MICROBIAL PATHOGENICITY

Factors affecting haemolysin production and Congo red binding in *Salmonella enterica* serovar Typhimurium DT 98

R. P. TIWARI, KAMALDEEP DEOL, PARVEEN RISHI and J. S. GREWAL*

Department of Microbiology, Panjab University, Chandigarh-160014 and *Malaria Group, International Centre for Genetic Engineering & Biotechnology, PO Box 10504, Aruna Asaf Ali Marg, New Delhi-110067, India

Differences in haemolysin expression were observed in a strain of *Salmonella enterica* serovar Typhimurium definitive phage type (DT) 98 cultured under various conditions. Haemolysin expression was optimal in cultures grown micro-aerobically. The zones of haemolysis were wider after longer periods of incubation. Haemolysin production varied after growth in the following media (greatest to least): brain heart infusion (BHI) broth > nutrient broth (NB) > trypticase soy broth (TSB) > M-9 glucose medium. Haemolysin production correlated directly with Congo red binding in nutrient broth. On Congo red blood agar, colonies were smaller, with dark centres and wider zones of haemolysis. Culture-cell-free haemolysin activity was higher, but cell-bound haemolysin activity was very low in growth medium supplemented with Congo red. Boiled tea extract at 25% v/v (of 25% w/v tea infusion) in PBS and nutrient broth was bactericidal to *S. Typhimurium* DT 98. The addition of boiled tea extract to growth medium inhibited haemolysin production by *S. Typhimurium* DT 98 at higher concentrations (6–12.5% v/v) but stimulated haemolysin production at lower concentrations (1.5–3% v/v). The pre-treatment of bacterial cell suspensions with lower concentrations of tea extract (1.5–3% v/v) also altered the Congo red binding, which showed an inverse correlation in nutrient broth.

Introduction

Salmonellosis is a multistage, multifactorial, host-dependent and often host-restricted infection [1]. Despite intensive studies, the number, nature and role of salmonella toxins in disease production remain unclear. The pathogen enters the gastrointestinal tract in contaminated food and water, attaches to and penetrates the small intestinal epithelium and then spreads to other internal organs [2, 3].

Exposure of *Salmonella enterica* serovar Typhimurium to moderate stress results in the synthesis of several proteins [4], including haemolysin, previously called cytolysin [5, 6]. At least six regulatory classes of nutrient, starvation-inducible loci have been characterised for the salmonellae [7]. To assign biological significance to any putative character or virulence

determinant, it is necessary to demonstrate its expression and sometimes its extracellular release in relation to host environmental cues or in appropriate ecological settings.

The present study investigated the expression of one such putative factor, haemolysin, together with the ability of *S. Typhimurium* definitive phage type (DT) 98 to bind Congo red. Cultural conditions are known to affect the induction of virulence traits [8, 9]. Enteropathogenicity has been correlated with the initial invasion of the intestinal epithelium that is necessary for infection to become established, and with increased haemolysin production [10–12]. The regulatory effect of Congo red on membrane protein is mediated through induction, and experiments with *Shigella flexneri* phage type 2a have suggested that Congo red mimics host tissue factor *in vitro* [13]. The Congo red binding technique has been used to determine the cell surface protein array involved in the virulence of *Aeromonas salmonicida* [14]. The appropriate regulation of genes enables *S. Typhimurium* to adapt to the intracellular environment of the host [15]. The initial penetration

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Corresponding author: Dr R. P. Tiwari (e-mail: ramptiwari@yahoo.com; jasvirgrewal@usa.net).

and destruction of M cells by invasive *S. Typhimurium*, followed by secondary invasion of adjacent enterocytes, has been attributed to the balanced production of haemolysin by *S. Typhimurium* [16, 17].

Tea extracts have been shown to have antibacterial properties [18–20]. Toda *et al.* [18] have reported that black or green tea inhibits the growth of a number of enteric bacteria. This prompted the investigation of the effects of tea extract on the viability and haemolysin production of *S. Typhimurium* DT 98.

Materials and methods

Bacterial strains

S. Typhimurium DT 98 was selected for this study from 18 strains of *S. Typhimurium* and 11 of *S. Typhi* because it showed the greatest haemolytic activity [21]. The strain was maintained on Le Minor medium agar slopes [22] in screw-capped tubes at 4°C, and sub-cultured bimonthly.

