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ORIGINAL ARTICLE

Effects of repeated cycles of acid challenge and growth on the phenotype and virulence of *Salmonella enterica*

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Abstract

Aims: The aim of the study was to investigate how stresses like low pH, which may be encountered in farms or food preparation premises, shape populations of *Salmonella enterica* by the selection of stress-resistant variants.
Methods and Results: Stationary-phase cultures of *S. enterica* serovar Enteritidis and serovar Typhimurium (one strain of each) were exposed to pH 2·5 for up to 4 h, followed by growth at pH 7 for 48 h. This process was repeated 15 times in two separate experiments, which increased the acid resistance of the three out of four populations we obtained, by three- to fourfold. Sustainable variants derived from the populations showed changes in colony morphology, expression of SEF17 fimbriae, growth, increased heat resistance and reduced virulence.
Conclusions: The study demonstrates that low pH environments can select for populations of *S. enterica* with persistent phenotypic changes such as increased acid resistance and occasionally increased SEF17 expression and lower virulence.
Significance and Impact of the Study: There is a common belief that increased acid resistance coincides with increased virulence. This study demonstrates for the first time that increased acid resistance often impairs virulence and affects the general phenotype of *S. enterica.*

Introduction

It is well established that cells of *Salmonella enterica* in common with other foodborne pathogens, can respond to potentially stressful environments with changes in their physiology, enhancing their survival (Humphrey 1990; Foster 1991; Humphrey *et al.* 1993; Segal and Ron 1998; Hecker and Volker 2001). The responses are normally transient and are no longer expressed once the stress is removed (Humphrey 1990). Little is known about how populations respond and if permanent changes occur, when cells are exposed to repeat or multiple stresses encountered in both natural and food production environments. For example, in agriculture, cells of *S. enterica* may experience frequent changes in levels of water activity, temperature, pH and exposure to disinfectants. *Salmonella enterica* can be isolated with regularity from the farm environment, even when animal houses have been cleaned and disinfected using approved compounds and methods (Rose *et al.* 2000). Such persistence poses a threat to biosecurity and may reflect selection of a more resistant population.

Microbial populations are not homogeneous, even if of clonal origin (Rocha *et al.* 2002). Mutants occur randomly and according to the conditions, they might be able to survive, increase their numbers and even outgrow the other cells. It is important to study the phenotype of these mutants to obtain information regarding the

variability of populations of pathogens and the impact of stress resistance on the overall phenotype. Hypermutable regions have been reported to be over-represented in stress genes and possibly play an important role in the generation of stress-resistant mutants in high frequencies (Rocha *et al.* 2002). The impact of this phenomenon has been demonstrated experimentally in *Listeria monocytogenes* where a hypermutable region in *ctsR*, the regulator of class III stress genes resulted in the appearance of stress-resistant mutants within hours of growth in clonal pure cultures of *L. monocytogenes* (Karatzas *et al.* 2005).

Several studies have highlighted the important role of stress resistance mechanisms in virulence. RpoS plays a central role in acid and general stress resistance but also in pathogenicity of *Salmonella* Typhimurium (Jørgensen *et al.* 2000). *Salmonella* Typhimurium containing mutations in *atrC* displayed lower acid tolerance response (ATR) and was highly attenuated in bone marrow-derived macrophages (Wilmes-Riesenberg *et al.* 1996). The DnaK/DnaJ chaperone machinery of *S.* Typhimurium is also essential for stress resistance and invasion of epithelial cells and survival within macrophages (Takaya *et al.* 2004). These mechanisms are probably enhancing survival of the pathogen in the low pH of the stomach or the oxidative environment of the macrophages. It has been suggested that acid stress might enhance virulence by triggering adaptive mutations, increasing the likelihood of disease (Archer 1996). Research in *S.* Typhimurium has shown that there could be a positive correlation between acid resistance and pathogenicity (Bearson *et al.* 1997; Bark *et al.* 2005). In line with this, a *L. monocytogenes* acid-tolerant mutant isolated following prolonged exposure to low pH, demonstrated increased virulence, but it was not shown if these effects were a result of a single or multiple mutations (O'Driscoll *et al.* 1996).

In this work, the risk of emergence of a more stress-resistant and possibly more virulent population of *S. enterica* after exposure to repeated acid stress was examined. We also investigated the effects of the repeated exposure to low pH on acid, heat and desiccation resistance, colony morphology, expression of SEF17 fimbriae and virulence.

