransferrin is a key antimicrobial activity of egg albumen [33]. *Salmonella* has iron-acquisition systems for both ferric and ferrous iron. The *ent* operon is responsible for the synthesis of enterobactin, the main siderophore of *Salmonella*. Among the *ent* genes, *ent*F encodes a subunit of enterobactin synthase essential for enterobactin biosynthesis. The *ton*B gene encodes part of the TonB–ExbB–ExbD complex, which is required for the energy-dependent transport of ferric siderophores across the outer membrane of Gram-negative bacteria [34]. The *feo*A and *feo*B genes encode the ferrous uptake system [35]. Whether the ferric or ferrous iron-acquisition system is necessary for SE to survive in egg albumen has not been reported previously.

In a previous analysis, we had demonstrated that isolates of SE survived better in egg albumen than those of *S. enterica* serovar Typhimurium and *E. coli*, which may contribute to its epidemiological association with chicken eggs [36]. In this study, we sought to determine factors of egg albumen and conditions that affect the survival of the contaminating SE in egg albumen.

MATERIALS AND METHODS

Bacterial strains, plasmids, oligonucleotides and reagents

Isolates of SE, and plasmids and oligonucleotides for mutagenesis are listed in the Table. Oligonucleotides were purchased from Sigma-Genosys (The Woodlands, TX, USA). Media for bacterial cultures were purchased from Becton, Dickinson and Company (Franklin Lakes, NJ, USA).

Survival of SE in egg albumen

Survival of both wild-type and mutant SE in egg albumen was assayed as described previously [37]. Organic, antibiotic-free eggs from a local farm were bought and stored at 4 °C for up to 1 week until use. Storage of eggs for 1 week was not found to have an appreciable effect on the survival of bacteria (data not shown). Overnight culture of SE was mixed with egg albumen to a concentration of approximately $2–4 \times 10^3$ colony-forming units/ml (c.f.u./ml) unless specified otherwise. This concentration was used in all assays because it is sufficiently low for egg albumen to kill bacteria (high concentrations of SE overcome the bactericidal activity of egg albumen; see Results section) and still would allow us to quantify the bacterial killing of approximately 150- to 300-fold by egg albumen. Bacteria–egg albumen mixtures were incubated at 37 °C or at specified temperatures for different periods of time and plated on LB agar plates to determine the concentration of surviving bacteria. Since different batches of eggs vary in their bactericidal activity, the time-course of survival of SE in egg albumen varies slightly in different assays. All assays were repeated at least three times, and data from one representative assay are presented.

The effect of iron restriction of egg albumen on SE survival was assayed by determining the survival/growth of SE in egg albumen supplemented with 127 μg/ml of ferric ammonium citrate (110% of the concentration necessary to saturate ovotransferrin) [33].

Construction of SE mutants and assay for siderophore production

Deletion mutants of *ent*F, *ton*B and *feo*AB were constructed by homologous recombination with the Red recombinase system [38, 39]. Since *feo*A and *feo*B are immediately adjacent to each other in an operon, we deleted both genes in the *Δfeo*AB mutant. Oligonucleotides containing sequences homologous to the target genes were used to amplify the kanamycin or chloramphenicol resistance gene cassettes from plasmids pKD4 and pKD3 respectively, and PCR products were electroporated into SE2472 transformed with plasmid pKD46 (Table). The kanamycin resistance marker was used to generate the *Δent*F and *Δton*B mutants and the choloramphenicol resistance marker was used to generate the *Δfeo*AB mutant. Homologous recombinants were selected on LB agar plates supplemented with kanamycin (75 μg/ml) or chloramphenicol (34 μg/ml) and confirmed by PCR with primers outside of the homologous region [39]. All mutations were transduced into fresh SE by general transduction by phage P22 before further analysis

https://doi.org/10.1017/S0950268806006054 Published online by Cambridge University Press

Table. *Bacterial strains, plasmids and oligonucleotides used for mutagenesis*

		Ref.
Bacterial strains		
S. enteritidis		
SE2472	Clinical isolate, Phage type 4	[42]
SE6782	Clinical isolate, Phage type 4	[42]
SE0718	Clinical isolate, Phage type 4	This study
SE2472 *ΔentF*	*entF::kan* derivative of SE2472	This study
SE2472 *ΔfeoAB*	*feoAB::cm* derivative of SE2472	This study
SE2472 *ΔtonB*	*tonB::kan* derivative of SE2472	This study
SE2472 *ΔentF/ΔfeoAB*	*entF::kan, feoAB::cm* derivative of SE2472	This study
Plasmids		
pKD3	$Ap^rCm^roriR_\gamma$	[38]
pKD4	$Ap^rKan^roriR_\gamma$	[38]
pKD46	Ap^r, containing the Red recombinase of λ phage	[38]
Primers for mutagenesis in SE2472		
*ent*F	5′ primer	5′-AGTCGCCGCC CAGCCGGGGA TCTGGATGGC GGAAAAACTC TCTGATTTAC CCTCCGCCTG GAGCGTGGCG CACTATGTGG AACTGAATGG CGAGCTGGAT GTGTAGGCTG GAGCTGCTTC
	3′ primer	5′-CAAACGGTGC GCTATGCGCC GTCGTCAGCA AACGGACGGC ATCCGCATAA TTGCCTTCAA TGGCGGTAAA TAAGGCGTCG GAGGCATTTC CCTGCTGCGC CATATGAATATCCTCCTTAG
*ton*B	5′ primer	5′-TCCTTTCCGT AGGCATTCAT GGTGCTGTAG TGGCAGGTTT GCTCTATACC TCGGTACATC AGGTTATTGA ATTGCCTGCG CCAGCGCAGC CTATCACGGT GTGTAGGCTG GAGCTGCTTC
	3′ primer	5′-CTGCGCCGCA CCGTTCAGAC GGAAGATAAT ATTGACCACC AGCCCGGAAC CCGGCTTGCC TGCTTCATAG CGCCATTTTC GCATCGCATT CTTAACTTCA CATATGAATATCCTCCTTAG
*feo*AB	5′ primer	5′-TCCTTCGTTG GCTAAACATC GGGTCTCCTG CCGCCCCCCT GAGCGCCGCA TGAGGTATAC ATCCAGTTAG TAAGAAACAA GTAGGTCGTA GTGTAGGCTG GAGCTGCTTC
	3′ primer	5′-CCAGTAGATC GCGAACCTGT ATCAATGAAG CCATTTTTTA CATCCCTTAG TGACAATTGC CCGCAGTGCC CGAACAACAG GAACTGACGC CATATGAATATCCTCCTTAG

