Invasiveness in chickens, stress resistance and RpoS status of wild-type *Salmonella enterica* subsp. *enterica* serovar Typhimurium definitive type 104 and serovar Enteritidis phage type 4 strains

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The heat and acid resistance and the ability to survive airdrying on commonly used kitchen surfaces were assessed for clinical and environmental strains of *Salmonella enterica* subsp. *enterica* serovar Typhimurium, definitive type (DT) 104. Three out of thirty-eight strains of DT 104 were found to be more sensitive in stationary phase to the stresses examined than the other strains. This compares to a previous study by the authors which showed that seven out of forty serovar Enteritidis phage type (PT) 4 strains were more sensitive. RpoS activity was examined indirectly in selected strains of DT 104 and PT 4. In those with normal stress resistance a 100-fold induction of an RpoS-dependent *spvRIA'*::*luxCDABE* fusion was observed upon entry into stationary phase. The sensitive strains examined showed either no induction or a reduced level of *spvRIA'*::*luxCDABE* expression. The *rpoS* gene was sequenced from these strains and three were found to harbour mutations including one deletion, one base-pair substitution resulting in a nonsense codon, and one insertion causing a frameshift resulting in an early stop codon. Strains with negligible or reduced *spvRIA'*::*luxCDABE* expression had low stress resistance. All strains of DT 104 could be recovered from liver and spleen tissues of infected hens 14 d post-infection, but one with no induction of *spvRIA'*::*luxCDABE* expression was significantly less likely to be recovered from chicken reproductive tissues, liver or spleen than the majority of other strains, including one with reduced *spvRIA'*::*luxCDABE* expression. This work has demonstrated that clinical and environmental strains of DT 104 and PT 4 not infrequently harbour mutations in the *rpoS* allele. It is possible that the *rpoS* mutations may have occurred during the initial isolation of the strains. The ability of a strain to cause infection, however, also depends on factors such as host susceptibility and dose.

Keywords: *spv-lux* reporter, chicken reproductive tissue

INTRODUCTION

Within the past 10 years, the prevalence of salmonellosis

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Abbreviations: DT, definitive type; PT, phage type; RLU, relative light units (per ml).

has shown a marked increase in Europe and much of the rest of the developed world. *Salmonella enterica* subsp. *enterica* serovar Enteritidis (*S. enteritidis*) has become the predominant strain. One particular phage type (PT) of *S. enteritidis*, PT 4, has been the most common cause of foodborne salmonellosis for nearly a decade in Western Europe and has also been linked to an increasing number of cases of food poisoning in the USA (Altekruse *et al.*, 1997). Another very prevalent *Sal-*

monella strain associated with human infections worldwide is *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S. typhimurium*) definitive type (DT) 104 (Anonymous, 1997). Together PT 4 and DT 104 currently account for over 70% of all confirmed cases of salmonellosis in England and Wales (Anonymous, 1999).

More than 90% of DT 104 strains show multiple antibiotic resistance and ~15% carry resistance to quinolones (Wall *et al.*, 1994; Threlfall *et al.*, 1996). This may cause therapeutic problems, as fluoroquinolones are the drugs of first choice for treating extra-intestinal and serious intestinal complications of human and animal salmonellosis. DT 104 has a broad spectrum of hosts and can easily spread to many different domestic and wild animals. Outbreaks of DT 104 and PT 4 infection have been associated with a number of food vehicles including poultry meat and eggs (Wall *et al.*, 1994; Coyle *et al.*, 1988; Rampling *et al.*, 1989). A better understanding of the mechanisms by which these foodborne pathogens survive in the environment and colonize chickens will provide the basis for designing more effective intervention strategies.

In previous studies, the resistance of 40 PT 4 strains to heat, acid and hydrogen peroxide, as well as the ability to survive airdrying on commonly used kitchen surface materials was examined (Humphrey *et al.*, 1995). The studies identified a small subgroup of strains that were much more sensitive to these stresses than the majority. These strains also showed differences in their ability to invade chicken tissues, particularly those of the reproductive tract (Humphrey *et al.*, 1996). Like PT 4, DT 104 has been shown, under experimental conditions, to be able to contaminate egg contents and can also be isolated from muscle tissue in laying hens (Williams *et al.*, 1998; Leach *et al.*, 1999). Little is known about the ability of DT 104 to invade other tissues, or whether a spectrum of invasiveness exists.

