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Pathogenicity in isolates of *Salmonella enterica* serotype Enteritidis PT4 which differ in RpoS expression: effects of growth phase and low temperature

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SUMMARY

Experiments with 2 wild type isolates of *Salmonella enterica* serotype Enteritidis PT4, which differed in RpoS expression, tolerance to certain hostile environments and pathogenicity, found that changes in *in vitro* acid, heat, or peroxide tolerance had no effect on the ability of the isolates to multiply in the spleens of C57/BL7/J mice infected orally. Thus, with the pathogenic RpoS-positive isolate, the infectivity of log phase chilled cells, which are profoundly acid-sensitive, was the same as that of non-chilled stationary phase cells which are acid-tolerant. Similarity the infectivity of the RpoS-negative, sensitive isolate, was not enhanced by increases in any tolerance. The ability to survive on surfaces, like infectivity, was also largely unaffected by either growth phase or cold exposure. These two attributes may thus be related and, given that the pathogenic PT4 isolate is capable of prolonged survival and the non-pathogenic isolate survives poorly, survival could serve as a potential marker of pathogenicity. Although the pathogenicity of the two isolates was very different, they showed an almost identical increase in acid tolerance following culture at pH 4·0 for up to 60 min.

INTRODUCTION

Salmonella enterica serotype Enteritidis phage types (PTs) continue to be internationally important human pathogens [1]. PT4 predominates in Europe [1] but is now also found in the USA, for example [2]. Contaminated eggs and poultry meat are frequent vehicles of infection and acidified and/or cooked dishes are also often implicated [1, 3]. Studies on one wild type human isolate of PT4, chosen at random, demonstrated that this bacterium responded to changes in either temperature or pH in an essentially similar manner to that reported for other salmonellae [4–7]. Further work on over 50 wild type isolates has shown, however, that PT4 can be divided into at least 2 populations on the basis of tolerance [8] and pathogenicity [9]. The majority of isolates so far studied (*c.* 80%) survive well in either aerosols or on surfaces in both log or stationary phase, will become much more tolerant to heat, acid and H_2O_2 on culture to stationary phase, are highly pathogenic in C57/BL6/J mice infected orally and have the ability to infect the reproductive tissues of chickens with high frequency [8, 9]. A minority of isolates (*c.* 20%) survive poorly, show a muted tolerance response on culture to stationary phase and are essentially non-pathogenic in chickens and mice [8, 9]. Subsequent work has found that the tolerant/pathogenic isolates of PT4 have normal RpoS expression whereas expression is abolished in non-pathogenic isolates [10]. The identification of 2 very different, but closely

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related, wild type populations of PT4 provides an opportunity to study the relationships between *in vitro* tolerance and pathogenicity. Much research activity has been concerned with this topic in other salmonella. It is known that salmonella virulence loci *spv* are activated by products of stress response genes and work, largely with mutants of either Dublin or Typhimurium, has shown that salmonellae able to respond to potentially hostile environments *in vitro* are better able to cause infection [see 11–13 for reviews]. For example, isolates of Typhimurium with raised pathogenicity become more acid-tolerant following extended exposure to mildly acidic media (ATR). Isolates which are less pathogenic do not show such behaviours [14–17]. Whether cells with increased tolerance are more pathogenic has yet to be fully established.

The work presented in this paper had 3 principal objectives. The first was to use spleen counts in infected C57/BL6/J mice [18, 19] to differentiate between PT4 isolates of different pathogenicity rather than mortality [9]. The second objective was to examine ATR in isolates from the two PT4 populations. It is possible that the isolate chosen for the previously reported work [4–7] and which was capable of ATR [6] came from the majority, tolerant/pathogenic RpoS-positive population and its response to low pH, for example, may not be representative of all PT4 isolates. This was examined with the expectation that if Enteritidis behaves in the same manner as certain strains of Typhimurium the sensitive/non-pathogenic RpoS-negative isolate chosen for this study and earlier work [8, 9] would show a difference in ATR to the tolerant/pathogenic isolate. The principal objective was to explore the possibility that some of the phenotypic traits seen in the tolerant/pathogenic isolates may have potential as markers of pathogenicity in PT4. For this part of the study we chose to alter tolerance by either manipulation of bacterial growth phase or exposure of cells to low temperature pre-infection. We thus explored the thesis that changes in *in vitro* tolerance may have a direct effect on the ability of salmonellae to cause infection.

