BACTERIAL PATHOGENICITY

The effect of environmental pH on the physiology and surface structures of *Salmonella* serotype Enteritidis phage type 4

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The incidence of food-poisoning caused by *Salmonella* serotype Enteritidis PT4 has increased. Implicated food products display pH levels between 4 and 9. Accordingly, the effect of growth at extremes of pH on the presence of surface structures and the carriage of a 38-MDa plasmid was determined by growing a clinical isolate of Enteritidis PT4 in a chemostat. Steady-state growth was possible over the pH range 4.35–9.45, corresponding to the pH extremes associated with key reservoirs implicated in outbreaks. Without pH control, cultures stabilised at pH 7.10. Growth at extremes of pH had significant effects on the distribution of cell surface structures; at pH 9.45, only 3% of cells were fimbriate compared with 52% at pH 7.10 and 20% at pH 4.35. The proportion of motile cells and the presence of flagella was also reduced at extremes of pH. A 38-MDa plasmid was present in cells grown in the chemostat at pH 7.10, but not in cells grown at pH 4.35 or pH 9.45. Thus, environmental pH may have a significant impact on the virulence potential of Enteritidis PT4.

Introduction

Salmonellae are a major cause of foodborne disease in most developed countries. In recent years a significant increase in the number of reported cases of salmonellosis in man has been observed [1]. This has been associated mainly with an increase in infections caused by *Salmonella enterica* serotype Enteritidis (*S. enteritidis*), especially of phage type (PT) 4, in the UK, Europe and some other areas of the world [2].

Epidemiological investigations of foodborne salmonellosis have identified an important role for egg and poultry products as vehicles of sporadic outbreaks of disease [3]. Enteritidis PT4 causes invasive disease in poultry that leads to chronic infection of various organs. If the reproductive tissues become infected, transmission to eggs may occur [4, 5]. Consequently, Enteritidis PT4 may be found in the contents of eggs and within poultry muscle, as well as contaminating the surface of these products.

Implicated food products display a wide range of environmental conditions in which Enteritidis PT4 must be able to survive and grow. For example, infected chicken meat has a pH around neutrality,

whilst the pH of egg albumin is >9 [6]; furthermore, during the production of mayonnaise, the addition of acetic acid or citric acid to raw eggs reduces the pH of the product to 3.2–4.5 [7]. Since environmental conditions dictate the phenotypic properties of bacteria, the source from which Enteritidis is derived may influence the virulence potential of cells, and modify their ability to survive subsequent stresses. Continuous-culture techniques were used to provide reproducible, steady-state bacterial growth under defined, pre-determined environmental conditions. Steady states can be maintained for long periods making it possible to study the effect of growth at selected pH values on the physiology of Enteritidis PT4, with other environmental conditions and growth rate remaining constant.

The aim of this study, therefore, was to determine the range of pH over which Enteritidis PT4 is able to grow and to determine the effect of growth at extremes of pH on selected properties of cells such as the presence of surface structures and the carriage of a plasmid, that might be related to pathogenicity.

Materials and methods

Bacterial strain and growth conditions

The strain of Enteritidis PT4 used (reference no. 226 405) was isolated at the Public Health Laboratory, Salisbury, from the faeces of a patient with food

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poisoning. To determine the pH range for growth and the effect of environmental pH on bacterial cell properties, it was grown in a chemostat (LH Engineering, Inceltech, Reading, Berkshire) with a 500-ml working volume, in Vogel Bonner minimal medium [8] supplemented with vitamins [9] and glycoprotein (hog gastric mucin, Sigma) 0.25% w/v in an atmosphere of CO₂ 5% and N₂ 95% at 37°C. The medium flow-rate was set to give a dilution rate of 0.1/h, equivalent to a mean generation time of 6.9 h. The pH range for growth was determined by increasing or decreasing the pH value until steady-state cultures could no longer be established. The pH in the culture vessel was maintained at the required values by automatic addition of 2 M NaOH or 2 M HCl.

