Low tyrosine content of growth media yields aflagellate *Salmonella enterica* serovar Typhimurium

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Identification of *Salmonella* serotypes is based on flagellar and somatic antigens. The absence of flagella may consequently affect complete identification of the serotype; here it is shown that *Salmonella enterica* serovar Typhimurium exhibits morphological differences dependent on the peptone constituents of the culture medium. Aflagellate salmonella were produced in certain media where the nutritional ingredient was casein-based peptone or gelatin-based peptone; in gelatin-based peptone, aggregates of salmonella were observed. However, in media containing soy-based peptone as the primary nutrient, salmonella displayed a normal flagellated morphology. Transfer of aflagellate salmonella from nutritionally poor media, with casein- or gelatin-based peptone, into rich nutrient broth allowed flagella synthesis, indicating that the aflagellate form is still able to produce flagella. Amino acid sequencing of the peptones producing aflagellate organisms showed a relatively low tyrosine concentration: only 0.03 ± 0.01 g l⁻¹ for gelatin-based buffered peptone water, compared to 0.21 ± 0.01 for soy-based buffered peptone water. Tyrosine is essential for flagellin, which is the subunit of the salmonella flagellar filament. The addition of 200 µM tyrosine to casein-based peptone media produced flagellate salmonella; 2 mM glucose was needed in addition to tyrosine to achieve a similar morphology in gelatin-based media. Therefore, culture media containing less than 1.20 g tyrosine l⁻¹, and of limited carbohydrate source, when used for serological testing of clinical isolates, may result in an incomplete serological identification.

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INTRODUCTION

Salmonella enterica serovar Typhimurium is a common species responsible for salmonellosis in the UK. Transmission may occur by ingestion of contaminated food, mainly meats, or by the faecal–oral route from an infected individual. Salmonellosis is characterized by watery and sometimes bloody diarrhoea, abdominal pain, headache, nausea, vomiting and fever. Septicaemia or focal infections are possible complications.

Pre-enrichment of food samples for low numbers of sublethally injured salmonella before growth selection has been recognized as a crucial step in positive identification (Andrews, 1986). The most common pre-enrichment medium for salmonella enumeration and recovery is buffered peptone water (BPW) comprising salts, phosphate buffers and the complex, undefined ingredient peptone. Performances of commercial preparations of BPW were

compared (Baylis *et al.*, 2000), and differences found in their ability to sustain the recovery of injured organisms.

Media composition affecting the degree of flagellation has been previously observed: certain basic constituents of solid media, such as different agars, have produced organisms with a novel agar-penetrating flagellar structure (Guard-Petter, 1997). Addition of glucose to Hektoen enteric agar, a selective differential medium for salmonella, causes hyper-flagellated salmonella capable of swarming (Harshey & Matsuyama, 1994). *Proteus* species have been observed to alter the wavelength of flagella (i.e. distance from one wave-crest to the next) in response to media variations (Leifson *et al.*, 1955).

This study shows how different peptones from various origins and sources, as a constituent of BPW, affect salmonella morphology, most notably the flagellation.

METHODS

Media. BPW was made according to the directions provided by the manufacturers: 10 g peptone l⁻¹ (various peptones were used, as

Abbreviations: BPA, buffered peptone agar; BPW, buffered peptone water.

Table 1. Peptones employed in this study

Code	Medium	Digest	Manufacturer	Product no.	Batch no.
C ₁	Casein	Pancreatic	Merck	1.07213	91
C ₂	Casein	Pancreatic	Merck	1.07213	2LO75
G ₁	Gelatin	Pancreatic	Merck	1.07284	2LO74
G ₂	Gelatin	Pancreatic	Merck	1.07284	K90912484
S ₁	Soy	Papain	Merck	2.80227	K32373027
S ₂	Soy	Papain	Becton-Dickinson	243620	2108720

described in Table 1), 5 g sodium chloride l⁻¹, 3.5 g disodium hydrogen phosphate l⁻¹ and 1.5 g potassium dihydrogen phosphate l⁻¹ (BDH). Supplementary medium contained 100 µM tryptophan and 200 µM tyrosine (Sigma). Glucose (2 mM) (Fisher Scientific) was also added where indicated. 'Sloppy' buffered peptone agar (BPA) plates were made similarly to BPW but with 0.3% agar added (Difco). Nutrient agar (NA), nutrient broth (NB), tryptone soya agar (TSA) and tryptone soya broth (TSB) were all made in accordance with manufacturer's instructions (Oxoid). All media were sterilized by autoclaving.