Salmonella enterica serovar Enteritidis strains and plasmids used in this study are listed. Primers used to generate deletion mutants through homologous recombination are listed. Sequences underlined are homologous to those of plasmid pKD4 or pKD3 and were used to amplify the kanamycin resistance cassette (pKD4) or chloramphenicol resistance cassette (pKD3).

[40]. The double mutant *ΔentF/ΔfeoAB* was generated by transduction of *ΔentF* mutation into the *ΔfeoAB* mutant with phage P22 [40].

The production of siderophores was detected by chrome azurol S (CAS) agar plates as described previously [41]. Overnight cultures were plated onto CAS agar plates, and incubated at 37 °C. Siderophore production was detected by a change of colour of the CAS agar from blue-grey to orange in regions surrounding a bacterial colony that appears as a halo around bacterial colonies. Since the *ΔentF* and *ΔtonB* mutants grow more slowly on the CAS agar plates, they were incubated at 37 °C for longer periods of time to allow bacterial colonies to reach approximately the same size as the *ΔfeoAB* mutant and the wild-type SE.

RESULTS

Iron restriction is important for the control of SE by egg albumen

Three isolates of SE, SE2472, SE6782 and SE0718, were assayed for their survival in egg albumen in the presence and absence of iron supplementation (Fig. 1). Without iron supplementation, the concentration of SE steadily decreased (Fig. 1*a*). With iron supplementation bacteria grew extensively from

https://doi.org/10.1017/S0950268806006054 Published online by Cambridge University Press

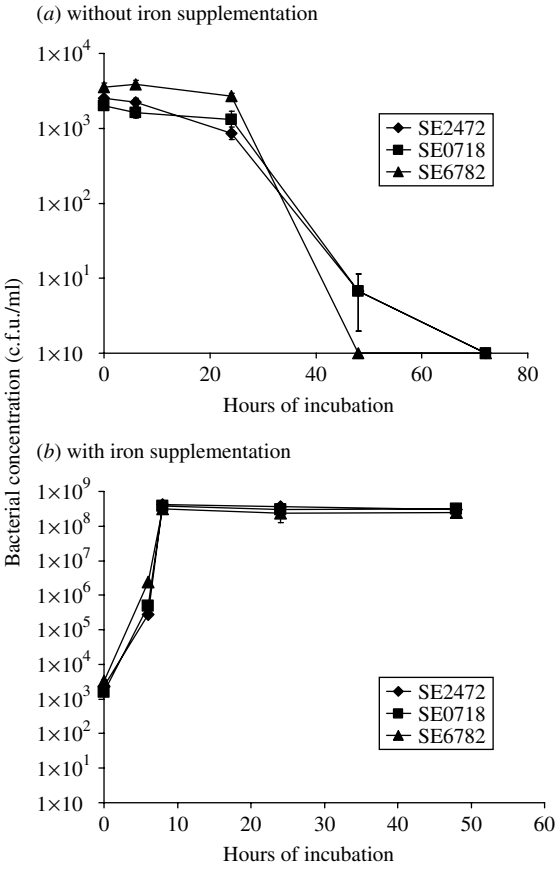

Fig. 1. Effect of iron supplementation on the survival of *Salmonella enterica* serovar Enteritidis (SE) in egg albumen. Survival of SE without (*a*) and with (*b*) iron supplementation was assayed. For the sample with iron supplementation, egg albumen was modified with 127 μg/ml of ferric ammonium citrate before being incubated with bacteria. After incubation at 37 °C, concentration of surviving bacteria was determined by plating and plotted against the incubation time period. At least three experiments were performed, and results from a representative experiment performed in triplicate are shown. Error bars indicate standard deviation and lie within the data labels in some instances.

approximately 10^3 c.f.u/ml. to over 10^8 c.f.u./ml in 24 h (Fig. 1*b*). All three strains displayed similar survival in both unsupplemented and iron-supplemented egg albumen (Fig. 1).