The *rpoS*-encoded σ^S subunit of RNA polymerase is an important regulator of the general stress response and is also required for virulence in *S. typhimurium* in orally infected mice (Loewen & Hengge-Aronis, 1994; Mahan *et al.*, 1996; Fang *et al.*, 1992) but less is known about the role of *rpoS* in the systemic infection of chickens.

The principal aim of the present study was to assess the ability of naturally occurring DT 104 strains to invade tissues in laying hens and relate this to their RpoS status and ability to survive exposure to drying, low pH and heat. We demonstrate variation in the ability to invade and/or persist in different chicken tissues among the DT 104 strains. Stress-sensitive strains were present among the DT 104 strains but these strains were not all less invasive. A majority of stress-sensitive DT 104 and PT 4 strains harboured mutations in the *rpoS* gene or showed reduced expression of the RpoS protein as measured indirectly using the RpoS-dependent *spvR/A':luxCDABE* fusion. The significance of the *rpoS* gene for the contamination of egg contents is discussed.

METHODS

Bacterial strains and phenotypic screens. The DT 104 strains described in Table 1 originated from unrelated human cases, various environmental sources and food animals and are held in a strain collection at the Public Health Laboratory Service (PHLS) Food Microbiology Research Unit, Exeter, UK. The PT 4 strains examined have previously been described (Humphrey *et al.*, 1995). All strains contain the *Salmonella* virulence plasmid which carries the virulence genes *spvRABCD* (Gulig *et al.*, 1993). The strains received very limited subculture after initial isolation and were maintained as frozen stocks at -70°C on Cryobeads (Prolab Diagnostics). Cultures were grown at 37°C to stationary phase in nutrient broth (NB, Oxoid CM 67) as described previously (Humphrey *et al.*, 1995). Phenotypic screens of 38 DT 104 strains were essentially performed according to the techniques described by Humphrey *et al.* (1995), with some minor modifications. Heat resistance was assessed after 15 min at 52°C instead of 10 min; survival on Formica surfaces at 20°C in 0.02 ml blood was assessed after 72 h instead of 24 h; acid resistance was assessed after 5 min at pH 2.8 instead of pH 2.6. Experiments determining heat and acid resistance were performed in duplicate while experiments to determine survival in blood on Formica were performed in triplicate. Differences in stress resistance of the strains were examined for statistical significance using ANOVA in Microsoft Excel.

Oral infection of chickens. Point-of-lay Hisex Brown pullets (17 weeks old) were obtained from a commercial *Salmonella*-free source. Faecal samples were tested prior to infection of the birds to confirm that they were free of *Salmonella* spp. The inoculum for each strain was grown in NB to stationary phase as previously described (Humphrey *et al.*, 1996). Fifteen birds were infected with each strain via the crop with 0.5 ml of culture diluted to contain approximately 10^7 c.f.u. (confirmed retrospectively) as described previously (Humphrey *et al.*, 1996). The birds were housed singly in cages following infection. Birds were examined at least daily for signs of illness, and faeces were collected every other day. On day 14 post-infection, the chickens were killed and entire liver, spleen, oviduct and ovary samples collected from each bird. All the work necessitating the involvement of animals was carried out with due concern for their welfare, in strict accordance with the Home Office Animals (Scientific Procedures) Act 1986 and subject to Local Ethical Committee approval.

Care was taken to ensure the aseptic removal of each organ. Birds were first surface-decontaminated using sodium hypochlorite solution. The body cavity was opened and the tissues removed in the following order: liver, spleen, oviduct and ovary. Fresh sterile surgical instruments were used for each bird and, on removal, each organ was immediately transferred to an individual sterile stomacher bag. Faeces and tissue samples were examined for the presence of *Salmonella* spp. using previously described methods (Humphrey *et al.*, 1996). Briefly, tissue samples were weighed and then homogenized using a stomacher in Maximum Recovery Diluent (MRD; Oxoid CM733). Liver and spleen homogenates were then serially diluted in MRD and plated onto xylose lysine desoxycholate (XLD; Oxoid CM 469) agar for microbiological counts. Remaining homogenates of liver and spleen, and those of ovary and oviduct, were also examined for the presence of *Salmonella* spp. using standard pre-enrichment and enrichment techniques. Faecal samples were selectively enriched in selenite broth (Oxoid CM 699) and plated onto XLD agar. The infection experiment was performed twice with strains 30, 11, 10 and 41 and once with strains 27, 16, 34