Materials and methods

Bacterial strains and culturing

The strains used in this study were *S. enterica* serovar Enteritidis phage type 4 strain 66045 (SE 66045) and *S. enterica* serovar Typhimurium definitive type 104 strain 30 (ST 30). SE 66045 was isolated from human faeces at Exeter Public Health Laboratory, UK in 1996; ST 30 was originally isolated from cattle faeces (Williams *et al.* 1998). Both strains that have functional RpoS, were purified by streaking on 5% horse blood agar (BA, Columbia Agar Base; Oxoid, Basingstoke, UK) and following incubation at 37°C for 24 h agar one representative colony was stored in Microbank cryovials (Pro-Lab Diagnostics, Wirral, UK) at −80°C. Prior to experiments, they were recovered from storage and stationary phase cultures were prepared in buffered peptone water (BPW; Oxoid).

Exposure to repeated acid stress

Pure cultures were stored at −80°C in Microbank cryovials and inoculated into sterile BPW, prior to each experiment. Cultures were incubated at 37°C until log phase (*c.* 3 h) and 10 μl from each was inoculated into 10 ml of fresh BPW and incubated at 37°C overnight under static conditions. Three aliquots from each culture were used to inoculate (1% inoculum) three bottles containing BPW (pH 2·5) acidified with hydrochloric acid and incubated at 37°C for various times. Subsequently, bacteria in each culture were harvested by centrifugation at 3600 ***g*** for 10 min and the pellets were resuspended in three corresponding bottles containing BPW (pH 7·0) followed by incubation at 37°C for 48 h. From the three cultures, the one that survived the longest acid challenge was selected and it was used to provide the three inocula for the next acid-challenge cycle. Owing to the increased acid resistance of the surviving population following each acid cycle, the acid treatment times were also increased progressively ranging from 60 to 240 min as the number of acid cycles increased. This was repeated 15 subsequent times and the populations obtained from the last acid challenge-growth cycle, were streaked onto BA and confirmed as salmonellae by growth on xylose lysine deoxycholate agar (XLD) and agglutination with *Salmonella*-specific antibodies. All populations were stored on Microbank cryovials as described before, for subsequent experiments. All the acid cycling processes were performed in duplicate for each strain, resulting in two final populations for each one of the two strains. Thus, the populations were: SE 66045 Cycl1, SE 66045 Cycl2, ST 30 Cycl1 and ST 30 Cycl2.

Isolation of acid-tolerant variants from populations

In order to investigate the makeup of the acid-cycled populations, representative individual isolates were obtained from them. Isolates SE 66045 CI and CII were obtained from populations SE 66045 Cycl1 and Cycl2, respectively. Similarly, isolates were obtained from ST 30 populations and named accordingly. Each isolate came from random selection of a single colony, following streaking of the corresponding population on BA. More isolates were obtained, but all the ones isolated from the same population had a similar phenotype. Therefore, only

a representative isolate from each population is presented to avoid redundancy. All isolates were identified as *Salmonella* spp. using the aforementioned methods.

Stability of phenotypes

Stability of acid-resistant phenotypes of all isolates was assessed by sub-culturing 15 times in BPW (pH 7) with 1% inocula at 37°C for 48 h, each time (2·5–3 generations). The acid resistance of each strain was assessed, as described next, after every five subcultures (12·5–15 generations). In addition, all other phenotypic characteristics were assessed.

Determination of acid resistance

Acid resistance of populations and isolates was measured in BPW (pH 2·5) prepared as described before. Overnight stationary phase cultures were diluted to *c.* 10^6 cells ml^{-1} by inoculating 100 μl into 9·9 ml of BPW (pH 2·5) and held at 37°C. Samples were taken at regular time intervals and diluted in maximum recovery diluent (MRD; Oxoid) containing 10% lysed horse blood in the first dilution. Dilutions were enumerated using the method described by Miles and Misra (1938) onto BA and incubated at 37°C for 48 h (Mattick *et al.* 2001). Acid resistance was expressed in *D*-values representing the time in minutes needed to reduce the population by 90% or 1 log.

Analysis of growth kinetics

Growth characteristics of all isolates were assessed at 37°C under static conditions. Five microlitres from an overnight culture of each survivor were inoculated into 200 μl of fresh BPW. Samples were placed in a Sero-well microtitrate plate (Sterilin, Staffs, UK) and bacterial growth was assessed by measuring the OD_{600} of the samples in a microplate reader, Bio-Rad Model 680 (Bio-Rad, Hercules, USA) at 37°C for 25 h. Growth curves were constructed in triplicate by taking OD measurements every hour.