Generation of deletion mutants of SE that are deficient in iron acquisition

To study the role of iron assimilation in the survival of SE in egg albumen, we generated deletion mutants in both the ferric (*ent*F and *ton*B) and ferrous iron (*feo*AB) uptake systems of SE. The production of siderophores of the *Δent*F, *Δton*B and *Δfeo*AB mutants and the wild-type SE2472 was tested on CAS agar plates [41]. While the wild-type SE2472 produced an orange halo on CAS agar plates, the *Δent*F mutant of SE2472 produced no halo, indicating that it does not produce detectable amounts of siderophore for ferric iron (Fig. 2*a*). A halo was present around colonies of *Δton*B and *Δfeo*AB as expected since they have an intact enterobactin biosynthesis system and are expected to secrete enterobactin as the wild-type SE (Fig. 2*a*).

Survival of iron-acquisition mutants in egg albumen

We tested the survival of the ferric and ferrous iron acquisition mutants in egg albumen to determine if SE needs to actively assimilate iron to survive. The *Δent*F, *Δton*B and *Δfeo*AB mutants and *Δent*F/*Δfeo*AB double mutant of SE2472 showed defective survival in egg albumen (Fig. 2*b*). Among the mutants, the *Δent*F mutant was the most defective in surviving in egg albumen, although the difference between the mutants was not statistically significant. With iron supplementation, all mutants and the wild-type SE2472 were able to grow in egg albumen (Fig. 2*c*). The mutants, however, demonstrated different growth curves in iron-supplemented egg albumen. While the *Δton*B and *Δfeo*AB mutants displayed the same growth curve as the wild type SE2472, the *Δent*F and *Δent*F/*Δfeo*AB mutants displayed a significant delay in their growth in the iron-supplemented egg albumen. Iron-acquisition mutants were also generated on the background of isolate SE6782 [42], and similar results were obtained on the siderophore production and survival in egg albumen as described above for SE2472 (data not shown).

Effect of the pH of egg albumen, initial bacterial concentration and incubation temperature on the survival of SE in egg albumen

Egg albumen has a basic pH of 9·0. To test if the basic pH of egg albumen contributes to its bactericidal activity, we assayed the survival of SE isolates in egg albumen with its pH adjusted to 8·0. Compared to the unadjusted egg albumen, SE demonstrated more growth in egg albumen of pH 8·0 (Fig. 3).

We also assayed the effect of the level of contamination (starting concentration) of SE on its survival in egg albumen (Fig. 4). Bacterial survival at 10^3, 10^5 or

https://doi.org/10.1017/S0950268806006054 Published online by Cambridge University Press