MATERIALS AND METHODS

Bacterial isolates

Two isolates of Enteritidis PT4, which have been described in detail elsewhere [8–10] were used. Isolate E comes from the tolerant/pathogenic PT4 population and fully expresses RpoS [10]. Isolate I is sensitive and largely avirulent by the oral route. This isolate has a deletion of 393 base pairs in the *rpoS* gene [10].

The two bacteria were maintained on blood agar and were cultured for the experiments reported here in lemco broth (Oxoid CM67). The inocula were produced in the following manner: for stationary phase cultures, colonies from a 5% blood agar plate previously incubated overnight at $37 \pm 0{\cdot}2$ °C were emulsified in 9 ml lemco broth. The optical density of this suspension was standardized to 0·2 at 600 nm. 0·01 ml was inoculated into 9 ml^{-1} of fresh medium which was incubated at $37 \pm 0{\cdot}2$ °C for 15 h. Cultures entered stationary phase at 14 h and contained approximately $3{\cdot}0 \times 10^8$ cells of PT4 per ml. To obtain log phase cells, 0·1 ml^{-1} of the stationary phase culture was inoculated into 9 ml of fresh lemco broth which was incubated at $37 \pm 0{\cdot}2$ °C for 2 h. Cultures entered log phase after 90 min and after 2 h counts were approximately 5×10^7 cells of PT4 per ml of medium. The above cultures were used in all the experiments described below.

Mice

Experiments used the C57/BL6/J mouse strain. Experimental protocols were as described previously [9]. In essence, for each experimental group, five mice were infected at 4 weeks of age into the oesophagus with 10^5 or 10^7 cells of PT4. Infected mice were caged individually and were observed at least daily. Mice showing signs of distress were euthanased by carbon dioxide asphyxia. Instead of using mortality as an indication of salmonella pathogenicity [9] we used the ability of the PT4 isolates to multiply in the mouse spleen [18, 19]. Mice were euthanased 6 days post-infection, spleens were removed using aseptic techniques, emulsified in Maximal Recovery Diluent (MRD) and salmonellae enumerated using standard plating techniques.

Measurement of heat, acid and H_2O_2 tolerance and the ability to survive on surfaces

Death rates at 52 °C and at pH 2·6, in the presence of H_2O_2 (10 mM) or in lysed horse blood droplets (0·02 ml) on formica surfaces at 20 °C, were measured as before [8] and studies were performed using both

log (2 h) and stationary (15 h) phase cells either before or after exposure to 4 °C for 24 h.

The effect of shift to pH 4·0 on acid tolerance

In addition to the above experiments, tolerance was also measured, using previously published techniques [6], in log phase cultures which were either maintained in lemco broth at pH 7·0 ± 0·02 or shifted to fresh medium which had been adjusted to pH 4·0 ± 0·02 with HCl. One ml^{-1} of a 2 h broth culture was placed in 9 ml^{-1} of the above media and incubated for up to 60 min at 37 °C. Samples were removed at 0, 5, 15, 30 and 60 min. Death rates at pH 2·6 were measured as described above.

Measurement of catalase activity

The catalase activity of isolates E and I was measured using the following technique. 100 ml of cells were grown to early stationary phase at 30 °C with shaking in Tryptic Phosphate Broth (TPB). Ten ml was removed and added to a small (100 ml) conical flask with a side arm. The arm was connected to a calibrated U-tube which contained 15 ml of coloured water and which was open to the atmosphere at one end. Two hundred ml of hydrogen peroxide (30% w/w) was added to the flask and the top was quickly sealed with a rubber bung. The gas (oxygen) produced in the flask displaced the column of coloured water and this displacement was recorded with time (for a duration of 130 s). The rate of evolution of gas was taken as an indication of catalase activity.