Determination of heat resistance

The heat resistance of the isolates was determined. Overnight cultures were grown as described before and inoculated into BPW (pH 7·0) at 52°C to a final concentration of *c.* 10^6 cells ml^{-1}. Samples were taken at regular intervals and enumerated as described before.

Desiccation resistance

Fifty microlitres of overnight cultures from each of the individual isolates were inoculated in 5 ml of lysed blood to give a final concentration of *c.* 10^6 cells ml^{-1}. Numbers of cells in the suspension were enumerated as described before. Subsequently, 30 μl of the suspension were placed onto sterile Formica (kitchen grade) squares (1 cm^2) in triplicate, which were placed in sterile petri dishes and were allowed to dry at 37°C for 24 h. Bacteria were recovered by soaking each square for 15 min in 5 ml of MRD in a 25-ml Universal bottle, followed by vortexing. Samples were diluted in MRD and enumerated as before.

Colony morphology

Colony morphology was examined by transferring randomly selected colonies from all acid-cycled populations, onto the surface of colonization factor antigen (CFA; Walker *et al.* 1999) and modified brilliant green agar (BGA; Oxoid). The plates were incubated at either 25°C or 37°C for 72 h and colony morphology was monitored during incubation. Similarly, the colony morphology of the individual isolates was also examined.

SEF17 expression

Enzyme-linked immunosorbent assays (ELISA) were used to assess SEF17 fimbrial expression in all single isolates obtained, as described by Walker *et al.* (1999), with the exception that strains were grown in CFA liquid media at 37°C for 18 h and at 20°C for 48 h.

Virulence assays

All animal studies were conducted under the jurisdiction of the Animals Scientific Procedures Act (1986) and were reviewed by the local ethical review committee. Experiments were conducted using C57/BL6/J mice at 20 g weight (*c.* 4 weeks of age). Mice caged in groups, were infected into the oesophagus with 10^6 and 10^4 cells for SE 66045 and ST 30, respectively. Mice were observed at least daily and euthanized five days postinfection. Samples of faeces, spleen, liver and blood were taken using aseptic techniques, emulsified in MRD and cells of *S. enterica* were enumerated using standard techniques (Baskerville *et al.* 1992; Humphrey *et al.* 1992). Virulence assays were also carried out using mice infected with lower doses, of 10^4 and 10^3 CFU ml^{-1}, with pre- and postcycled ST 30 CI cells.

Statistical analysis

All assays were performed in triplicate (using three independent cultures) with data presented as means with error bars showing the SEM. Data were presented as line graphs and as *D*-values, which were calculated using

linear regression, or by time to 3 log reduction in CFU ml^{-1}. Significant differences between data sets were determined using *t*-test (two-tail) analysis in Microsoft Excel®. In mouse virulence assays, significant differences were determined by using Fisher's exact probability test. In all cases, statistically significant result was when $P < 0{\cdot}05$.

Results

Acid resistance

Repeated exposure of *S.* Enteritidis and Typhimurium to pH 2·5 followed by growth of surviving cells at pH 7 after each challenge, resulted in populations with significantly increased acid resistance with the exception of ST 30 Cycl2 (Fig. 1). In general, acid-cycled populations demonstrated 2–5·2-fold increases in *D* (pH 2·5) values over those of the precycled cells. In most cases, after each successive acid cycle, populations showed increased acid resistance over the previous one (data not shown).

As before, the individual isolate, ST 30 CII, was not more acid-tolerant than its parent strain, while all other isolates showed 1–3 log lower reduction in numbers compared with the corresponding wild-type (Fig. 2).