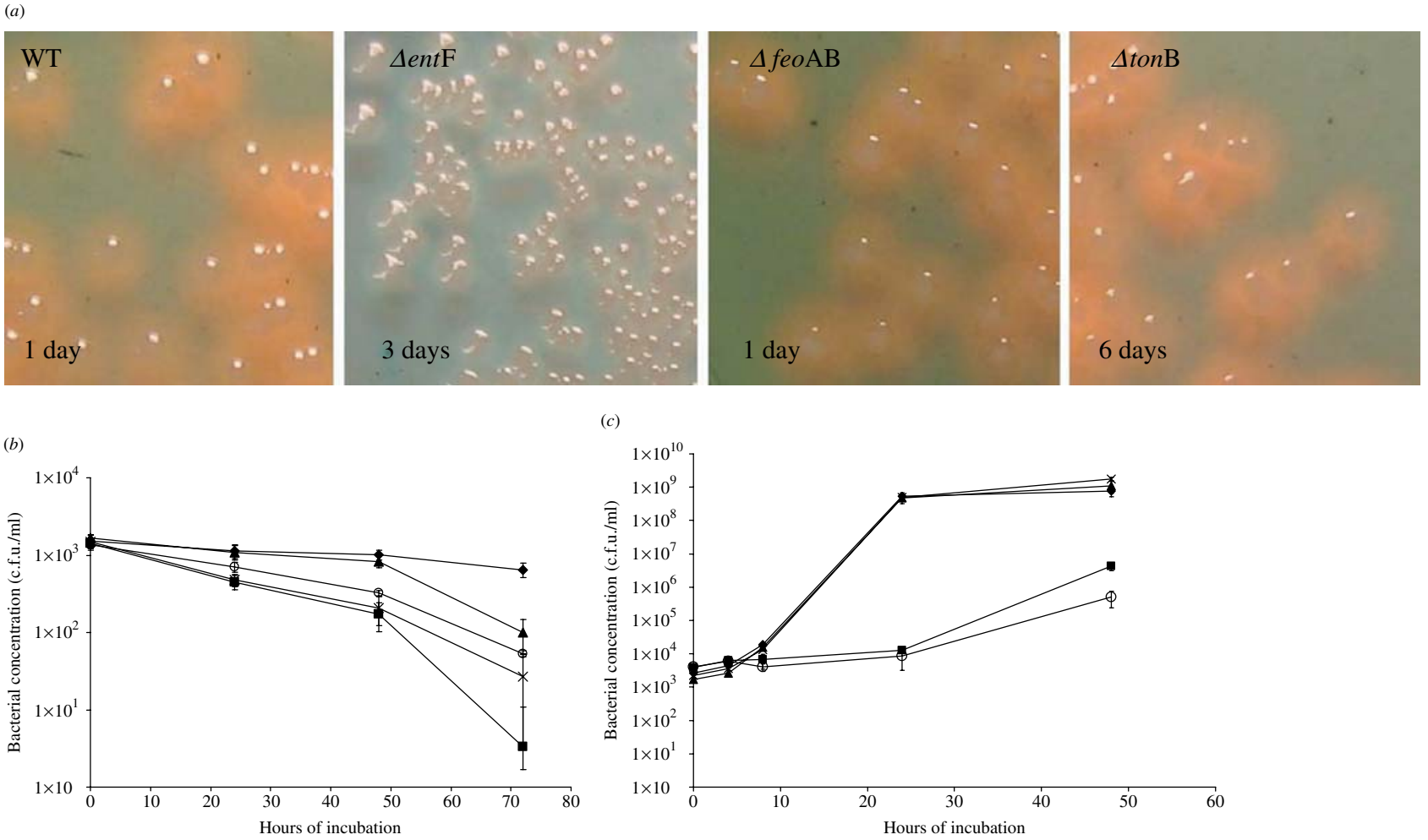

Fig. 2. Survival of iron metabolism mutants in egg albumen. (*a*) Growth of *Δent*F, *Δfeo*AB, *Δton*B and wild-type *Salmonella enterica* serovar Enteritidis (SE) SE2472 on CAS agar plates. The production of siderophore is demonstrated by the orange halo surrounding the colonies. Since the *Δent*F and *Δton*B mutants grow slower than the *Δfeo*AB and wild-type SE SE2472, they were incubated for 3 days and 6 days at 37 °C respectively, while *Δfeo*AB and wild-type SE SE2472 were incubated for 1 day. (*b*) Survival of *Δent*F (–■–), *Δfeo*AB (–×–), *Δton*B (–▲–), *Δent*F/*Δfeo*AB (–○–) and wild-type SE SE2472 (–◆–) in egg albumen. (*c*) Growth of *Δent*F (–■–), *Δfeo*AB (–×–), *Δton*B (–▲–), *Δent*F/*Δfeo*AB (–○–) and wild type SE SE2472 (–◆–) in egg albumen supplemented with ammonium ferric citrate. Overnight culture of each bacterial strain was incubated with egg albumen at 37 °C. The survival of bacteria was determined by plating and plotted against the indicated incubation time periods. At least three experiments were performed, and results from a representative experiment performed in triplicate are shown. Results in Figure 2 are not directly comparable to those in Figure 1 due to variability between batches of eggs used for the assays. However, variability in eggs does not alter the relative survival between different bacterial strains or the conclusion of each assay. Error bars indicate standard deviation and lie within the data labels in some instances.

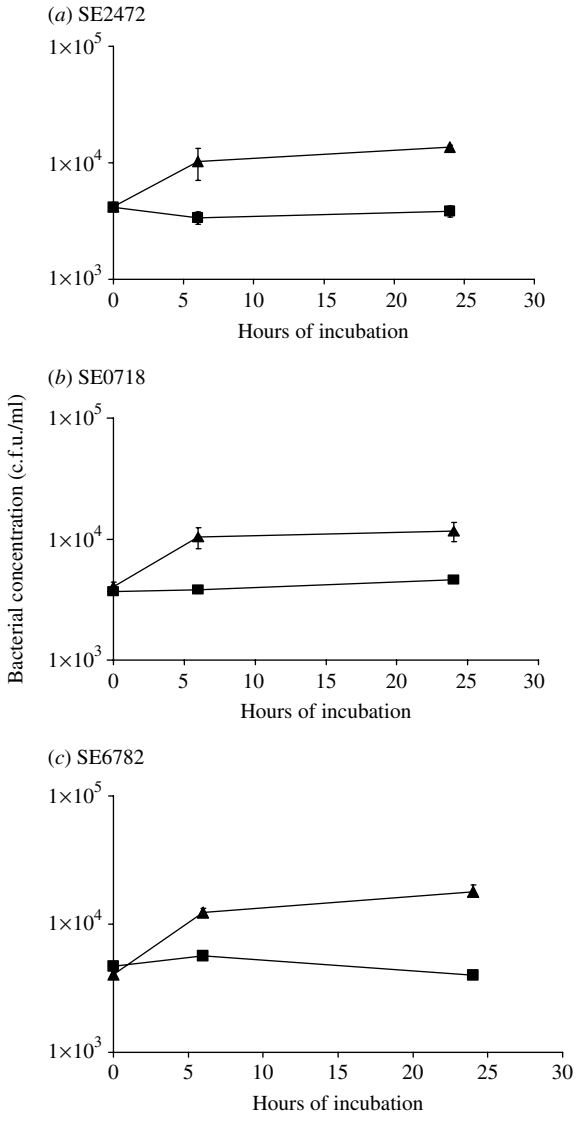

Fig. 3. Survival of *Salmonella enterica* serovar Enteritidis (SE) at different pH. SE isolates SE2472 (*a*), SE 0718 (*b*) and SE 6782 (*c*) were incubated with egg albumen and pH-adjusted egg albumen at 37 °C. For the pH-adjusted egg albumen, hydrogen chloride (HCl) was added to the egg albumen to lower the pH from the natural pH of 9·0 (–■–) to pH 8·0 (–▲–). Surviving bacteria were quantified by plating and plotted against the incubation time period. At least three experiments were performed, and results from a representative experiment performed in triplicate are shown. Error bars indicate standard deviation and lie within the data labels in some instances.