Screening for haemolysin activity

Each test strain was cultured under micro-aerobic conditions on overlaid blood agar at 37°C [21]. The strain was spread on blood agar plates, which were incubated at 37°C for 4 h, overlaid with an additional layer of nutrient agar, and incubated at 37°C for a further 72 h, during which they were examined daily for haemolysis.

Preparation of cell-free supernate and bacterial cell suspension

S. Typhimurium DT 98 was cultured for 16 h at 37°C in nutrient broth (30 ml/100 ml flask), which was then centrifuged at 7000 *g* for 15 min at 4°C. The clear, culture-cell-free supernate (CCFS) was separated carefully with a sterile pasteur pipette, taking care not to disturb the bacterial pellet. The supernate was filter-sterilised through a membrane filter (Millipore; 0.45 µm) and stored in a sterile vial for subsequent use. The bacterial cell pellet was washed twice with phosphate-buffered saline (PBS) before suspension in the same buffer to an optical density (OD) of 0.3 at 600 nm.

Haemolysin assay

Preparation of red blood cell suspension. A washed suspension of sheep red blood cells (3% v/v in 0.02 M PBS, pH 7.2) was prepared from sheep blood [21]; 300-µl volumes of CCFS or bacterial cell suspension (BCS) were mixed gently with 6 ml of this sheep cell suspension in sterile test tubes, which were incubated at 37°C for 3 h with occasional shaking. The tube contents were then centrifuged at 5000 *g* for 15 min. The supernates were collected in separate tubes and used for absorbance determination (A_{545} values) against

controls. In the control tubes, sterile nutrient broth and PBS were added instead of CCFS and BCS, respectively. Haemolytic activity was expressed as haemoglobin release, mg/ml of solution, calculated from the haemoglobin standard absorbance curve.

Congo red binding. The method of Qadir *et al.* [23] was used. Congo red (0.003% w/v) was incorporated into nutrient agar (NA) before autoclaving. Plates streaked with test strains were incubated at 37°C for 18 h. The next day, colonies were examined for the presence (red, crb^+) or absence (white, crb^-) of Congo red binding.

Congo red uptake assay. *S. Typhimurium* DT 98 cells were cultured in volumes of 50 ml/100 ml flask at 37°C for 16–18 h in various growth media, i.e., brain heart infusion broth (BHI), M-9 glucose medium (M-9) [24], nutrient broth (NB), peptone water (PW), or trypticase soy broth (TSB). The culture was then centrifuged at 10 000 *g* for 10 min at 4°C. The bacterial cell pellet obtained after decanting the CCFS was washed twice with PBS, before suspension in PBS to an OD of 0.3 at 600 nm. The bacterial suspension (5 ml/tube) was mixed with Congo red (0.003% w/v), kept at 37°C for 10 min, then centrifuged at 10 000 *g* for 10 min at 4°C. The supernate was collected in separate tubes to determine its absorbance at 480 nm against a PBS control. The amount of dye binding to the cells was calculated from the Congo red standard curve as the difference between the amount added to the mixture and the amount remaining in the solution.

Factors affecting haemolysin expression

Growth medium: Each 100-ml flask, containing 30 ml of growth medium (BHI, M-9, NB, PW or TSB), was inoculated with 100 µl of an overnight culture of *S. Typhimurium* DT 98 in NB. Inoculated flasks were kept at 37°C for 18 h and cultures were screened the next day for cell-bound and cell-free haemolysin activity.

Effect of Congo red. The plates were taken out after 4 h to overlay a thin layer of nutrient agar, while the *S. Typhimurium* DT 98 was cultured on blood agar containing Congo red (0.001–0.005% w/v) at 37°C. The plates were examined visually after 48 h for zones of haemolysis. In another experiment, two 50-ml flasks, containing nutrient broth 20 ml either with or without Congo red (0.003% w/v), were inoculated with 100 µl of a 16–18-h culture of *S. Typhimurium* DT 98. These flasks were incubated at 37°C for 24 h and then centrifuged at 13 000 *g* for 15 min. CCFS was separated and stored in separate tubes as described above, to determine haemolytic activity. The cell deposits were washed twice with PBS (0.02 M, pH 7.2) before suspension in PBS to an OD_{600} of 0.3, for the determination of cell-bound haemolytic activity.