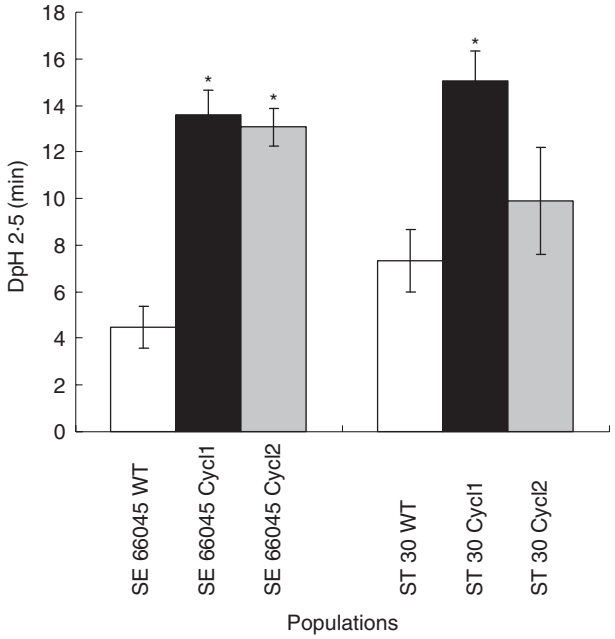

Figure 1 Acid resistance of populations, SE 66045 WT, SE 66045 cycl1 and SE 66045 cycl2; ST 30 WT, ST 30 cycl1 and ST 30 cycl1. Acid resistance is expressed in *D*-values representing the time in minutes needed to reduce the population by 90% or 1 log. White bars represent wild-type strains, while black and grey bars represent the whole populations obtained from the corresponding wild-type strain subjected in duplicate (cycles I and II, respectively) to 15 cycles of repeated acid challenge followed by growth in neutral buffered peptone water (BPW). Acid challenges were performed in BPW with pH 2·5, which was achieved by addition of hydrochloric acid. Error bars represent SE. Asterisk denotes statistically significant difference with the corresponding wild-type ($P < 0{\cdot}05$).

Stability of the acid-resistant phenotypes

All isolates demonstrated a stable phenotype for at least five overnight subcultures (12·5–15 generations) in the absence of acid stress but SE 66045 CI and CII lost acid resistance after 10 subcultures or 25–30 generations (data not shown). The acid resistance of ST 30 CI expressed as *D*-values (time in minutes required to reduce the population by 90% or 1 log) at pH 3 was reduced from 31·86 min to 19·33 min after 15 subcultures (37·5–45 generations), but this still remained higher than that of the corresponding wild-type, which was *c.* 10·07 min.

Analysis of growth kinetics

All acid-resistant isolates showed impaired growth with lower population densities compared with their corresponding wild-type (Fig. 3). Previously, we had confirmed that cells of all isolates were similar in size compared with those of their corresponding wild-type and in addition, we experimentally proved that viable numbers were corresponding to the OD_{600} regardless of the strain used. SE 66045 CI (Fig. 3a) also showed growth impairment accompanied with a specific growth rate at an exponential phase of 0·041 h^{-1} compared with 0·067 h^{-1} for the corresponding wild-type SE 66045 WT.

Heat resistance and desiccation resistance of acid-cycled cultures

The strains did not show any increased thermotolerance as a result of acid cycling, with the exception of an increase for ST 30 CI. For this strain, the log reduction in CFU ml^{-1} was 1·6 (±0·09 SE) showing a statistically significant difference ($P < 0{\cdot}05$) compared with 2·05 (±0·03 SE) for the wild-type following 90 min at 52°C (Fig. 4). Furthermore, all isolates did not show increased desiccation resistance after acid-cycling.

Colony morphology

Acid-cycling selected strains with changes in colony morphology in the ST 30 Cycl1 and ST 66045 Cycl1 populations. Colonies of ST 30 CI (Fig. 5b) were convoluted at 25°C and highly convoluted at 37°C (Fig. 5c) within 24 h, whereas the wild-type strain produced convoluted

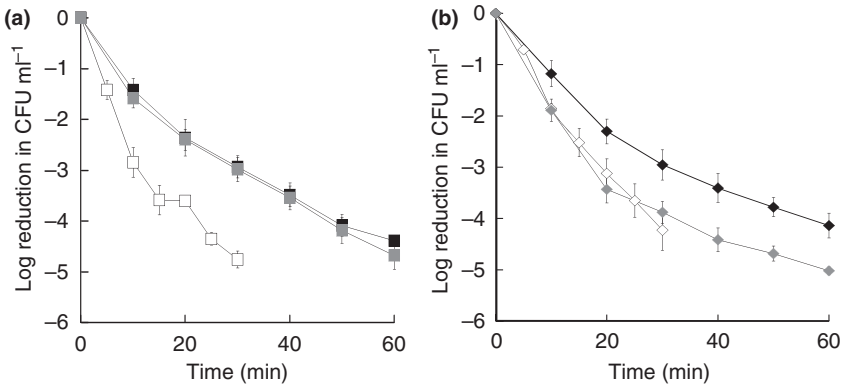

Figure 2 Acid resistance of: SE 66045 WT (□), CI (■) and CII (▩) (a); ST 30 WT (◇), CI (◆) and CII (◈) (b). White symbols represent wild-type strains, while black and grey symbols represent isolates from the corresponding wild-type strain subjected in duplicate (CI and CII) to 15 cycles of repeated acid challenge followed by growth in neutral buffered peptone water (BPW). Acid challenges were performed in BPW with pH 2·5, which was achieved by addition of hydrochloric acid. Error bars represent SE. All strains with the exception of ST 30 CII demonstrated significant statistical difference with their corresponding wild-type ($P < 0{\cdot}05$).