Growth parameters

At each pH value studied, cultures were allowed to achieve steady-state conditions before use. Ten pot-volume changes of medium were allowed to occur (5 days at this dilution rate) before a culture was considered to have reached steady state. Purity of culture was confirmed daily by spreading a sample on nutrient agar plates which were incubated at 37°C in air; any variant colonies were confirmed as Enteritidis on selective media: XLD (Oxoid), Hektoen Enteric (HE, Lab M) and DCA Hynes (Lab M). Cell yields were determined daily by estimation of viable counts on nutrient agar after overnight incubation at 37°C. The maximum specific growth rate ($\mu_{\max}$) was determined by the wash-out method of Tempest [10]. Several estimations of $\mu_{\max}$ were made at different dilution rates and the maximum observed value was selected as being the best estimate of $\mu_{\max}$ under the test environmental conditions.

Motility estimations

Motility was assessed by light microscopy in a Thoma counting chamber (Hawksley, London). Fresh samples from each steady-state culture were examined for motile cells; formaldehyde 1% w/v was added before a total count was made. Motility was expressed as a proportion of the total cell count. Values from the chemostat cultures were compared with those of cells grown in Vogel Bonner medium (pH 7) at 37°C for 18 h in batch cultures.

Surface structures

Cell morphology and surface structures were viewed by transmission electron microscopy. Culture samples were fixed by the addition of formaldehyde 0.5% w/v, applied to formvar-carbon filmed, 400-mesh EM specimen grids with a disposable plastic loop which avoided cell damage from shearing forces. Grids were negatively stained with phosphotungstic acid 1% w/v.

Growth environment transitions

The proportion of motile cells and the distribution of surface structures were also calculated after switching of cultures from one pH value to another. Initially, the chemostat culture was allowed to reach steady state at pH 9.45; pH control was then discontinued and, after 24 h, the pH stabilised at pH 7.10 and a new steady state was established. Cells were taken directly from the chemostat, before, during and after the transition, and were assessed for motility and surface structures, as described above.

Plasmid content

A 38-MDa plasmid, carried by some strains of Enteritidis, has been associated with virulence in mice [11]. After growth for 10 days at each steady state, plasmid profiles of cultures taken directly from the chemostat were determined and the presence of the 38-MDa plasmid was assessed. A modification of the method described by Portnoy [12] was used, with extra care taken in the preparation of the alkaline denaturing solution [13], and the plasmid was precipitated from the neutralised, cleared lysate with polyethylene glycol [14]. Plasmid DNA was analysed by agarose-gel electrophoresis on agarose 0.7% w/v gels containing ethidium bromide 0.05 µg/ml and performed in Tris-acetate buffer [15]. Gels were examined by UV transillumination. Plasmids in *Escherichia coli* strain 39R861 were also included as plasmid-size reference molecules [16]. Enteritidis PT4 strain p132344 and an isogenic variant strain p132344/1, which is plasmid free, were also included as controls [11].

Data analysis

Data on cell yields between chemostat runs at the same pH value and between different pH values were analysed by two-way analysis of variance, with a Statgraphics package (STC Inc., Rockville, MD, USA). Pairwise comparisons between cell yields at the three pH values were made by the Scheffé test. Differences were assumed to be significant at $p < 0.05$.

The proportions of cells with surface structures at the three different pH values were compared by use of the large-samples proportions test (assuming a normal distribution) [17]; again, differences were assumed to be significant at $p < 0.05$.

Results

Growth characteristics of Enteritidis PT4 strain 226 405

Growth occurred in the chemostat over the pH range 4.35–9.45 under the conditions described; steady-state growth was not possible outwith this pH range. Without pH control, the culture stabilised at pH 7.10 which, with the two extremes of pH, were selected for

more detailed studies. The reproducibility of repeat chemostat runs was good with no significant difference being detected between repeat runs by two-way analysis of variance ($F_{[10,82]} = 1.59$, $p = 0.125$). Cell yields at the different pH levels were compared by two-way analysis of variance and were highly significantly different ($F_{[2,10]} = 404.1$, $p < 0.0001$). Following analysis of variance, pair-wise comparisons of each combination of pH values were made by the Scheffé test. Each pair-wise comparison of cell yields was significantly different, with most of the variation due to greatly reduced cell yields being obtained at the extremes of pH compared to pH 7.10 (Table 1).