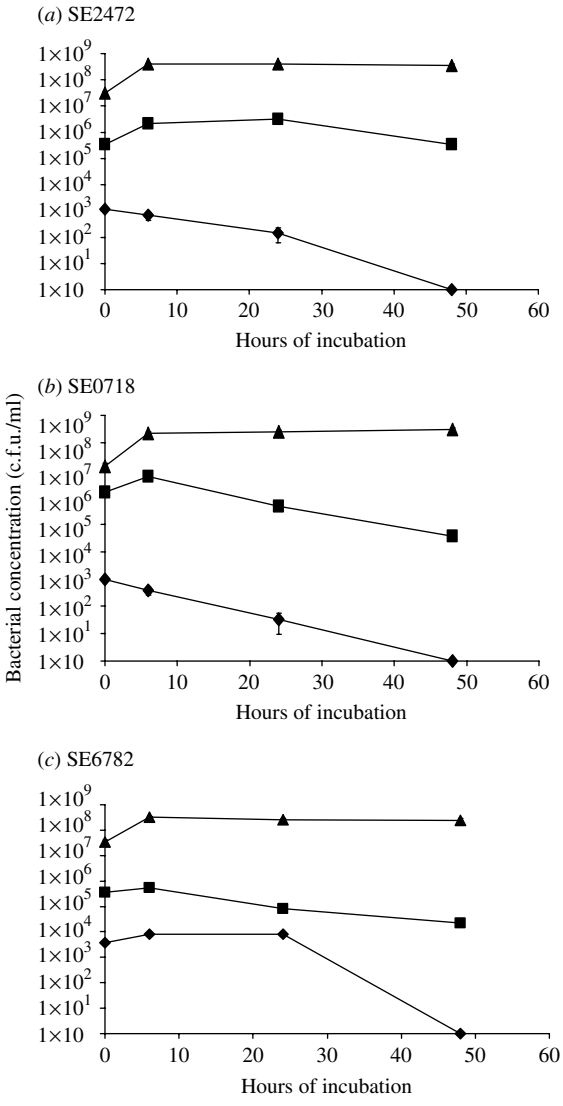

Fig. 4. Survival of *Salmonella enterica* serovar Enteritidis (SE) isolates SE2472 (*a*), SE0718 (*b*) and SE6782 (*c*) in egg albumen with starting concentrations at ~10^3 c.f.u./ml (–◆–), 10^5 c.f.u./ml (–■–) and 10^7 c.f.u./ml (–▲–). Each isolate was incubated with egg albumen at 37 °C at the three concentrations shown above. The number of surviving bacteria was determined by plating and plotted against the incubation time. At least three experiments were performed, and results from a representative experiment performed in triplicate are shown. Error bars indicate standard deviation and lie within the data labels in some instances.

10^7 c.f.u./ml was determined. While SE was killed by egg albumen when the starting bacterial concentration was ~10^3 c.f.u./ml, it was able to survive longer and sometimes maintained its concentration when the starting concentration was ~10^5 c.f.u./ml and was able to multiply when the starting concentration was ~10^7 c.f.u./ml (Fig. 4).

We tested SE's survival under different incubation temperatures. As shown in Figure 5, bacterial concentrations remained largely unchanged when SE was incubated with egg albumen at 4 °C or 25 °C. In contrast, bacterial concentrations decreased dramatically when SE was incubated at 37 °C with egg albumen. The bactericidal effects of the egg albumen were even

https://doi.org/10.1017/S0950268806006054 Published online by Cambridge University Press

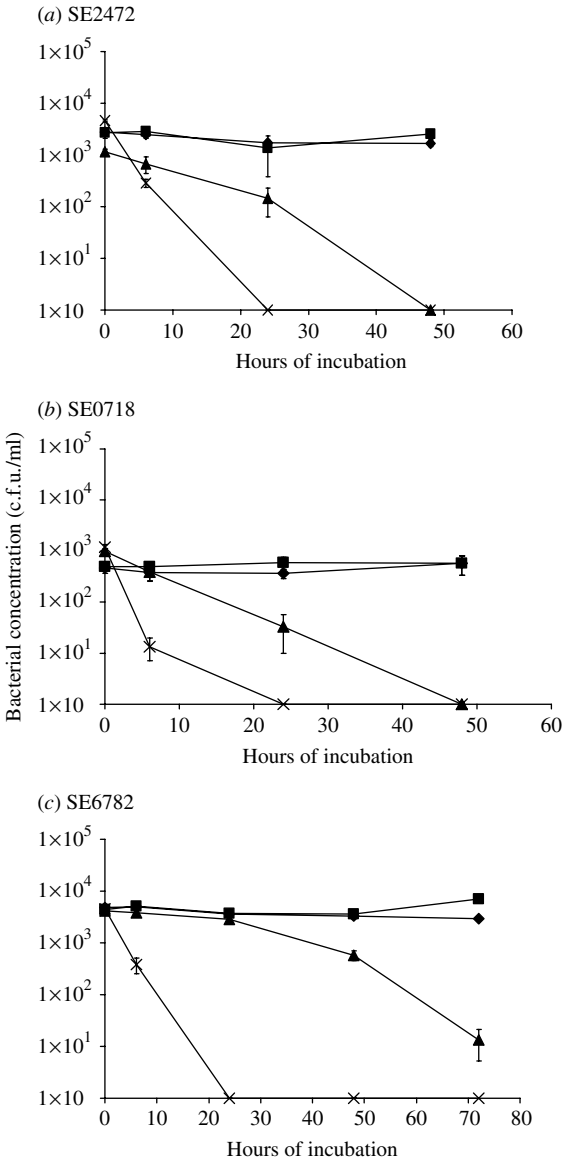

Fig. 5. Survival of *Salmonella enterica* serovar Enteritidis (SE) in egg albumen at different temperatures. SE isolates SE2472 (*a*), SE0718 (*b*) and SE6782 (*c*) were incubated with egg albumen at 4 °C (–◆–), 25 °C (–■–), 37 °C (–▲–) and 42 °C (–×–). The concentration of surviving bacteria was determined by plating and plotted against the incubation times. At least three experiments were performed, and results from a representative experiment performed in triplicate are shown. Error bars indicate standard deviation and lie within the data labels in some instances.

more pronounced at 42 °C, at which temperature SE was quickly eliminated (Fig. 5).