Cultures. The strain used was *Salmonella enterica* subspecies *enterica* serovar Typhimurium (ATCC 14028); growth in the chemically defined minimal medium M9 (Neidhardt *et al.*, 1974), containing ammonium chloride as the sole nitrogen source, indicates a normal amino acid metabolism for enterobacteria. Cultures were maintained on NA (Oxoid) slopes at 4 °C and subcultured every 14 days. Freezer stocks on beads (Prolab) were kept at -20 °C. Experimental inocula were prepared by incubating a single colony picked from a NA slope and transferred to 20 ml BPW (Merck) in a 100 ml culture flask overnight in a reciprocating water bath (Gallenkamp), 100 strokes min⁻¹, at 37 °C. Experimental media were then inoculated with 20 µl of the resulting culture and incubated as above for 24 h, unless otherwise specified.

Electron microscopy. Negatively stained preparations were obtained by transferring 10 µl culture onto a 3 mm 200 mesh copper grid, and blotting dry after 1 min. Methylamine tungstate (3% w/v; Emscope Laboratories) was applied for 1 min and blotted dry. *Salmonella* cultured in different types of BPW were examined on three separate occasions under a transmission electron microscope (JEOL JEM-1210). Observations were noted, e.g. the presence/absence of flagella, and representative electron micrographs taken.

Swimming rate of salmonella. Sloppy BPA plates, as previously described, were centrally inoculated with salmonella using 10 µl sterile inoculation loops (VWR). These plates were then incubated at 37 °C for 5 h, after which the distance between central inoculation point and the periphery of outward growth was measured. The experiment was performed in triplicate and the mean speed of migration from the inoculation point (mm h⁻¹) calculated.

Amino acid composition of peptones. Peptone samples (50 µl, containing 0.5 g peptone l⁻¹) incorporating an internal standard of 15 nM norleucine were hydrolysed with 6 M HCl at 110 °C for 24 h, dried under vacuum, filtered using a 0.2 µm membrane (Millipore) and then resuspended in 150 µl sodium citrate loading buffer, 0.20 M at pH 2.20 (Biochrom). Samples (100 µl) of these were inoculated onto an amino acid analyser that utilized ion-exchange HPLC (Biochrom 20; Pharmacia Biotech). These were run in triplicate and resultant values adjusted to the internal standard. Tyrosine concentrations for NB (Oxoid) and TSB (Oxoid) were calculated from the Oxoid Manual (Bridson, 1995).

Phenol sulphuric acid method for determination of carbohydrates. Peptone samples, at a concentration of 0.5 g l⁻¹, suspended in distilled H₂O were analysed using the phenol sulphuric acid method, as described by Dubois *et al.* (1956).

RESULTS

Growth in BPW made up using different peptones gave distinct morphological variations revealed on examination by transmission electron microscopy. *Salmonella* cultured in casein-based BPW were markedly lacking in flagella: 1 flagellum or less was observed on most organisms and many were aflagellate (Fig. 1a, b). Gelatin-based BPW (Fig. 1c, 1d) gave organisms that were completely aflagellate and observed only as aggregates. Soy-based BPW (Fig. 1e, f) produced organisms with normal flagellate morphologies. Flagellation is similarly affected (data not shown) in *Salmonella enterica* subspecies *enterica* serovar Poona (NCTC 4840), *Escherichia coli* (NCTC 10418) and *Vibrio fischeri* (NRRL-B-11177).

Swimming rates of salmonella grown with different peptones varied greatly. Fig. 2(a) shows a sloppy BPA plate, where the peptone constituent was gelatin. Growth of the organisms occurred at the point of inoculation, but individuals failed to swim outwards. Fig. 2(b) shows salmonella growing and swimming outward from the inoculation point to form a pattern of concentric rings on soy-based BPA. Swim rates (mm h⁻¹) of salmonella were tested on BPA with different constituent peptones, and also on a plate containing 0.3% NA for comparison (Fig. 2c). This experiment clearly shows that salmonella were more motile upon soy- and casein-based agar compared to gelatin-based agar. Also noticeable are the differences between the swim rates on the media containing two different manufacturers' soy-based peptones, and upon the two gelatin-based peptone media.