Effect of tea extract. Tata brand blended tea (20 g) was suspended in 100 ml of PBS (0.02 M, pH 7.2) in two

separate beakers. One beaker was kept at room temperature for 3 h while the contents of the other beaker were boiled for 5 min. Tea infusions were then centrifuged at 6000 *g* for 15 min in separate tubes. Supernates thus obtained were collected in sterile tubes and added as supplements to NA agar (3–25% v/v) before autoclaving. NA containing tea extract was used for making blood agar plates. These plates were used to determine haemolytic activity as described above.

Effect of tea extract on Congo red binding. *S. Typhimurium* DT 98 was cultured for 18 h at 37°C in various growth media (BHI, NB or TSB). The cultures were then centrifuged and cell deposits were washed in PBS. The bacterial cell density was adjusted to an OD₆₀₀ of 0.3. Bacterial cell preparations were mixed with boiled tea extract (1.5–25% v/v) in separate tubes, which were kept at 37°C for 10 min. The tubes were then centrifuged at 13 000 *g* for 15 min and the supernate was decanted. Bacterial cell deposits were suspended in PBS. These were divided into two equal portions. The test portion was mixed with Congo red (0.003% w/v) and incubated at 37°C for 10 min, together with a control (no Congo red), and then centrifuged. The supernates obtained from each tube were collected into separate tubes and scored for absorbance at 400 nm against PBS. Congo red bound to bacterial cells was calculated as follows: bound Congo red = A₄₈₀ control supernate with 0.003% Congo red – A₄₈₀ test supernate. The amount in µg was determined from the Congo red standard absorbance curve.

Effect of tea extract on bacterial cell viability. A bacterial cell suspension in PBS adjusted to an OD₆₀₀ of 0.3 and tea extract (3–25% v/v) were mixed in equal proportions in different tubes and incubated at 37°C. Samples were withdrawn after incubation for 0, 3, 6, 9 and 24 h, diluted in sterile blanks and spread-plated on to NA. The plates were incubated at 37°C for the determination of viable counts. Controls were processed similarly except that an equivalent amount of PBS was added instead of tea extract.

Effect of tea extract on bacterial growth. Sterile NB (75 ml) was mixed with tea extract (25 ml of 25% w/v infusion in PBS) or PBS (control) in 250-ml flasks, which were then inoculated with 100 µl of a 16–18-h culture of *S. Typhimurium* DT 98 (OD₆₀₀ of 0.3). Inoculated flasks were incubated at 37°C on a rotary shaker (180 rpm). Samples (100 µl) were withdrawn after incubation for 0, 3, 6, 9, 12 and 24 h. Each sample was suitably diluted in PBS before spread-plating on NA for the determination of viable counts at 37°C.

Minimal inhibitory and bactericidal concentrations of tea extract. The boiled tea extract (25% w/v tea infusion in PBS) was diluted two-fold serially in NB broth (10 ml/tube). The tubes were inoculated with 20 µl from overnight cultures of *S. Typhimurium* DT

98 and incubated at 37°C for 18 h. Next day, a loopful of culture from each tube was streaked on to an NA plate. The plates were examined for bacterial growth after incubation at 37°C for 18 h. The lowest concentration of tea extract that showed no growth was taken as the minimal bactericidal concentration.

Statistical analysis

Karl Pearson's coefficient of correlation and the unpaired Student's *t* test were used for the analysis of data.

Results

Of the 29 strains of salmonellae screened for haemolytic activity, some were found to be very weakly haemolytic and produced a barely visible, narrow zone of haemolysis around the colonies on bilayered blood agar plates at 37°C. However, these strains produced wider zones of haemolysis when cultured under micro-aerobic conditions. An increase in the size of the zone of haemolysis was also observed if the incubation at 37°C was prolonged to 48 or 72 h [21]. The Congo red binding and haemolysin activity were highest for *S. Typhimurium* DT 98. *S. Typhimurium* DT 98 colonies (24 h) on Congo red blood agar were small and discrete, with a dark red centre, lighter outer zone and a zone of haemolysis around the colonies (Fig. 1). In contrast, the colonies on layered blood agar (24 h) were larger, with a narrow zone of haemolysis. The haemolytic zone around the colonies widened on further incubation (48 h) at 37°C (Fig. 1).