DISCUSSION

SE is the major bacterium that causes human infection through chicken eggs [9, 17–21]. In this study, we analysed the factors of chicken egg albumen and conditions that influence the survival of the contaminating SE. Our results indicate that egg albumen effectively controlled bacterial growth, while egg yolk failed to restrict bacterial proliferation (data not shown). One major factor contributing to the difference of bacterial growth in egg albumen and egg yolk was likely to be the iron restriction caused by ovotransferrin in the egg albumen [33]. This notion is supported by our results that iron-supplemented egg albumen lost its bactericidal activity of SE instead (Fig. 1). The role of iron restriction in controlling SE was further demonstrated in our results that iron-acquisition mutants of SE showed decreased survival in egg albumen (Fig. 2). Both ferric and ferrous iron was important for survival in egg albumen, since both *Δent*F and *Δfeo*AB mutants demonstrated less survival than that of the wild-type SE (Fig. 2*b*). SE with deletion in *ent*F also showed decreased survival in iron-supplemented egg albumen (Fig. 2*c*). This is probably due to their inability to use enterobactin to acquire iron efficiently regardless of the abundance of iron in the environment. Alternatively, *ent*F or enterobactin may have yet unidentified functions in addition to their role in iron acquisition. It is believed that at least some SE strains produce aerobactin, a hydroxymate type of siderophore. Since the aerobactin-encoding gene of SE has not been characterized, we have not been able to determine if the wild-type clinical strain SE2472 produces aerobactin. However, the absence of halo around the *Δent*F mutant of SE2472 indicated that the production of aerobactin is probably negligible (Fig. 2*a*).

Although iron restriction is important for egg albumen to control SE, other factors in egg albumen contribute to its bactericidal activities. M9 minimal or Vogel Bonner media (pH 9) supplemented with ovotransferrin slowed the growth of SE, but failed to control or eliminate SE (data not shown), suggesting that other components of egg albumen are also important for its bactericidal activities. We have recently carried out a systematic analysis of bacterial factors involved in the survival of SE in egg albumen [36]. A transposon mutant library of SE was constructed and screened for mutants that were susceptible to egg albumen. Characterization of genes inactivated by Tn insertions indicated that a majority of them are involved in the structural and functional integrity of bacterial cell wall, including transporters, membrane proteins of the Type III secretion system, fimbrial usher proteins and enzymes of the LPS biosynthesis

https://doi.org/10.1017/S0950268806006054 Published online by Cambridge University Press

pathway [36]. These results suggest that components of egg albumen may interact directly with SE cell wall to kill the bacteria. This notion is consistent with previous reports that both ovotransferrin and lysozyme can form pores in Gram-negative bacteria through their cationic interaction with the bacterial cell wall, and their pore-forming activities are independent of their activity as iron chelator or as muramidase respectively [43–46]. This notion is further supported by our results in this study that the pH of the egg albumen and the incubation temperature of bacteria and egg albumen affect the bactericidal activity of egg albumen (Figs 3 and 5), since these changes may alter the interaction of egg albumen components with bacteria and reduce its bactericidal activity.

The bactericidal activity of egg albumen was dependent on the initial bacterial concentration (Fig. 4). The egg albumen was capable of killing bacteria when the bacterial concentration was low ($\sim 10^3$ c.f.u./ml), but failed to control SE when the bacterial concentration was higher (Fig. 4). Although the bacterial concentrations used in this study are likely to be much higher than that in a natural infection, this result indicated that high concentrations of bacteria may saturate the antimicrobial factors of the egg albumen. A high bacterial concentration might result in an insufficient local concentration of antimicrobial components on the bacterial cell wall leading to reduced bacterial killing. Alternatively, it is also possible that as some of the bacteria died in egg albumen, they released their content including stored iron, which enabled the surviving bacteria to grow.

In addition to the egg albumen components that act on the bacterial surface to mediate bacterial killing, egg albumen may also contain enzymatic antibacterial activities. For example, we have discovered previously that egg albumen has nuclease activities that nick both naked and intracellular bacterial DNA [37]. The nuclease activities are likely to be protein based [37] and were temperature dependent, with activities lower at 4 °C and 25 °C, and higher at 37 °C and 42 °C (data not shown). This is consistent with our results of the effect of incubation temperature on the survival of SE (Fig. 5). Although lower temperatures were expected to curtail bacterial growth [29], we found surprisingly that higher temperatures of 37 °C and 42 °C were more detrimental to bacterial survival in egg albumen (Fig. 5). This may be because the nuclease activities and other possible enzymatic antimicrobial activities are more active at higher temperatures and thus lead to more bacterial killing. These results raise an intriguing possibility that it is preferable to store eggs at 37 °C for a certain period of time instead of 4 °C to allow the endogenous bactericidal activities of egg albumen to kill the contaminating SE. We had tested the brief heating of egg albumen and SE mixtures (up to 2 h) at a moderate temperature (50 °C) followed by storage at 4 °C, and found that the treatment was sufficient to kill all contaminating SE up to 10^7 c.f.u./ml in egg albumen (data not shown). Since the concentration of contaminating SE in eggs from naturally infected chickens is believed to be very low, the heat treatment should be effective in killing contaminating SE in egg albumen. It must be pointed out that further studies with intact eggs are necessary to determine if the heat treatment is effective in eliminating SE in whole eggs.