Salmonella cultured in casein- and gelatin-based BPW for 24 h, which were aflagellate or markedly lacking in flagella, were then inoculated into NB. After 24 h incubation the resultant cultures were flagellate (Fig. 3a-d).

The amino acid composition of each peptone was established; a representative full analysis of the amino acids in the G₁ peptone sample is shown in Fig. 4(a). Peptones that did not produce normal flagellate organisms were lacking

Fig. 1. Electron micrographs of salmonella displaying various morphologies dependent upon the peptone constituent of BPW, (a) C₁, (b) C₂, (c) G₁, (d) G₂, (e) S₁ and (f) S₂.

in tyrosine (Fig. 4b) compared to the absolute value of tyrosine required for flagellation, which was determined to be 0.06 g l^{-1} in the presence of carbohydrate, and 1.3 g l^{-1} in its absence. However, C₂-cultured salmonella were exceptional: aflagellate organisms cultured in peptone containing relatively high concentrations of tyrosine. Glutamic acid and leucine are known to be essential for synthesis of flagellin, the protein subunit of the flagellar filament (Kerridge, 1959), and so the values for these two amino acids are also shown for each peptone (Fig. 4c, d).

The overall compositions of dehydrated culture media were determined with regard to protein and carbohydrate content (Fig. 5). Great variation in the percentage of carbohydrate was found; gelatin- and casein-based BPW contained little to no carbohydrate, whilst soy-based BPW contained up to $19.06 \pm 0.00 \%$ (w/w). The percentage of protein was also variable: from $29.95 \pm 0.03 \%$ (w/w) in soy-based BPW to $47.56 \pm 0.04 \%$ (w/w) in casein-based BPW. NB and TSB are also shown for comparison.

Flagellated salmonella were produced in casein-based BPW on the addition of tyrosine ($200 \mu\text{M}$) and tryptophan ($100 \mu\text{M}$) supplements. Gelatin-based BPW supplemented with tyrosine and tryptophan alone did not produce flagellated salmonella. The addition of tyrosine, tryptophan and glucose (2 mM) to gelatin-based BPW produced

Fig. 2. Swim rates of *Salmonella enterica* serovar Typhimurium in various BPA media. Radii of swim circles were measured after 5 h growth at 37°C , and photographed for (a) G₁ and (b) S₁ media, and the swim rate calculated as the rate of growth obtained upon experimental sloppy BPA, nutrient rich '0.3% agar' NA and tryptone soya agar (TSA) (c). Error bars indicate the mean $\pm$ SD ($n=3$).

Fig. 3. Aflagellate inocula from (a) C₁, (b) C₂, (c) G₁ and (d) G₂ media transferred into NB produce flagellate organisms, suggesting that the process is not irreversible.

Fig. 4. Full amino acid analysis for G₁ media (a). Amino acid composition of peptones (not BPW) showing the amino acids important for flagellin synthesis: tyrosine (b), glutamic acid (c) and leucine (d). Bars indicate the mean composition $\pm$ SD, where $n=3$. The peptones are represented as follows: C₁, hatched bars; C₂, white bars; G₁, bars with dots; G₂, cross-hatched bars; S₁, bars with horizontal lines; S₂, grey bars.

flagellated salmonella. The addition of tryptophan, glucose and high concentrations of tyrosine (20 mM) produced aflagellate salmonella.

Fig. 5. (a) Carbohydrate (%) as a constituent of BPW. (b) Protein (%) as a constituent of BPW. The percentages of carbohydrate and protein in TSB and NB are also shown for comparison; these were calculated from the Oxoid Manual (Bridson, 1995). Bars indicate the mean composition $\pm$ SD, where $n=3$.

DISCUSSION

The variation of salmonella morphology, in relation to peptone source and origin, in this work can be attributed to the amino acid composition of the peptone: low concentrations of tyrosine ($0.03 \pm 0.01 \text{ g l}^{-1}$) in gelatin-based peptones and in some casein-based peptones correspond with aflagellate morphology of the salmonella thereby cultured (Table 2). A high protein content, as in casein- and gelatin-based BPW, does not correlate with sufficient tyrosine for flagellin synthesis. Often where there is a limited source of carbohydrate in combination with relatively low tyrosine concentrations the aflagellate phenotype is exacerbated. Soy-based peptones contain over sevenfold higher tyrosine concentrations ($0.21 \pm 0.01 \text{ g l}^{-1}$) and a higher concentration of carbohydrate compared with gelatin-based peptones, and consistently produce highly flagellated organisms.