Table 1. *S. typhimurium* DT 104 strains

Strain	Tolerance profile*	Antibiotic resistance profile†	Source
30	R	SuT	Bovines
10	S	ACSSuT	Human
11	S	SuT	Human
41	S	ACSSuT	Human
27	R	ACSSuTTm	Chicken
34	R	ACSSuT	Human
16	R	SuT	Sewage
31	R	SuT	Bovines
12	R	ACSSuT	Human
13	R	ACSSuT	Sewage
14	R	ACSSuT	Milk
15	R	ACSSuT	Human
17	R	ACSSuT	Environment
18	R	ACSSuT	Sewage
19	R	ACSSuT	Human
20	R	ACSSuT	Water
21	R	ACSSuTTm	Water
22	R	ACSSuT	Sewage
23	R	ACSSuTTm	Water
24	R	ACSSuT	Bovines
25	R	ACSSuT	Bovines
26	R	ACSSuT	Bovines
28	R	ACSSuT	Sewage
29	R	SuT	Bovines
32	R	SuT	Human
35	R	ACSSuT	Human
36	R	ACSSuT	Human
37	R	ACSSuT	Human
38	R	ACSSuT	Human
39	R	ACSSuT	Human
40	R	SuT	Human
43	R	ACSSuT	Human
44	R	ACSSuTTm	Human
45	R	ACSSuTTm	Human
46	R	ACSSuTTm	Human
47	R	ACSSuTCp	Human
48	R	ACSSuTCp	Human
49	R	ACSSuTCp	Human
50	R	ACSSuT	Human

* The classification of the strains as overall resistant (R) or sensitive (S) was based on the following tests. Acid resistance (% survival after 5 min at pH 2.8) was determined in 39 strains; strains were classified as acid resistant when $\geq 25\%$ survived and as sensitive when $< 10\%$ survived. Heat resistance (% survival after 15 min at 52 °C) was determined in 37 strains; strains were classified as heat resistant when $> 20\%$ survived and as sensitive when $< 4\%$ survived. Survival on surfaces (% survival after 72 h at 20 °C on a Formica surface) was determined in 22 strains; strains were classified as resistant when $> 20\%$ survived and as sensitive when $\leq 6\%$ survived.

† A, ampicillin 10 µg; C, chloramphenicol 2.5 µg; S, streptomycin 25 µg; Su, sulphamethoxazole 25 µg; Tm, trimethoprim 10 µg; Cp, ciprofloxacin 0.1 µg.

and 31. The proportion of *Salmonella* spp. positive tissues for each strain was compared using the χ^2 test with Yates' corrections for small numbers (Statgraphics 5, STSC Inc.).

Expression of bioluminescence. The ability of the DT 104 and PT 4 strains to activate transcription of the σ^s -dependent *spvR* and *spvA* promoters was determined by measuring expression of bioluminescence during growth of strains transformed with the plasmids pSB367 or pSB368 (Swift & Stewart, 1994). Both pSB367 (high copy number) and pSB368 (medium copy number) contain the *spvR* gene and a transcriptional fusion *luxCDABE* (from *Photobacterium luminescens*) to the *spvA* promoter. Expression of the SpvR protein requires an active RpoS protein (Guiney *et al.*, 1995) and the expression of *luxCDABE* results from the activation of the *spvA* promoter by SpvR and RpoS. The bioluminescence of these strains is, therefore, a measure of active RpoS sigma factor in the cell. The functionality of this system as a reporter for active RpoS levels was shown in *Escherichia coli* BJ4, where the introduction of a mutation in *rpoS* reduced light levels by 3 log (Swift & Stewart, 1994). Preparation of competent cells, electroporation and isolation of bioluminescent colonies was performed according to previously published techniques (Swift *et al.*, 1993). Transformed strains were grown with shaking (200 r.p.m.) in LB broth, Lennox (LB; Difco) at pH 7.3. These cultures were standardized to an initial OD₆₀₀ of 0.025 from overnight cultures and grown at 37 °C for up to 15 h. Bioluminescence was measured by determining the number of relative light units per ml (RLU) in scintillation vial inserts (4 ml; FSA Laboratory Supplies) with a Turner Designs TD-20E luminometer (Streptech) (Swift *et al.*, 1993). Growth of the cultures was measured simultaneously as OD₆₀₀. Measurement of RpoS expression and OD₆₀₀ was repeated on two separate occasions for each strain. The σ^s -dependency of the bioluminescence expressed by the *spvR/A'::luxCDABE* fusion was validated by measuring the light expressed in three *S. typhimurium* strains with identical backgrounds (*S. typhimurium* LT2) but with different *rpoS* alleles (strain χ 4996, *rpoS::bla*; strain χ 3000, *rpoS*^{LT2}; strain χ 8000, *rpoS*^{SL1344}; Wilmes-Riesenberg *et al.*, 1997). In order to confirm that light expression was dependent on the RpoS-dependent promoter, light measurements using a constitutive promoter fused to the *lux* operon (pSB311) were performed with otherwise isogenic σ^s -positive and σ^s -negative *S. typhimurium* strains (Hill *et al.*, 1993). In these experiments expression of bioluminescence was constitutively high and showed no growth-phase-dependent induction (results not shown).