The observed maximum growth rate was reduced at pH 4.35; such a reduction was not observed at pH 9.45, at which the doubling time was 35 min, the same as that measured at pH 7.10 (Table 1).

Cell motility

A reduction in the proportion of motile cells was observed during continuous culture; 90% of batch-culture cells were motile, compared with only *c.* 10% in continuous culture at pH 7.10. The proportion of motile cells was reduced still further ($\sim 1\%$) during growth at the extreme pH values, and this reduction in motility correlated with the number of cells expressing flagella, as determined by electron microscopy (Table 2).

Surface structures

Growth at the extremes of pH in the chemostat had marked effects on the distribution of cell surface structures (Table 3). The number of cells with fimbriae was considerably reduced at pH 9.45. A typical cell from a culture at pH 9.45 is shown (Fig. 1); at this pH value, 95% of the bacterial cells had no surface structures, whereas only 42% of cells were devoid of structures when grown at a constant pH of 7.10. A cell from a culture grown at pH 7.10, and expressing fimbriae but not flagella, is illustrated in Fig. 2. A

Table 1. Characteristics of Enteritidis strain 226 405 grown in continuous culture at different pH values

Growth pH	Cell numbers ($\log_{10}$ cfu/ml)	Maximum growth rate/h ($\mu_{\max}$)	Maximum doubling time (min)
4.35	7.24 (0.19)* [27]†	0.99	42
7.10	8.63 (0.15) [45]†	1.20	35
9.45	6.96 (0.45) [22]†	1.20	35

*Standard deviation values shown in parentheses.

†Number of determinations shown in square brackets.

Table 2. Motility and presence of flagella on cells of Enteritidis PT4 grown under different culture conditions

Growth condition	pH	Percentage of bacterial cells that were	
		motile	flagellate*
Batch	7.10	>90	81 (9)* [4]†
Continuous	4.35	1	2 (1) [3]
Continuous	7.10	10	8 (4) [2]
Continuous	9.45	<1	2 (2) [2]

*Standard deviation values shown in parentheses.

†Number of determinations shown in square brackets.

reduction in surface structures was also observed at pH 4.35, but to a lesser extent; 69% of the cells in this population were 'bald'. The proportion of cells expressing fimbriae was shown to be significantly different at the three pH values when the data were analysed by the large-sample proportions test, with $p < 0.001$ for all comparisons; most of the difference occurred between the extremes of pH (4.35 and 9.45) and pH 7.10; comparison of pH 7.10 with pH 4.35 and with pH 9.45 gave $Z = 9.17$, $p < 0.00001$ and $Z = 23.4$, $p < 0.00001$, respectively. The proportion of cells expressing fimbriae was also shown to be significantly different between pH 4.35 and 9.45 but at a lower level: $Z = 4.30$, $p < 0.00002$. A greater variation in the estimation of surface structures was observed with the low-pH culture, perhaps because mucin is less soluble at low pH values and coats the

Table 3. Surface structures detected on cells of Enteritidis PT4 grown in chemostat cultures at different pH values

Steady state growth pH	Percentage bacterial cells with			
	fimbriae only	flagella only	both fimbriae and flagella	neither
4.35	20 (15)* [2]†	11 (12)* [2]†	0 [2]†	69 (41)* [2]†
7.10	52 (16) [4]	6 (4) [4]	1 (1) [4]	42 (14) [4]
9.45	3 (2) [4]	2 (2) [4]	0 [4]	95 (1) [4]
9.45 → 7.10‡	90 (5) [3]	2 (1) [3]	0.3 (0.2) [3]	8 (4) [3]

*Standard deviation values are shown in parentheses.

†Number of determinations are shown in square brackets.

‡Culture, grown initially at pH 9.45, then switched to pH 7.10.