Statistical analysis

With 1 exception (see below) all experiments were repeated on at least 3 occasions. The significance of differences between strains and the effects of the various culture/storage conditions were examined using statistical programs on Microsoft Excel.

RESULTS

Preliminary studies revealed that levels of salmonellae in spleens were maximized by 6 days post-infection and changed little over the next 3 days (data not shown). As some of the mice infected with isolate E were beginning to show signs of distress by 7–8 days after infection it was decided to terminate the experiments at 6 days.

With the exception of log phase cells subjected to overnight storage at 4 °C and used at a dose level of 10^7 cells per mouse, where only 2 replicate experiments were performed, all variables were examined using at least 3 separate experiments.

Mice were infected with either 10^5 or 10^7 cells of PT4 and data from the 2 dose levels are presented separately. In all experiments the numbers of isolate E were significantly higher in spleens than those of isolate I ($P < 0·0001$). Table 1 shows 2 simple analyses which compare spleens with high infection levels ($> 10^6$ salmonellae) and those from which no salmonellae were isolated by direct culture. As the data illustrate, increasing the infective dose from 10^5 to 10^7 cells increased the number of spleens which were salmonella-positive (Table 1: $P < 0·01$). With isolate E the number of heavily infected spleens also increased ($P < 0·05$). This was not the case with the other isolate and in all experiments only 2/140 spleens (1·4%) contained $> 10^6$ salmonellae. Even when the level of discrimination was increased by using 10^5 salmonellae per spleen as the cut off point the behaviour of the 2 bacteria was still markedly different. In all experiments only 4/140 (2·9%) of spleens from mice infected with I contained $> 10^5$ salmonellae. The comparative figure for spleens from mice given E was 111/139 (80%; $P < 0·0001$). Isolate I was capable of a degree of persistence in spleens but was largely unable to multiply. An increase in the infective dose also increased mean salmonella levels (Fig. 1) but the effects were not significant with either bacterium ($P > 0·05$).

Changes in growth phase or pre-exposure of the salmonellae to 4 °C for 24 h had little effect on spleen salmonella levels, irrespective of the PT4 isolate (Fig. 1). Thus differences were not significant with 1 exception. When mice were infected with non-chilled cells of isolate I at an infective dose of 10^7 cells per mouse, mean spleen counts (± S.E.) were $\log_{10} 2·1 \pm 0·1$ when stationary phase cells were used and $\log_{10} 3·4 \pm 0·2$ when mice were infected with log phase cells ($P = 0·017$).

Tolerance and survival profiles

Both isolates were highly acid- and heat-sensitive in

Table 1. *Differences between Enteritidis PT4 isolates in infection levels in the spleens of C57/BL6/J mice**

Parameter measured	Infective dose per mouse	Number of spleens salmonella-positive/ total examined (%) E	I	Significance level of differences between isolates†
Number of spleens salmonella-positive‡	10^5	55/70 (78·6%)	36/70 (51·4%)	< 0·001
	10^7	65/69 (94·2%)	54/70 (78·3%)	< 0·01
Number of spleens containing > 10^6 salmonellae	10^5	39/70 (55·7%)	0/70 (−)	< 0·0001
	10^7	51/69 (73·9%)	2/70 (2·9%)	< 0·0001

* Data from all experiments have been combined in this analysis.
† Chi-square test used.
‡ Presence of salmonellae established by direct plating. Detection limit was 10 cells per spleen.