Sequence analysis of *rpoS* genes. The *rpoS* genes were amplified from *S. typhimurium* DT 104 and *S. enteritidis* PT 4 strains using RPO5' (5'-CGGAATTCTTATTATCATC-AAACATAAC-3') and RPO3' (5'-CCTTGCCCGGGCTG-TGCCGATGCAC-3') amplimers made at the Biopolymer Synthesis and Analysis Unit, University of Nottingham. Template genomic DNA was purified using the ISO-Quick Kit (ORCA Research). PCR amplification was carried out in triplicate, for 30 cycles, each consisting of 30 s denaturation at 95 °C, 1 min of reannealing at 55 °C and 2 min of DNA synthesis at 72 °C. PCR products were separated by electrophoresis, extracted from the gel using a QiaQuick Gel Extraction Kit (QIAGEN), dialysed (Millipore VS filters) and sequenced at the University of Nottingham Sequencing Facility. Each gene was sequenced from at least two independent PCR products and DNA sequences from both strands of the *rpoS* genes were obtained.

RESULTS

Resistance to drying, heat and low pH

In the present investigation, 38 strains of DT 104 were examined for their ability to survive environmental stresses and were classified as being either sensitive or resistant (Table 1). Three DT 104 strains were classified as sensitive (Table 1). In a previous study of forty PT 4

strains, seven were identified as being significantly more sensitive to these stresses (Humphrey *et al.*, 1995). Strains from the two groups of DT 104 and PT 4 were compared with respect to survival after exposure to heat and acid stress as well as survival on surfaces (Table 2). By ANOVA, no significant differences were observed between the strains classed as resistant, with regard to either heat resistance ($F_{34,35} = 0.86$, $P > 0.67$) or acid

Table 2. Comparison of the ability to survive airdrying on surfaces or exposure to heat or acid stress in stationary-phase cultures of *S. typhimurium* DT 104 and *S. enteritidis* PT 4

Strain	Survivors (%)*		
	Blood, 20 °C, 72 h	52 °C, 15 min	pH 2.8, 5 min
DT 104 30	33 (2.5)	28 (2.0)	27 (2.0)
DT 104 10	2.8 (0.8)	0.17 (0.1)	6.5 (0.5)
DT 104 11	6.0 (0.9)	3.9 (1.0)	9.0 (1.4)
DT 104 41	5.4 (1.0)	2.7 (0.8)	8.5 (0.5)
DT 104 27	39 (1.0)	23 (2.0)	25 (2.5)
DT 104 34	23 (4.0)	33 (2.0)	35 (2.0)
DT 104 16	40 (11)	29 (5.0)	36 (3.0)
DT 104 31	32 (5.0)	25 (3.0)	32 (1.5)
PT 4 E	36 (0.9)	36 (4.0)	42 (11)
PT 4 I	1.1 (0.4)	2.5 (2.0)	6.0 (1.0)
DT 104 12	27 (3.0)	28 (6.0)	41 (9.0)
DT 104 13	ND	28 (1.0)	48 (5.0)
DT 104 14	44 (5.0)	33 (5.0)	46 (14)
DT 104 15	ND	26 (6.0)	38 (14)
DT 104 17	41 (10)	28 (5.0)	35 (6.0)
DT 104 18	42 (10)	22 (1.0)	43 (12)
DT 104 19	ND	41 (1.0)	41 (1.0)
DT 104 20	ND	ND	38 (13)
DT 104 21	52 (11)	53 (12)	53 (9.0)
DT 104 23	49 (8.0)	ND	34 (14)
DT 104 24	ND	30 (6.0)	32 (3.0)
DT 104 25	ND	29 (3.0)	44 (1.0)
DT 104 26	ND	30 (4.0)	42 (16)
DT 104 28	ND	33 (10)	47 (18)
DT 104 29	38 (6.0)	30 (4.0)	35 (1.0)
DT 104 32	35 (5.0)	31 (3.0)	43 (9.0)
DT 104 35	ND	26 (4.0)	37 (3.0)
DT 104 36	ND	32 (4.0)	47 (2.0)
DT 104 37	46 (4.0)	24 (2.0)	29 (3.0)
DT 104 38	ND	28 (0.0)	37 (8.0)
DT 104 39	ND	32 (2.0)	37 (5.0)
DT 104 40	ND	36 (7.0)	29 (5.0)
DT 104 43	56 (14)	24 (3.0)	40 (19)
DT 104 44	ND	27 (2.0)	57 (11)
DT 104 45	ND	31 (5.0)	56 (4.0)
DT 104 46	43 (8.0)	25 (3.0)	68 (11)
DT 104 47	33 (8.0)	27 (2.0)	49 (13)
DT 104 48	ND	23 (3.0)	56 (5.0)
DT 104 49	39 5.0	ND	39 (5.0)
DT 104 50	36 5.0	ND	59 (11)