Fig. 1. Transmission electron micrograph of *Salmonella* serotype Enteritidis PT4 grown in chemostat culture at pH 9.45 showing a bacterial cell with no surface structures. Bar = 1 μ m.

Fig. 2. Transmission electron micrograph of *Salmonella* serotype Enteritidis PT4 grown in chemostat culture at pH 7.1 showing cells with many fimbriae. Bar = 1 μ m.

cell surface, so leading to less efficient staining and viewing. The proportion of flagellate cells was significantly higher at pH 7.10 than at pH 9.45 ($Z = 4.96$, $p < 0.00001$); however, there was no significant difference when compared to pH 4.35: $Z = 0.55$, $p < 0.60$. The proportion of cells with flagella was marginally higher at pH 4.35 than at pH 9.45: $Z = 2.18$, $p < 0.03$.

pH transition studies

The surface structures of cells were monitored during a transition from pH 9.45 to pH 7.10. No cells expressing flagella were visible when viewed by electron microscopy at pH 9.45 before the transition. The proportion of motile bacterial cells estimated by light microscopy was $<1\%$. Within 1.5 h of discontinuing pH control, it had fallen to pH 8.79 and the viable count was 6.0×10^6 cfu/ml. After a further 1.5 h, the pH was 8.4, the viable count had increased to 1.5×10^7 cfu/ml and 18% of the cells were flagellate. When a steady state was achieved at pH 7.10, the majority of cells were fimbriate (Table 3).

Plasmid content

A 38-MDa plasmid was present in cells grown continuously for 10 days in steady state and in overnight batch cultures at pH 7.1. However, this plasmid was not detected in cultures grown continuously at pH 4.35 or at pH 9.45 (Fig. 3).

Discussion

A clinical isolate of Enteritidis PT4 was able to sustain growth over the pH range 4.35–9.45, a range of relevance since it corresponds with the extremes of pH believed to be encountered in its environment in food products and during infection. For example, the pH of ovalbumin reaches pH 9.5 during egg storage [6] whilst mayonnaise, which has repeatedly acted as the vehicle of Enteritidis outbreaks, may have a pH of *c.* 4 [18, 19].

Generally, most bacteria of importance in foodborne diseases are unable to grow below pH 4.5, which means that acidic foods do not normally present major health risks as far as the common bacterial agents causing food-transmitted disease are concerned [20]. The minimum pH for growth of *E. coli* and *Salmonella* serotype Paratyphi was estimated to be 4.4 and 4.5, respectively, similar to those determined in this study. However, the maximum pH allowing growth of these two species was 9.0 and 7.8, respectively; the pH range allowing growth of these two species is narrower than those determined for Enteritidis in this study. Comparable data on the pH range for the growth of other foodborne pathogens showed *Bacillus cereus* and *Staphylococcus aureus* to

Fig. 3. Detection of plasmid DNA by agarose electrophoresis. Lanes: 1, mol.-wt ladder; 2, *Pseudomonas aeruginosa* strain RP4 containing a 36-MDa plasmid; 3, Enteritidis strain p132344; 4, Enteritidis strain 226405, sample stored at -20°C , from chemostat run at pH 9.45; 5, Enteritidis strain p132344/1; 6, Enteritidis strain 226405 from L broth batch culture; 7, *E. coli* strain 39R861; 8, 9, Enteritidis strain 226405, sample stored at -20°C , from chemostat run at pH 7.10; 10, Enteritidis strain 226405, sample direct from chemostat run at pH 9.45; 11, as lane 10 but run at pH 4.35; 12, as lane 10; 13, as lane 11; 14, as lane 10 but run at pH 7.10.

have ranges similar to those detected in this study, i.e., 4.9–9.3, and 4.0–9.8, respectively. Wider ranges of pH tolerance were observed for alkali-tolerant bacteria such as *Vibrio parahaemolyticus* for which the limiting pH values for growth were between 4.8 and 11.0 [21].