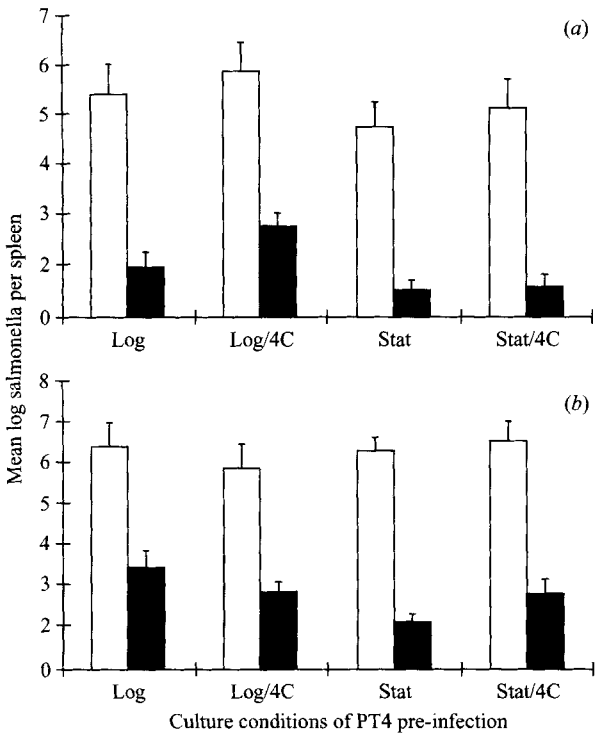

Fig. 1. Influence of growth phase and/or pre-exposure to 4 °C on the pathogenicity of Enteritidis PT4 isolates in C57/BL6/J mice. □, spleen counts from mice infected with isolate E; ■, counts from mice infected with isolate I. Standard errors are shown as vertical lines. (*a*) Spleen counts from mice infected with 10^5 cells. (*b*) Counts from mice given 10^7 cells.

log phase, particularly after chilling, which also reduces tolerance to these conditions in stationary phase cells (Figs. 2*a*, 2*b*). H_2O_2-tolerance was also lower in log phase cells, particularly with isolate I. This bacterium was also essentially H_2O_2-sensitive in stationary phase, in contrast to isolate E (Fig. 2*c*). Chilling had no effect on peroxide tolerance (Fig. 2*c*) and neither growth phase nor pre-exposure to 4 °C had any effect on the ability to survive in small blood droplets at 20 °C and in all experiments the survival of isolate E was markedly better than that of the other isolate (Fig. 2*d*; $P < 0{\cdot}001$).

The acid tolerance response

Log phase cultures of both isolates showed a marked increase in acid tolerance following culture in a mildly acid environment. Acid tolerance, for this experimental protocol, was maximized after about 15 min at pH 4·0 and remained largely unchanged for a further 45 min at this pH value (Fig. 3). There was no significant difference between the isolates.

Catalase activity

Catalase activities of stationary phase cultures of isolates E and I were investigated. In a quantitative assay the rates of oxygen evolution after the addition of substrate (30% w/w H_2O_2) were measured for each isolate. Over the period of the assay isolate E consistently showed 2–3 fold higher rates of oxygen generation than isolate I. For example, after 100 s O_2 was being released from H_2O_2 mixed with a culture of isolate E at a rate of $0{\cdot}051 \pm 0{\cdot}002$ ml per s. With isolate I the rate was $0{\cdot}016 \pm 0{\cdot}001$ ($P < 0{\cdot}001$).