ND, Not determined.

* The numbers in parentheses represent the standard error of the mean.

Fig. 1. Invasiveness of *S. typhimurium* DT 104 strains into liver (open bars), spleen (solid bars), ovary (striped bars) and oviduct (hatched bars). Results show the number of birds with *Salmonella*-positive tissues, as determined by enrichment culture, out of the 15 in each group that were infected. An asterisk (*) denotes repeat experiments which were performed with strains 30, 10, 11 and 41 only.

resistance ($F_{35,36} = 0.94$, $P > 0.58$), or the ability to survive on surfaces ($F_{19,40} = 1.4$, $P > 0.17$). For the DT 104 strains the mean percentage survival values for resistant strains were 28 (SE 0.6), 40 (SE 1.5) and 39 (SE 1.8), respectively, for heat resistance, acid resistance and ability to survive on surfaces, while the corresponding figures for the sensitive strains were 2.2 (SE 1.1), 8 (SE 0.8) and 5.7 (SE 2.1). The difference between the stress-sensitive and -resistant strains was highly statistically significant by ANOVA for both heat ($F_{1,76} = 173$, $P < 0.00005$), acid ($F_{1,78} = 54$, $P < 0.00005$) and drying on surfaces ($F_{1,70} = 89$, $P < 0.00005$).

Invasiveness of DT 104 strains in chicken tissues

All strains were able to invade liver and spleen tissue in laying hens as determined by enrichment but frequency of recovery from every tissue differed between the strains (Fig. 1). In liver, the numbers of organisms were below the detection limit of the direct count method (i.e. < 10 cells per g tissue) for all strains except strain 31, where a mean count of $\log_{10} 1.4$ per g of tissue (SD 0.3) was obtained from the three livers which had counts above the detection limit. The number of organisms recovered from spleens was, however, more often sufficiently high to be detected by the direct count method (Table 3). Mean levels of organisms ranged from less than 10 c.f.u. per g, where no direct counts were obtained (strains 10 and 41), to a mean level ranging between $\log_{10} 1.2$ and $\log_{10} 3.5$ c.f.u. per g in those tissues where it was possible to establish direct counts (Table 3). Similar levels of *Salmonella* have been recovered from spleen and liver of chickens infected orally by other workers (Rajashekara

Table 3. Numbers of *S. typhimurium* DT 104 recovered from spleens, 14 d post-infection, of chickens orally infected with 10^7 cells

Strain	No. of samples positive by direct count ($n = 15$)	Mean $\log_{10}$ c.f.u. per g tissue of samples positive by direct count (SD)
30	4	1.2 (0.3)
30*	4	1.4 (0.6)
10	0	—
10*	0	—
11	4	1.2 (1.0)
11*	3	1.6 (0.6)
41	0	—
41*	0	—
27	4	1.2 (0.7)
34	5	3.5 (0.9)
16	10	2.0 (0.5)
31	2	1.9 (0.9)

* Denotes repeat experiments which were performed with strains 30, 10, 11 and 41 only.