Based on our analysis of factors of egg albumen that affect the survival of SE and the genetic analysis of SE factors important for SE to persist in egg albumen, we propose that egg albumen uses multiple mechanisms to control contaminating SE. First, the high concentration of ovotransferrin in egg albumen chelates iron to make it unavailable to contaminating SE and prevent SE from proliferating. Second, ovotransferrin, lysozyme and maybe other as yet unidentified antimicrobial peptides interact with the surface of SE and form pores in the cell wall. Third, egg albumen penetrates SE and kills bacteria through nuclease activities and other as yet unidentified mechanisms. Characterization of the chemical nature of the antimicrobial components of egg albumen in addition to ovotransferrin and lysozyme will provide direct evidence if the hypothesis proposed above is correct.

ACKNOWLEDGEMENTS

We thank Sharon Abbott of the State of California Department of Health Services for the *Salmonella* isolates. This study was supported by USDA 2002-35201-11543 (to L.W.R.) and USDA 2004-35201-14125 (to S.L.).

DECLARATION OF INTEREST

None.

REFERENCES

1. **Baumler AJ, Hargis BM, Tsolis RM.** Tracing the origins of *Salmonella* outbreaks. *Science* 2000; **287**: 50–52.
2. **Centers for Disease Control and Prevention.** *Salmonella* – 2002 Annual Summary; 2003.

3. **Centers for Disease Control and Prevention.** Surveillance for foodborne-disease outbreaks – United States, 1993–1997. *Morbidity and Mortality Weekly Report* 2000; **49**: 1–72.
4. **Gomez TM, *et al.*** Foodborne salmonellosis. *World Health Statistics Quarterly* 1997; **50**: 81–89.
5. **Guard-Petter J.** The chicken, the egg and *Salmonella enteritidis*. *Environmental Microbiology* 2001; **3**: 421–430.
6. **Herikstad H, Motarjemi Y, Tauxe RV.** *Salmonella* surveillance: a global survey of public health serotyping. *Epidemiology and Infection* 2002; **129**: 1–8.
7. **Angulo FJ, Swerdlow DL.** *Salmonella enteritidis* infections in the United States. *Journal of the American Veterinary Medical Association* 1998; **213**: 1729–1731.
8. **Centers for Disease Control and Prevention.** Outbreaks of *Salmonella* serotype enteritidis infection associated with eating shell eggs – United States, 1999–2001. *Journal of the American Medical Association* 2003; **289**: 540–541.
9. **Hogue A, *et al.*** Epidemiology and control of egg-associated *Salmonella enteritidis* in the United States of America. *Review of Science and Technology* 1997; **16**: 542–553.
10. **Marcus R, *et al.*** Dramatic decrease in the incidence of *Salmonella* serotype Enteritidis infections in 5 FoodNet sites: 1996–1999. *Clinical Infectious Diseases* 2004; **38** (Suppl 3): S135–S141.
11. **Mason J.** *Salmonella enteritidis* control programs in the United States. *International Journal of Food Microbiology* 1994; **21**: 155–169.
12. **Mumma GA, *et al.*** Egg quality assurance programs and egg-associated *Salmonella enteritidis* infections, United States. *Emerging Infectious Diseases* 2004; **10**: 1782–1789.
13. **Patrick ME, *et al.*** *Salmonella enteritidis* infections, United States, 1985–1999. *Emerging Infectious Diseases* 2004; **10**: 1–7.
14. **Morales RA.** Farm-level costs for control of Salmonella enteritidis in laying flocks. In: Caswell JA ed. *The Economics of Reducing Health Risk from Food*. University of Connecticut Press, Storrs, 1996.
15. **Morales RA, Mcdowell RM.** Economic consequences of *Salmonella enterica* serovar Enteritidis infection in the humans and the U.S. egg industry, pp. 307–312. In: Saeed AM ed. Salmonella enterica *Serovar Enteritidis in Humans and Animals*. Iowa State University Press, Ames, 1999.
16. **Centers for Disease Control and Prevention.** Outbreaks of *Salmonella* serotype Emteritidis infection associated with consumption of raw shell eggs – United States, 1994–1995. *Morbidity and Mortality Weekly Report* 1996; **45**: 737–742.
17. **Food Safety and Inspection Service.** *Salmonella enteritidis* risk assessment for shell eggs and egg products. Final report; 1998.
18. **Hennessy TW, *et al.*** A national outbreak of *Salmonella enteritidis* infections from ice cream. The Investigation Team. *New England Journal of Medicine* 1996; **334**: 1281–1286.
19. **Henzler DJ, *et al.*** *Salmonella enteritidis* in eggs from commercial chicken layer flocks implicated in human outbreaks. *Avian Diseases* 1994; **38**: 37–43.
20. **Hope BK, *et al.*** An overview of the *Salmonella enteritidis* risk assessment for shell eggs and egg products. *Risk Analysis* 2002; **22**: 203–218.
21. **St. Louis ME, *et al.*** The emergence of grade A eggs as a major source of *Salmonella enteritidis* infections. New implications for the control of salmonellosis. *Journal of the American Medical Association* 1988; **259**: 2103–2107.
22. **Gast RK, Beard CW.** Production of *Salmonella enteritidis*-contaminated eggs by experimentally infected hens. *Avian Diseases* 1990; **34**: 438–446.
23. **Keller LH, *et al.*** *Salmonella enteritidis* colonization of the reproductive tract and forming and freshly laid eggs of chickens. *Infection and Immunity* 1995; **63**: 2443–2449.
24. **Keller LH, *et al.*** Invasion of chicken reproductive tissues and forming eggs is not unique to *Salmonella enteritidis*. *Avian Diseases* 1997; **41**: 535–539.
25. **Okamura M, *et al.*** Differences among six *Salmonella* serovars in abilities to colonize reproductive organs and to contaminate eggs in laying hens. *Avian Diseases* 2001; **45**: 61–69.
26. **Okamura M, *et al.*** Differences in abilities to colonize reproductive organs and to contaminate eggs in intravaginally inoculated hens and in vitro adherences to vaginal explants between *Salmonella enteritidis* and other *Salmonella* serovars. *Avian Diseases* 2001; **45**: 962–971.
27. **Shivaprasad HL, *et al.*** Pathogenesis of *Salmonella enteritidis* infection in laying chickens. I. Studies on egg transmission, clinical signs, fecal shedding, and serologic responses. *Avian Diseases* 1990; **34**: 548–557.
28. **Gast RK, Holt PS.** Deposition of phage type 4 and 13a *Salmonella enteritidis* strains in the yolk and albumen of eggs laid by experimentally infected hens. *Avian Diseases* 2000; **44**: 706–710.
29. **Gast RK, Holt PS.** Influence of the level and location of contamination on the multiplication of *Salmonella enteritidis* at different storage temperatures in experimentally inoculated eggs. *Poultry Science* 2000; **79**: 559–563.
30. **Humphrey TJ, *et al.*** *Salmonella enteritidis* phage type 4 from the contents of intact eggs: a study involving naturally infected hens. *Epidemiology and Infection* 1989; **103**: 415–423.
31. **Gast RK, Beard CW.** Isolation of *Salmonella enteritidis* from internal organs of experimentally infected hens. *Avian Diseases* 1990; **34**: 991–993.
32. **Gast RK, Guard-Petter J, Holt PS.** Characteristics of *Salmonella enteritidis* contamination in eggs after oral, aerosol, and intravenous inoculation of laying hens. *Avian Diseases* 2002; **46**: 629–635.
33. **Baron F, Gautier M, Brule G.** Factors Involved in the inhibition of growth of *Salmonella enteritidis* in liquid egg white. *Journal of Food Protection* 1997; **60**: 1318–1323.