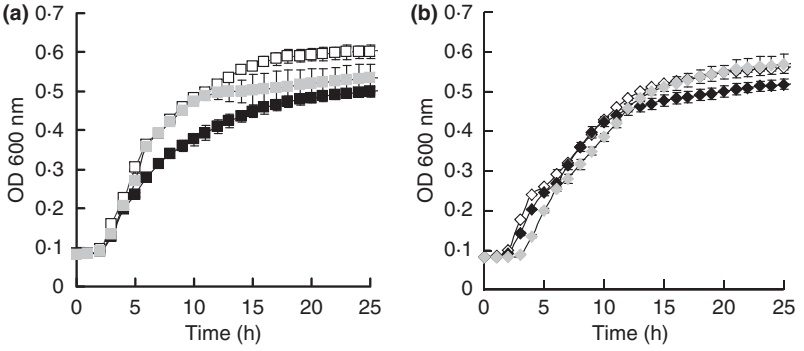

Figure 3 Growth of: SE 66045 WT (□), CI (■) and CII (▩) (a); ST 30 WT (◇), CI (◆) and CII (◈) (b) at 37°C and pH 7 monitored by OD_{600}. Error bars represent SE.

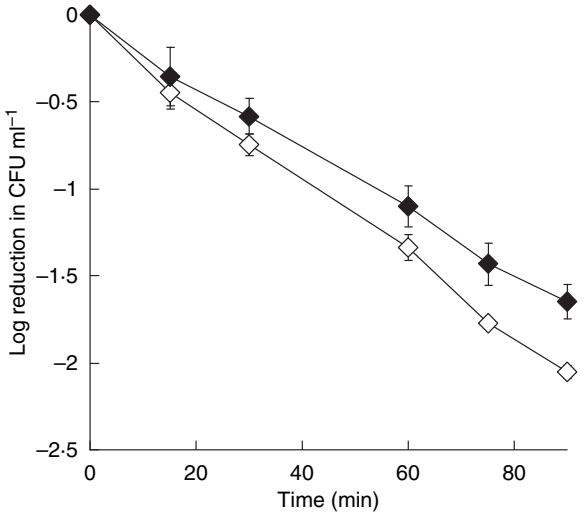

Figure 4 Heat resistance of: ST 30 WT (◇) and CI (◆) (a). Samples from overnight cultures in buffered peptone water (BPW) were inoculated into BPW (pH 7·0) at 52°C to a final concentration of *c.* 10^6 cells ml^{-1}. Error bars represent the SE. All strains presented demonstrated significant statistical difference with their corresponding wild-type ($P < 0{\cdot}05$).

colonies only at lower temperature (Fig. 5b) and smooth colonies at 37°C (Fig. 5a). Results with ST 66045 CI were similar.

SEF17 expression

Expression of thin aggregative fimbriae in both SE 66045 CI and ST 30 CI showed a statistically significant increase ($P < 0{\cdot}05$) by at least a 1·5-fold following acid cycling, whether cells were incubated at either 20°C or 37°C (Fig. 6). No such changes were seen in SE 66045 CII and ST 30 CII.

Virulence of *Salmonella enterica* strains after repeated exposure to acid

Changes in virulence in a mouse model were seen with three of the isolates that demonstrated acid-resistant phenotype. SE 66045 CI demonstrated statistically significant lower colonization of spleens and livers compared with the wild-type, as calculated with Fisher's exact probability