The strain used in the present study was isolated from the faeces of an infected patient. The ability of Enteritidis strains to grow over such a wide pH range may not be a universal property as cultures of other strains became non-viable at pH values <5.0 [22]. Previous exposure to environmental stress may markedly influence the subsequent ability of bacteria to grow and survive. For example, brief incubation of Enteritidis in media at pH values between 3 and 6 resulted in a marked and rapid increase in the acid resistance of some cells [23]. The variation in the reported tolerance of different strains of Enteritidis PT4 to pH challenge may indicate either a variation within the phage type itself or may be a reflection of the different techniques [24, 25].

Growth at extremes of pH had significant effects on surface structures and plasmid carriage of cells. A reduction in the proportion of motile cells during continuous culture in comparison to those from batch

cultures was observed, due perhaps to shearing forces, to the absence of nutrient gradients with continuous mixing or to the bacterial energetics, as organisms with the lowest energy requirements are selected in continuous cultures [26]. Synthesis of bacterial flagella and the accompanying array of chemotaxis receptors represents a major commitment of energy and resources for growing cells. The proportion of motile cells in cultures at extremes of pH was very low (~1%), and this value closely correlated with the number of cells possessing flagella. This observation suggests that the loss of motility was due to a shut down in the production of flagella; similar findings have been made with *Campylobacter jejuni*, in which the expression of sigma 54 *flaB* flagellin promoter was subject to environmental regulation. FlaB filament-protein production was affected by growth pH, the composition of the growth medium and the temperature of growth [27].

Environmental pH had significant effects on the distribution of fimbriae on cells in the present study. Only 3% of cells grown at pH 9.45 were fimbriate compared with 20% of cells grown at pH 4.35 and 52% at pH 7.10; these findings are in agreement with observations made on the production of fimbrial adhesins by enterotoxigenic *E. coli* during growth at various specific growth rates and at different pH values [28]. The production of fimbriae decreased at pH values above and below pH 7 [28] and was also dependent on the growth rate of cells, with a significant production of fimbriae detected only at specific growth rate values higher than 0.2/h; no significant differences were observed, however, between aerobic and anaerobic growth.

Salmonellae express many different types of fimbriae and Enteritidis has been shown to produce fimbrial structures differentiated as SEF 14, SEF 17 and SEF 21 [29]. In the present study, the effect of pH on the production of individual fimbrial types, was not determined and it is possible that particular fimbriae respond differently to specific environmental stimuli. The expression of SEF 14 has been shown to be influenced by the type of growth medium and, for one test strain, SEF 14 fimbriae were produced in peptone water at pH 7.2 but not at pH 6.0 [30].

A 38-MDa plasmid of Enteritidis has been associated with virulence in mice [11]. Despite the data from mouse studies, the relevance of this virulence plasmid in human infection remains unclear. For the strain tested in this study, the plasmid was detected in cells grown continuously at pH 7.10 but not at pH 4.35 or pH 9.45. The use of plasmid profiling for epidemiological investigation necessitates stable plasmids. Most studies of plasmid stability have been concerned with in-vivo stability of plasmid profiles for the duration of outbreaks. This 38-MDa plasmid is reported to be very stable [31], but it may be that extreme

environmental conditions of pH and nutrient availability induce changes in plasmid content and expression [32] and reduce the level of plasmid-carrying cells in the population to below the detection level of the assay. Even without direct selection, plasmid-containing bacteria may survive as a small minority of the population.

In order to determine whether the absence of motility and flagella during growth at pH 9.45 was a phenotypic rather than a genotypic response, the presence of flagella on cells and motility was monitored when the pH fell from 9.45 to 7.10. Flagella reappeared on cells almost immediately, suggesting that the response was, indeed, phenotypic and that expression of the relevant genes may be rapidly switched on or off in response to environmental stimuli.

In conclusion, Enteritidis was able to grow over a wide range of pH values, including those found in foods associated with outbreaks of disease. However, pH was also found to regulate markedly the expression of surface structures and the carriage of a 38-MDa plasmid implicated in cell virulence. Consequently, the pathogenic potential of cells grown at different pH values should now be compared in a mouse model. Such studies should allow further insights to be made into the factors contributing to the virulence of Enteritidis PT4.

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