34. **Postle K, Kadner RJ.** Touch and go: tying TonB to transport. *Molecular Microbiology* 2003; **49**: 869–882.
35. **Kammler M, Schon C, Hantke K.** Characterization of the ferrous iron uptake system of *Escherichia coli*. *Journal of Bacteriology* 1993; **175**: 6212–6219.
36. **Clavijo RI, *et al.*** Identification of genes associated with the survival of *Salmonella enterica* serovar Enteritidis in chicken egg albumen. *Applied Environmental Microbiology* 2006; **72**: 1055–1064.
37. **Lu S, Killoran PB, Riley LW.** Association of *Salmonella enterica* serovar Enteritidis YafD with chicken egg albumen resistance. *Infection and Immunity* 2003; **71**: 6734–6741.
38. **Datsenko KA, Wanner BL.** One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proceedings of the National Academy of Sciences USA* 2000; **97**: 6640–6645.
39. **Lu S, *et al.*** The global regulator ArcA controls resistance to reactive nitrogen and oxygen intermediates in *Salmonella enterica* serovar Enteritidis. *Infection and Immunity* 2002; **70**: 451–461.
40. **Maloy SR, Steward VJ, Taylor RK.** *Genetic Analysis of Pathogenic Bacteria*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1996.
41. **Schwyn B, Neilands JB.** Universal chemical assay for the detection and determination of siderophores. *Analytical Biochemistry* 1987; **160**: 47–56.
42. **Lu S, *et al.*** Analysis of virulence of clinical isolates of *Salmonella enteritidis* in vivo and in vitro. *Infection and Immunity* 1999; **67**: 5651–5657.
43. **Ibrahim HR, *et al.*** Identification of a distinct antibacterial domain within the N-lobe of ovotransferrin. *Biochimica et Biophysica Acta* 1998; **1401**: 289–303.
44. **Ibrahim HR, Sugimoto Y, Aoki T.** Ovotransferrin antimicrobial peptide (OTAP-92) kills bacteria through a membrane damage mechanism. *Biochimica et Biophysica Acta* 2000; **1523**: 196–205.
45. **Ibrahim HR, Thomas U, Pellegrini A.** A helix-loop-helix peptide at the upper lip of the active site cleft of lysozyme confers potent antimicrobial activity with membrane permeabilization action. *Journal of Biological Chemistry* 2001; **276**: 43767–43774.
46. **Pellegrini A, *et al.*** Effect of lysozyme or modified lysozyme fragments on DNA and RNA synthesis and membrane permeability of *Escherichia coli*. *Microbiology Research* 2000; **155**: 69–77.