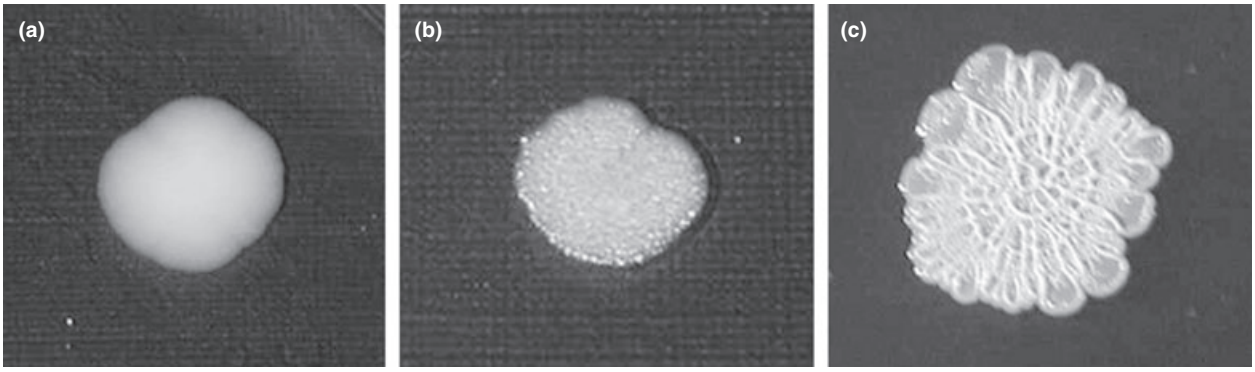

Figure 5 Examples of colony morphology exhibited by *Salmonella* Enteritidis and *Salmonella* Typhimurium strains following repeated cycles of acid challenge and growth. Colonies from wild-type ST 30 were smooth at 37°C (a) and convoluted at 25°C (b) while those of ST 30 CI were convoluted at 37°C (b) and very convoluted at 25°C (c). Colonies were grown on modified brilliant green agar.

test (Fig. 7). ST 30 CI also showed statistically significant lower colonization of spleens and faeces. ST 66045 CII did not show statistically significant differences in virulence but infected the spleens and livers of only 10% of the mice, compared with 50% for the wild-type. Non-acid-resistant isolate ST 30 CII did not show any changes in virulence as a consequence of acid-cycling (data not shown) but infection of mice with 10^5, 10^4 or 10^3 CFU ml^{-1} demonstrated a dose–response effect (data not shown).

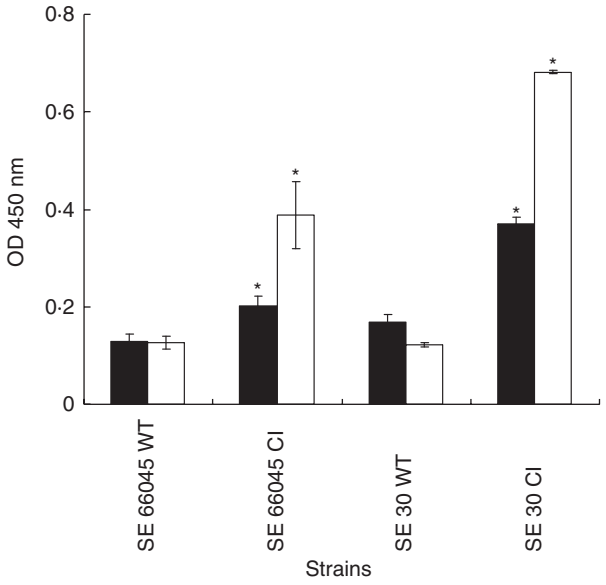

Figure 6 Expression of the thin aggregative fimbriae SEF17 in SE 66045 WT and CI and ST 30 WT and CI, measured by enzyme-linked immunosorbent assay using a monoclonal antibody. Black and white bars represent expression in cultures grown at 20°C and 37°C, respectively. Error bars indicate SE. Asterisk denotes statistically significant difference with the corresponding wild-type grown at the similar temperature ($P < 0{\cdot}05$).

Discussion

Salmonella enterica populations in both natural and food production environments are often exposed to repeat or multiple stresses and low pH is one of the most commonly encountered. Such exposures might select for strains with increased resistance to acid, disinfectants and possibly to other stresses, which could contribute to the survival of *S. enterica* following cleaning and disinfection in a farm environment (Rose *et al.* 2000). It has also been hypothesized that such mutants may display increased survival in the stomach, which could increase virulence (Archer 1996). Bark *et al.* (2005) have shown that acid-resistant mutants have a high dissemination between human isolates suggesting a positive correlation between acid resistance and pathogenicity.

In this work, the risk of emergence of more stress-resistant populations following exposure to repeated cycles of pH 2·5 followed by growth at pH 7 was investigated. Acid-cycling resulted in populations with increased acid resistance, suggesting that stresses like low pH select strains that may have a survival advantage in farm or food-processing environments. The isolation of wild-type cells in the case of ST 30 CII could be because of selection of unstable or unfit mutants, or wild-type cells protected from the lethal effects of acid owing to clumping. As experiments were performed with pure cultures of the strains we can conclude that the variants that survived the process and comprised the final populations appeared during the acid-cycling process. Increased acid resistance was not because of stress responses that can not be retained for several generations, but possibly owing to genetic alterations. This demonstrates the ability of