et al., 2000). Invasion and/or persistence in tissues was, therefore, more reliably established by enrichment techniques, since levels of organisms in tissues 14 d following oral administration of challenge strains were frequently, though not universally, low. As determined by enrichment (Fig. 1), strains 10 and 41 were found significantly (χ^2 test, $P < 0.001$) less frequently in liver and spleen tissue than strain 30. Strain 10 was not isolated from any ovaries or oviducts. The frequency of isolation from tissues with the stress-sensitive strain 11 was substantially greater than that of strain 10 and not significantly (χ^2 test, $P > 0.05$) reduced by comparison with the majority of the stress-resistant strains (30, 27, 34, 16 and 31). There was a strong positive correlation between the frequency of isolation from liver and isolation from spleen, ovary and oviduct as demonstrated by the significant P values for the linear regression analysis of each of these associations across the different strains ($P = 0.04$, 0.04 and 0.05 , respectively). This demonstrates that if a DT 104 strain is more frequently isolated from one of these tissues in poultry, such as the liver, it will also be more readily isolated from the others, including the reproductive tissues. Frequency of isolation from any tissue did not correlate with the faecal carriage rates in the DT 104 strains ($P > 0.05$; data not shown).

σ^S -dependent gene expression

The expression from the σ^S -dependent *spvR/A'lux::CDABE* fusion was determined in three resistant strains (16, 31 and 30) and a sensitive strain (11) of DT 104. It was not possible to determine RpoS expression using pSB367 or pSB368 in most of the DT 104 strains

Fig. 2. Expression of the σ^S -dependent *spvR/A'::luxCDABE* fusion: (a) in sensitive (Δ , I; $\circ$, C) and resistant ($\square$, E; $\times$, A) strains of *S. enteritidis* PT 4, and (b) in a sensitive strain ($\square$, 11) and three resistant ($\times$, 30; $\circ$, 16; Δ , 31) strains of *S. typhimurium* DT 104. Results from representative experiments are shown.

because of an inability to select for the plasmid marker and inherent resistance of these strains to chloramphenicol and ampicillin. Activation of the RpoS-dependent fusion was also determined in two resistant (E and A) and two sensitive (I and C) strains of PT 4. In strains classified as resistant, expression of the *spvR/A'lux::CDABE* fusion demonstrated an induction of promoter activity at the approximate transition between exponential and stationary phases (Fig. 2a, b). The extent of the induction was around 10–100-fold under the conditions used here. In PT 4 strains classified as sensitive, the initial level of light was at least 2 $\log_{10}$ lower than in resistant strains and, with no significant induction during the transition into stationary phase, up to a 4 $\log_{10}$ difference in light could be observed for cells in the stationary phase (Fig. 2a). DT 104 strain 11 showed a similar level of σ^S -dependent *spvR/A'luxCDABE* induction but the level of light emitted was 1–2 $\log_{10}$ less than in DT 104 strains classified as resistant (Fig. 2b). A semi-qualitative visual examination of RpoS expression was performed in DT 104 strain 41 by adding H₂O₂ to bacterial colony mass derived from a nutrient agar plate (Zambrano *et al.*, 1993). The extent of bubbling was similar to that of DT 104 strain 30, suggesting that a sufficient level of RpoS protein expression for hydrogen peroxidase II production was present in strain 41. When the expression of *spvR/A'lux::CDABE* encoded by pSB368 was determined in isogenic *S. typhimurium* strains of LT2 derivative harbouring different *rpoS* alleles, three levels of light

Fig. 3. Expression of the σ^S -dependent *spvR/A'::luxCDABE* fusion in *S. typhimurium* strains with an LT2 background but different *rpoS* alleles: γ 4996 (*rpoS::bla*) ($\square$), γ 3000 (*rpoS*^{LT2}) (Δ) and γ 8000 (*rpoS*^{SL1344}) ($\circ$). Results show one representative experiment.

emission were observed (Fig. 3). These results are analogous to three levels of β -galactosidase activity measured with a *spvA-lacZ* fusion by Wilmes-Riesenberger *et al.* (1997). However, the quantitative aspects of induction were different. A 100-fold induction was measured using the *lux* fusion (Fig. 3), as compared to the 18-fold induction seen with the β -galactosidase fusion (Wilmes-Riesenberger *et al.*, 1997).