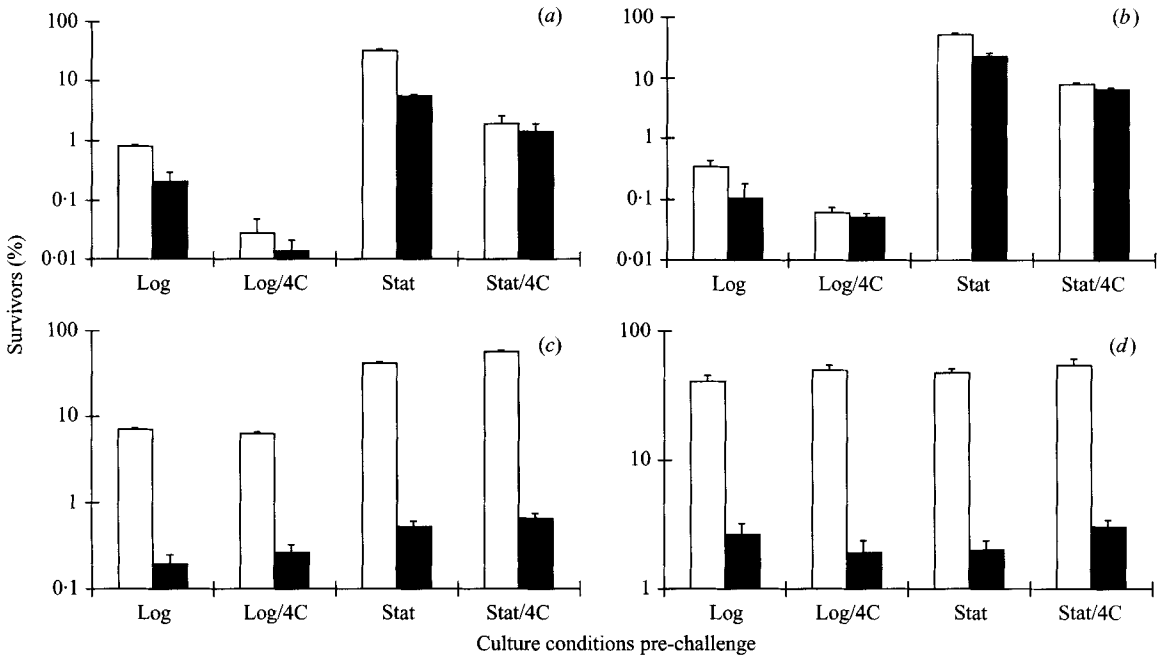

Fig. 2. Effects of growth phase and pre-exposure to 4 °C on tolerance in Enteritidis PT4 isolates. (*a*) Inoculum (%) surviving after exposure to pH 2·6 for 3 min; (*b*) survivors (%) after exposure to 52 °C for 10 min; (*c*) survivors (%) after exposure to 10 mM H_2O_2 for 15 min; (*d*) survivors (%) after 24 h at 20 °C in 0·02 ml drops of lysed blood exposed to air. □, values for isolate E; ■, values for isolate I. Data are mean values from at least three separate experiments. Standard errors are shown as the vertical lines.

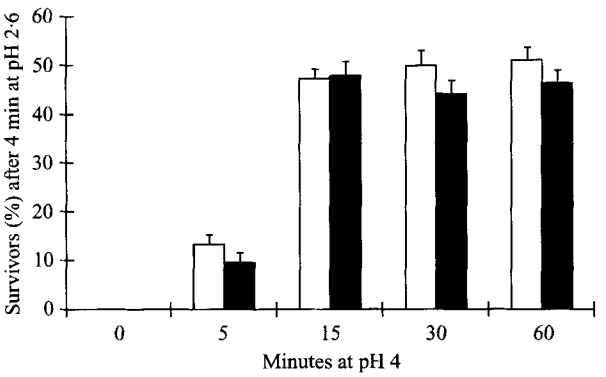

Fig. 3. Effects of culture at pH 4·0 for up to 60 min on acid tolerance in isolates of Enteritidis PT4. Log phase cells of isolates E or I were cultured at pH 4·0/37 °C for up to 60 min. At 0, 5, 15, 30 and 60 min samples of broth were transferred to pH 2·6/37 °C and death rates measured. □, values for isolate E; ■, values for isolate I. Data are mean values from three separate experiments. Standard errors are shown as the vertical lines.