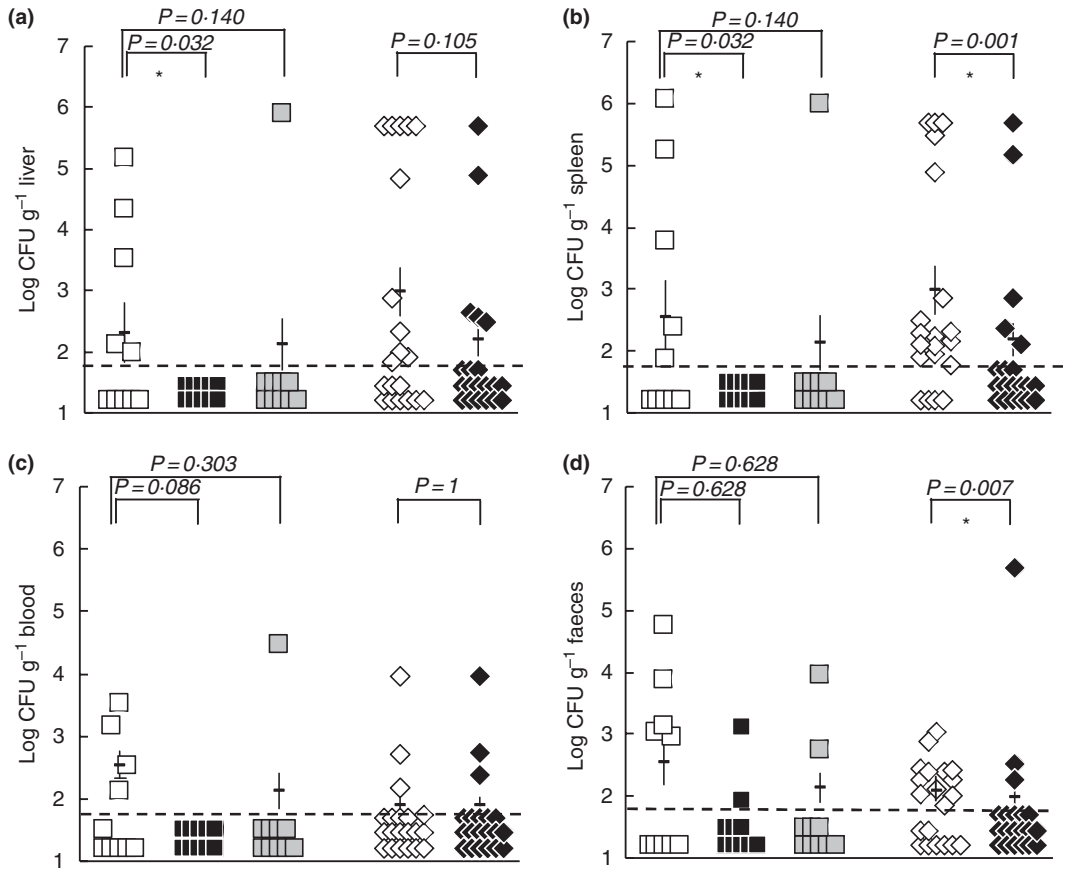

Figure 7 Bacterial numbers present in the liver (a), spleen (b), blood (c) and faeces (d) of C57/BL6/J mice, infected with 10^6 CFU ml^{-1} ($\pm 10^{0.5}$ CFU ml^{-1}) SE 66045 WT (□), SE 66045 CI (■) and SE 66045 CII (▩), or 10^4 CFU ml^{-1} ($\pm 10^{0.5}$ CFU ml^{-1}) ST 30 WT (◇) and ST 30 CI (◆). Data points represent bacterial counts from tissues isolated from different mice (n = 10 for SE 66035 and n = 20 for ST 30 strains) five days post-infection. Horizontal bars represent the mean bacterial CFU, while vertical bars represent the SEM. Horizontal dashed line represents detection limit. Brackets show comparisons with the corresponding wild-type strains while significant statistical difference compared with the wild-type strain, is denoted with an asterisk ($P < 0{\cdot}05$), as calculated with Fisher's exact probability test.

S. enterica to respond swiftly to environmental changes and adapt possibly through genetic variability as a result of the high mutation rates in this organism (LeClerc *et al.* 1996). Acid resistance of ST 30 CI persisted for at least 37·5–45 generations and those of the SE 66045 isolates for at least 12·5–15 generations in the absence of acid exposure. The reversion of SE 66045 isolates to the wild-type phenotype demonstrates that no major changes and extensive damage of the genetic material took place during acid cycling. Restoration of the wild-type phenotype could also denote that the acid-resistant phenotype was because of mutations in the hypermutable regions. These regions are known to switch from a wild-type to a mutated phase resulting in variability within a clonal population (Rocha *et al.* 2002; Karatzas *et al.* 2005).