Tyrosine, leucine and glutamic acid are essential amino acids present in the medium and required for the flagellar filament subunit flagellin (Kerridge, 1959). The absence of tyrosine thus prevents flagellar synthesis, e.g. in the case of salmonella cultured in gelatin-based BPW.

Tyrosine transport into the organism is by two permeases: uptake is by way of the aromatic permease that transports all

Table 2. Synthesis of salmonella flagella is dependent upon the concentration of tyrosine (Y) and the presence of carbohydrate in the final preparation of BPW

Tryptophan (W) was given as an inducer of tyrosine permease. *Typical value calculated from Oxoid manual.

Peptone in BPW	Tyrosine (g l ⁻¹)	Carbohydrate (% w/w)	Presence of flagella		
			Unsupplemented media	Supplemented media	
				Y + W	Y + W + glucose
C ₁	0.10 ± 0.07	0.00 ± 0.00	No	Yes	Yes
C ₂	0.27 ± 0.04	0.00 ± 0.00	No	Yes	Yes
G ₁	0.03 ± 0.01	0.00 ± 0.07	No	No	Yes
G ₂	0.04 ± 0.02	0.58 ± 0.00	No	No	Yes
S ₁	0.12 ± 0.00	19.06 ± 0.00	Yes	Yes	Yes
S ₂	0.21 ± 0.01	14.07 ± 0.00	Yes	Yes	Yes
TSB	0.14*	17.56 ± 0.00	Yes	Yes	Yes
NB	0.03*	0.64 ± 0.00	Yes	Yes	Yes

aromatic amino acids, or by the tyrosine-specific permease that shows absolute specificity for tyrosine (Ames, 1964). Aromatic amino acids present at relatively higher concentrations inhibit uptake of those present at lower concentrations (Ames, 1964); tyrosine is present at significantly lower concentrations than phenylalanine and histidine in the peptones used in this study. Tyrosine uptake via its specific permease is driven by a proton motive force (PMF) (Antonucci & Oxender, 1986), which requires ATP for its generation. We hypothesize that cultures with gelatin-based BPW do not produce flagellated salmonella for two reasons: (1) higher concentrations of phenylalanine and histidine inhibit tyrosine uptake by the aromatic permease, and (2) there is a lack of an energy source suitable for the production of a PMF that would power the tyrosine-specific permease. Thus, in supplemented gelatin medium containing tyrosine and glucose, flagellated organisms were produced. C₂ casein-based medium contains higher concentrations of tyrosine, phenylalanine and histidine in comparison with other casein-based media. Higher concentrations of tyrosine do not result in flagellated organisms as inhibition by high concentrations of phenylalanine and histidine prevents uptake of tyrosine by the aromatic permease. Lack of a suitable substrate for the synthesis of ATP renders the tyrosine-specific permease inactive, thus tyrosine is not easily taken up by the organism, and flagellin is not synthesized. Supplementing media with additional tyrosine, to overcome competition for uptake by the aromatic permease, results in flagella being produced.

The lack of flagella caused by growth in certain peptone-based broth was often correlated with low nutritional value of the medium; in G₁ medium aflagellate organisms also had slow exponential growth times, 54 min, and low yields, $3.95 \pm 2.59 \times 10^7$ c.f.u. ml⁻¹ at 48 h. Those media that contain soy-based peptone and yeast extracts produce flagellate organisms with normal exponential growth times and yields: 18 min and $8.85 \pm 1.92 \times 10^9$ c.f.u. ml⁻¹ in S₂

medium, respectively (data not shown). Most commercially available desiccated culture media list peptone as an ingredient with no reference to the source or origin; this is the basis of great variation, and hence considerable concern, as a false-negative identification may result due to the lack of recovery of sublethally injured salmonella (Andrews, 1986). Successful growth in a poor medium, resulting in aflagellate salmonella, may produce an incomplete serotype as flagellin presents the H antigen used for serological analysis, which along with the O antigen, categorize the serotypes of salmonella. These are important considerations for food monitoring and public health.

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