DISCUSSION

This study has extended previous work on the tolerance and pathogenicity of PT4 and the effects of growth phase [8, 9]. We decided to maximize the differences between log and stationary phase cells by using the former when they had just left lag phase as cells of this type have been shown to be particularly acid-sensitive, for example [20]. In addition to the expected differences in tolerance between the two isolates (Fig. 2), the data demonstrated that chilling markedly reduced heat and acid tolerance, particularly the latter (Fig. 2*a*). Prior exposure to low temperature had no effect, however, on the ability to cause infection (Fig. 1). Thus chilled log phase cells of isolate E, which are profoundly acid-sensitive (0·02% survival after 3 min at pH 2·6) were as pathogenic (Fig. 1) as stationary phase cells maintained at 37 °C (31% survival). Similarly, an increase in acid tolerance in isolate I as a consequence of culture to stationary phase (Fig. 2*a*) had no effect on spleen counts (Fig. 1). These data are in general agreement with other recent work [16] which showed that changes in *in vitro* acid tolerance brought about by mutations affecting ATR had no effect on the survival of Typhimurium in the mouse stomach.

The fact that log phase cells were at least as pathogenic as stationary phase cells is also in agreement with other studies using either tissue cultures [21–23] or mice infected intravenously [24]. These observations could suggest that none of the tolerances studied in this work has any direct relationship to the infection process. Non-pathogenic

isolates of Typhimurium have been shown to be more peroxide-sensitive [16, 25]. This probably indicates, as in this present study, that an alteration in a particular gene affects more than 1 cellular attribute. Data on chilling and H_2O_2 tolerance are in agreement with earlier work on Typhimurium and *Escherichia coli* which found that cells grown in complex media did not become more H_2O_2-sensitive unlike those grown in minimal media [26].

The principal objective of this study was to determine whether any phenotypic traits had value as markers of pathogenicity in Enteritidis PT4. Work on ATR and pathogenicity in Typhimurium LT2 [16, 25] indicated that non-pathogenic isolates, which differed in RpoS expression, did not show the same increased tolerance as isolates which were pathogenic. This was not the case in this present study and isolate I appeared able to develop sustained ATR when log phase cells were cultured at pH 4 (Fig. 3). However, the previous work on Typhimurium used other conditions for survival measurement, tolerance induction and defined media [16, 25]. Conflicting results have also been reported regarding the ability of *E. coli* RpoS-negative mutants to develop heat shock induced thermotolerance [27, 28]. The conditions of adaptation and challenge will clearly influence the end results.

It may be that markers of pathogenicity other than ATR are needed in PT4. Several candidates were examined in this present study. The ability to survive on surfaces (Fig. 2*d*), which is simple to measure, may be a candidate. This attribute, like pathogenicity, is largely unaffected by any of the experimental conditions chosen in this study. It is our intention to try and elucidate the factors responsible for greater survival. Many wild type PT4 isolates have now been studied in the Exeter laboratory and it seems that tolerant isolates, such as E, are common and all possess the ability to persist for a long time on surfaces. It is of interest that salmonella-free replacement poultry flocks have become infected with PT4 when put in cleaned houses that previously held infected birds [29]. A recent study of farms where chicken flocks had been infected naturally with PT4 found that the bacterium could be recovered from the farm environment many months after the birds had been removed [30].

Given the importance of chilled foods such as milk [1] or cheese [31] in outbreaks of human salmonellosis, it is perhaps not surprising that relatively short exposures to low temperature have no effect on virulence of PT4 (Fig. 2). Salmonellae are capable of prolonged survival at low temperature [32, 33] and there is a trend towards the longer term storage of chilled foods. Cells of *Vibrio vulnificus* held at 5 °C until they were non-culturable (> 32 days) became avirulent in albino mice [34]. Growth of *Listeria monocytogenes* at 4 °C, however, was found to increase its virulence [35]. More recent work has found that storage at 4 °C for 4 weeks had no effect on the pathogenicity of some strains of *L. monocytogenes* although others were affected [36].

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