Only ST 30 CI showed increased heat resistance, compared with its corresponding wild-type. It is known that acid stress can render cells of *S. enterica* and other bacteria, more thermotolerant (Farber and Pagotto 1992; Humphrey *et al.* 1993; Audia *et al.* 2001). One of the main cellular targets of heat and acid is proteins, which can be protected from denaturation by cellular mechanisms, like chaperones, resulting in a multiple stress-resistant phenotype (Rychlik and Barrow 2005). In general, acid cycling of *S. enterica* did not affect desiccation resistance of the isolates for the time the experiment lasted (24 h) and this is probably because this stress is different in nature from acid. The time of the experiment was restricted to 24 h because further reduction could allow the appearance of subpopulations or mutants within the population of the strains and subsequently affect the outcome of the experiment. It has a wider target range than acid, affecting even nucleic acids (Potts 1994). Desiccation resistance is also linked to DNA repair mechanisms and accumulation of compatible solutes that do not play such an important role in acid resistance.

In RpoS-positive cells, SEF17 fimbriae are normally expressed only at temperatures lower than 30°C (Walker *et al.* 1999). However, increased expression of SEF17 in acid-cycled cells was seen in SE 66045 CI and ST 30 CI at 37°C as well as at 20°C. The expression of thin aggregative fimbriae can be subject to phase variation, as mutations in the promoter region of *agfD* (*csgD*) can cause their expression in a temperature-independent manner (Romling *et al.* 1998). Although numerous factors can affect colony morphology, SEF17 expression is involved in the formation of convoluted colonies and this could explain their increased appearance in the acid-cycled populations from which the two isolates were obtained. The fact that these phenotypes occurred in two out of three acid-resistant populations, could link their characteristics with the effect that selected them (acid-cycling treatment). However, it is not known how these characteristics are linked and complex regulatory mechanisms might be involved.

It has been stated that acid-resistant strains might have enhanced survival in the stomach, increasing their numbers in the intestinal epithelium and allowing them to establish an infection (Archer 1996; Bearson *et al.* 1997; Bark *et al.* 2005). However, in this work we report for the first time that increased acid resistance might lead to lower ability of *S. enterica* to establish a systemic infection in mice. All acid-resistant isolates showed impaired virulence and although virulence impairment of SE 66045 CII was not statistically significant, it colonized only 10% of the spleens and livers, compared with 50% for the wild-type. All three isolates demonstrated impaired growth that could also be responsible for virulence impairment. This phenotype including all these characteristics has been previously described in piezo- and stress-tolerant mutants in various bacteria (Karatzas *et al.* 2003, 2007). The underlying mechanism linking increased stress resistance and impaired virulence is poorly understood. Although numerous reports in several bacterial species, have demonstrated that the presence of several stress proteins is essential for virulence, others have shown that their constitutive over-expression might be associated with attenuation of virulence (Stewart *et al.* 2001; Karatzas *et al.* 2003; Boddicker and Jones 2004). Stewart *et al.* (2001) suggested that in *Mycobacterium tuberculosis,* because many stress proteins might be recognized by the host immune system, their over-expression could lead to increased antigen load per cell, stronger immune responses and thus attenuation of virulence. However, evidence from *Shigella flexneri*, which is closely related to *S. enterica*, demonstrates that low pH leads to downregulation of virulence genes (Cheng *et al.* 2007). It seems that for virulence, the existence of functional regulatory mechanisms is important for upregulating stress proteins responsible for survival in the low pH of the stomach, but that also subsequently downregulates the ones that interfere with the normal expression of virulence-related proteins, or those targeted by the immune system of the host.

In this work we have shown that *S. enterica*, when exposed to repeated acid stress, can exhibit several morphological and phenotypic changes in short time. This demonstrates the flexibility of this organism to adapt to environmental changes, allowing occupation of a new niche inhospitable for the wild-type. Repeated cycles of acid challenge resulted in increased acid resistance and occasionally to the formation of convoluted colonies, at a temperature (37°C) previously nonpermissive and at increased heat resistance. In addition, we demonstrated for the first time that increased acid resistance in *S. enterica* might be linked with virulence attenuation. Further work in this direction will lead us to a better understanding of the mechanisms, by which *S. enterica* is able to survive acid stress and their implication in virulence.

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