Factors affecting haemolysin activity and expression

The haemolysin expression was minimal in synthetic medium, greatest in BHI and expressed in the other growth media in the order BHI > NB > TSB > M-9. Cell-bound haemolysin correlated with the haemolysin activity in the CCFS ($r = +0.89$). Any alteration (increase or decrease) in either type of haemolysin had the same effect on the other. The ratio between the cell-bound and cell-free haemolysin activities of strains cultured in different media was also similar (Table 1). However, the results of a comparison of the haemolysin activity of *S. Typhimurium* DT 98 cultured in NB with and without Congo red (0.003% w/v) showed greater haemolysin activity ($p < 0.05$) in broth supplemented with Congo red (CCFSH; 206 HU, 1.36 mg haemoglobin released/ml). In contrast, cell-bound haemolysin (CBH) activity was negligible (19 HU, 0.019 mg haemoglobin released/ml) in broth supplemented with Congo red (Table 2). Under similar conditions, the haemolysin activity was found to be 56 HU, or 0.041 mg haemoglobin released/ml, in CCFSH and 135 HU, or 0.080 mg haemoglobin released/ml, for CBH in NB cultures (control). The addition of Congo red appeared to facilitate the release of haemolysin into

Fig. 1. Haemolytic activity of *S. Typhimurium* DT 98 cultured under micro-aerobic conditions at 37°C. Colonies appearing on layered blood agar after incubation for 24 h are larger, with a narrow zone of haemolysis (**a**). The zone of haemolysis is wider in cultures incubated for 48 h (**b**), whereas colonies are smaller and have wider zones of haemolysis after incubation for 24 h on blood agar supplemented with Congo red, 0.003% w/v (**c**).

Table 1. Effect of growth medium on cell-bound and cell-free haemolysin production by *S. Typhimurium* DT 98 cultured in different growth media

Growth medium	Haemolysin activity			
	Haemoglobin released (mg/ml)		A_{545}	
	CBH	CCFSH	CBH	CCFSH
M-9 glucose	0.019	0.000	0.020	0.000
NB	0.060	0.020	0.088	0.019
TSB	0.057	0.019	0.086	0.021
BHI	0.080	0.049	0.138	0.068

CBH, bacterial cell density adjusted to OD_{600} 0.3; CCFSH, culture-cell-free supernate activity; NB, nutrient broth; BHI, brain-heart infusion broth; TSB, trypticase soy broth; CBH *versus* CCFSH, $p < 0.05$, $r = +0.89$.

the growth medium. The haemolysin that should have adsorbed on to the bacterial surface was also excreted. The expression of wider haemolytic zones by strains cultured on Congo red blood agar further confirms the above observation, whereby varying zones of haemo-

lysis were observed on blood agar containing different concentrations of Congo red (0.001–0.005% w/v).

The addition of tea extract to the growth medium at higher concentrations ($\geq 6\%$) inhibited haemolysin

Table 2. Effect of Congo red supplementation (0.003%) to NB on haemolysin expression by *S. Typhimurium* DT 98 cultured at 37°C for 24 h

Growth medium	Mean (SD) haemolysin activity			
	Haemoglobin released (mg/ml)		A ₅₄₅	
	CBH	CCFSH	CBH	CCFSH
NB with Congo red	0.019 (0.006)	1.360 (0.48)	0.019 (0.007)	0.026 (0.018)
NB	0.080 (0.015)	0.041 (0.023)	0.135 (0.074)	0.056 (0.024)

CBH, bacterial cell density adjusted to OD₆₀₀ 0.3; NB with Congo red *versus* NB CCFSH, *p* < 0.05.

production, whereas at lower concentrations (1.5–3%), haemolysin production was stimulated and the strains produced wider zones of haemolysis on blood agar supplemented with tea extract. Similarly, the differences in Congo red binding were also seen in bacterial cell suspensions cultured in different growth media that contained tea extract or differed in nutritional content. The degree of Congo red binding in different cultures corresponded to their haemolysin activities in the order BHI > NB > M-9 glucose medium. The Congo red binding was always higher for bacterial cells cultured in medium supplemented with tea extract than for those cultured in the same medium without tea extract. Characteristically, the Congo red binding was also highest in bacterial cells cultured in the presence of lower concentrations of tea extract (Table 3) ($r = -0.89$ in NB, -0.93 in TSB and -0.51 in BHI). Congo red binding and haemolysin activity in *S. Typhimurium* DT 98 appeared to be a directly correlated phenomenon ($r = +0.998$ in NB, $+0.69$ in TSB and $+1.0$ in BHI).