rpoS loci in DT 104 and PT 4 strains

The *rpoS* gene was sequenced in DT 104 strains 30, 11, 10 and 41 and PT 4 strains C, I and E. The *rpoS* gene in the stress-resistant PT 4 strain E was 100% identical in DNA sequence to the *rpoS* gene of *Salmonella* Dublin (GenBank accession no. X82129), whereas the two stress-sensitive PT 4 strains both harboured mutations. Strain C contained an insertion (A) at codon 220, causing a frameshift resulting in a stop codon after nucleotide 684. Strain I had undergone a 393 bp in-frame deletion beginning at nucleotide 540. The *rpoS* genes in DT 104 strains 30, 11 and 41 exhibited 100% identity in nucleic acid sequence to the *rpoS* gene of *S. typhimurium* C52 (GenBank accession no. X77752). The *rpoS* gene in DT 104 strain 10, however, contained a single base-pair substitution (G to T) at codon 33, changing a GAG codon, specifying glutamic acid to a TAG amber nonsense codon. It is interesting to note that several laboratory strains of *E. coli* including JC7623, AB1157 and RM4606 also contain a mutation at this codon (C to T), also resulting in a TAG amber nonsense codon (Visick & Clarke, 1997).

DISCUSSION

S. typhimurium DT 104 and *S. enteritidis* PT 4 are major foodborne enteric pathogens for humans, with poultry products as significant vehicles of infection. A clear understanding of the factors which determine the survival of the bacteria in relevant environments and their virulence in chickens is important in the identification of control measures. Estimating the possible

variation in these parameters will also help in risk assessment of salmonellosis.

Previous work on PT 4 demonstrated that naturally occurring strains differed with respect to their resistance to heat, acid, peroxide and airdrying on surfaces, and their virulence in mice and chickens infected orally (Humphrey *et al.*, 1995, 1996, 1998). The studies reported in this paper sought to take the investigations into resistance and virulence of *Salmonella* spp. further by examining the behaviours of *S. typhimurium* DT 104 strains and by correlating these with RpoS expression.

The results showed that as with PT 4, DT 104 comprises sensitive and resistant strains when cells are challenged in stationary phase. These results could suggest differences in RpoS expression, and this was explored in several DT 104 and PT 4 strains. In the five sensitive DT 104 and PT 4 strains examined, three harboured mutations in the *rpoS* gene, while two DT 104 strains had intact *rpoS* genes and one of those demonstrated a reduced level of RpoS-dependent *spvR/A'::luxCDABE* expression. In *E. coli*, expression of the RpoS protein is known to be primarily controlled post-transcriptionally and evidence for post-transcriptional regulation in *Salmonella* has also been found (Lange & Hengge-Aronis, 1994; Schweder *et al.*, 1996; Pratt & Silhavy, 1996; Bearson *et al.*, 1996). It is therefore possible that the reduced expression the RpoS-dependent *spvR/A'::luxCDABE* fusion in DT 104 strain 11 is due to mutations affecting the translational processing of the RpoS protein or, alternatively, to protein instability. The presence of mutant *rpoS* alleles has also been investigated in clinical and environmental strains of shiga-like toxin-producing *E. coli* (Waterman & Small, 1996). Ten out of 58 strains were acid sensitive, and acid resistance could be restored in six strains when complemented with a functional *rpoS in trans*, suggesting that at least 10% of the population could be *rpoS* mutants. The *rpoS* allele was sequenced in two acid-sensitive strains and one had undergone a single base-pair deletion resulting in a stop codon after amino acid 28 while the other contained an 11 base-pair duplication resulting in a stop codon after amino acid 77 (Waterman & Small, 1996). Laboratory strains of *E. coli* have also been shown to contain mutant *rpoS* alleles or acquire them through extended culture (Ivanova *et al.*, 1992; Visick & Clarke 1997; Zambrano *et al.*, 1993) and several of the typical laboratory LT2-derived strains of *S. typhimurium* contain an *rpoS* allele with a rare UUG start codon (Wilmes-Riesenberg *et al.*, 1997). The DT 104 strains 11 and 10, found in this study, were initially isolated from human cases of gastroenteritis, while the PT 4 strains C and I were isolated from chicken carcasses. The *rpoS* mutation in strain 10 may have arisen during the initial isolation of the strain, although it has maintained its stress sensitivity after trials of extensive subculturing in the laboratory. It is, however, also possible that strains lacking important virulence factors may cause infection, as the development of an infection will, among other things, depend on the status of the host and the amount of inoculum (Picard *et al.*,

1999). We are currently addressing this issue by examining the RpoS status of colonies arising from the initial isolation plates associated with cases of gastroenteritis.

The DT 104 and PT 4 *rpoS* mutants were very sensitive to airdrying on surfaces. The trehalose synthesis gene *otsA* is regulated by RpoS in *S. typhimurium* (Fang *et al.*, 1996) and trehalose is known to protect cells from drying by stabilizing membranes and enzymes, and this may explain the observation (Potts, 1994). PT 4 strain I, now identified as an *rpoS* mutant, has also been shown to survive poorly in aerosols (Humphrey *et al.*, 1996).

The pathogenicity of selected DT 104 strains was also examined in point-of-lay commercial hens infected orally. Strains with mutations in the *rpoS* gene were less frequently found in the tissues of experimentally infected birds and in previous work, the PT 4 strain I (identified as an *rpoS* mutant in this work) was also less frequently isolated from chicken tissues, particularly those of the reproductive tract (Humphrey *et al.*, 1996). Interestingly, the faecal carriage rate of stress-sensitive strains was not significantly different from those of stress-resistant strains and strain 11 was the only strain found in muscle tissue (Williams *et al.*, 1998). The data from these experiments demonstrate a potentially complex relationship between RpoS expression, pathogenicity and stress tolerance. The level of expression of the RpoS-dependent *spvR/A'::luxCDABE* fusion in strain 11 was lower than in the stress-resistant strain 30, but with a similar induction, while strain 10 encoded a highly truncated protein. However, strain 11 was not significantly less frequently isolated from spleen tissue compared to strain 30, and strain 11 was also able to invade the reproductive tissues of chicken, in contrast to strain 10. The stress-sensitive strain 41 was absent from ovarian tissues and was recovered from the oviduct of only one of 30 chickens, yet it carries a functional *rpoS* gene. This suggests it may well harbour mutation(s) in other stress response/virulence genes such as *rpoE* (Humphreys *et al.*, 1999). Together the data suggest that a functional *rpoS* gene is necessary but not sufficient for the full virulence of DT 104 in poultry and that molecular determinants other than the RpoS protein play a role.

The differences in the invasiveness into the reproductive tissues between the DT 104 strains did not correlate with the subsequent egg contamination rates reported by Williams *et al.* (1998). Indeed, the poorly invasive strain 10 produced egg contamination rates as high as many of the other strains, and the stress-sensitive strain 11 produced the highest rates. Clearly, while the RpoS protein is important for colonizing the reproductive tissue in laying hens, the contamination of egg contents appears to be dependent on other undefined molecular determinants.

This present study used chickens. Other work showed that expression of the RpoS protein had no effect on invasiveness into the tissues of day-old chicks (Allen-Vercoe *et al.*, 1998; Humphrey *et al.*, 1996). Strains with

normal expression of the RpoS protein are, however, significantly more virulent in orally infected mice (Fang *et al.*, 1992; Humphrey *et al.*, 1996, 1998; Wilmes-Riesenberger *et al.*, 1997). Thus, the role of the *rpoS* gene is dependent on the host environment, and while virulence in mice and survival in the environment is highly RpoS-dependent, invasion into day-old chicks may not require RpoS. In mice, the RpoS-dependent effect on virulence is mainly exerted via the activation of the virulence plasmid *spv* genes, but *rpoS*-regulated chromosomally encoded genes also have a contributory role (Fang *et al.*, 1992). The ability of *Salmonella* strains to invade the liver or colonize the caecum of chickens was, however, not affected by the absence of the virulence plasmid, suggesting that chromosomally encoded *rpoS*-regulated genes are responsible for the differences in invasiveness observed in chickens (Halavatkar & Barrow, 1993). Detailed investigation of the role of *rpoS* in the pathogenicity process has shown that *rpoS* does seem to be important for colonizing Peyer's patches (Nickerson & Curtiss, 1997). The process of infection is complex and depends on many factors including LPS, fimbriae, virulence plasmid, toxins and siderophores. Loss of one or more of these may result in loss of virulence depending on the host environment (Cox, 1995).

In this work we have demonstrated that within clinical and environmental strains of *S. typhimurium* DT 104 significant differences exist in the ability to invade and/or persist in chickens and to survive exposure to heat stress, low pH and airdrying on surfaces. It remains to be definitively determined whether *rpoS* nonsense mutations such as those identified in the clinical strains studied here and elsewhere are also present in strains at the point of host infection.

ACKNOWLEDGEMENTS

We would like to thank Mary Wilmes-Riesenberger for providing strains and Simon Swift for critical comments on the manuscript. This work has been sponsored by the Department of Health, the PHLS and the British Society for the Study of Infection.

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Received 6 December 1999; revised 20 June 2000; accepted 22 August 2000.