A successive decrease in bacterial viable count was observed as the exposure time of *S. Typhimurium* DT 98 to tea extract 25% v/v in PBS or NB increased. The decrease in viable count was slower in bacterial cells suspended in NB (Table 4). The viable count was <1 for bacterial cells suspended in PBS containing tea extract, whereas it was still 2×10^3 cfu/ml after 9 h in NB containing tea extract and became <1 only after 24 h. Boiled tea extract at 25% v/v was found to be bactericidal for *S. Typhimurium* DT 98.

Discussion

The pathogenic salmonellae often have a long passage within the host before they induce a symptomatic infection. Strains effectively compete with host defences at each phase, expressing phase-specific factors and inducing cell-surface changes [4, 15]. Entry through the intestine followed by transient bacteraemia leads to the establishment of foci of infection in the liver and spleen. In the present study, haemolysin, an important virulence factor [25, 26] that is expressed optimally in *S. Typhimurium* DT 98 under micro-aerobic conditions such as those found in its intestinal

phase, was examined. Haemolysin could be useful in epithelial cell invasion, the induction of cell-surface factors, survival in macrophages and the counteraction of host immune defences, particularly those non-specific defences encountered in the initial phases of infection [21, 27]. The direct correlation between the Congo red binding and haemolysin activity of *S. Typhimurium* DT 98 under conditions that favour haemolysin production is indicative of associated phenotypic changes in strains cultured under different conditions. Similar changes have been reported in strains of *Pseudomonas aeruginosa* cultured from the sputum of patients with cystic fibrosis, and in those cultured in iron-deficient conditions *in vitro* [28]. *S. Typhimurium* DT 98 cells cultured either in medium containing Congo red (0.003% w/v) or exposed to Congo red before haemolysin assay expressed very little cell-bound haemolysin activity. On the other hand, the haemolytic activity of CCFS from strains cultured in growth media containing Congo red was much higher than that shown by the same strain after culture in the absence of Congo red.

These results appear to show that: (1) some of the haemolysin adsorbs to the bacterial cell surface, and (2) the loci for binding Congo red and adsorbing haemolysin are either very similar or identical, as Congo red binding to bacterial cells resulted in the complete loss of the cell-bound haemolysin activity of the strain.

It is interesting that the Congo red binding appeared to activate rather than inhibit haemolysin synthesis or secretion, as the haemolysin activity of CCFS from cultures grown in the presence of Congo red was much higher than that from cultures grown in medium without Congo red. During synthesis and secretion of haemolysin, some of the haemolysin adsorbs to the bacterial cell surface. Congo red present in the medium seems to bind to these adsorption sites as well. Hence the amount of haemolysin synthesised continuously is excreted and, in addition, the haemolysin bound to receptors on the bacterial cell surface is also secreted [29] resulting in greater haemolysin activity in the CCFS. On Congo red agar, the haemolytic strains produced smaller colonies but with wider zones of haemolysis.

Table 3. Effect of boiled tea extract on Congo red binding by *S. Typhimurium* DT 98 cultured in NB at 37°C for 24 h

Tea extract concentration (% v/v)	NB				TSB				BHI			
	Test		Control		Test		Control		Test		Control	
	Test	Control	Congo red bound (μg)	Test control	Test	Control	Congo red bound (μg)	Test control	Test	Control	Congo red bound (μg)	Test control
12.5	1.480	1.493	0.390	0.013	1.470	1.490	0.600	0.020	1.460	1.524	1.920	0.064
6.25	1.420	1.440	0.600	0.020	1.450	1.400	1.500	0.050	1.410	1.492	2.460	0.082
3.00	1.370	1.420	1.200	0.040	1.400	1.410	1.800	0.100	1.390	1.450	1.800	0.060
1.50	1.370	1.310	1.800	0.060	1.370	1.300	2.100	0.700	1.290	1.390	3.000	0.100

Tea extract concentration *versus* Congo red bound, $r = -0.89$ in NB, -0.93 in TSB and -0.51 for BHI.
Congo red binding *versus* haemolysin activity, $r = +0.998$ in NB, $+0.69$ in TSB and $+1.0$ for BHI.

Table 4. Death rate kinetics of *S. Typhimurium* DT 98 cells suspended in NB and PBS (0.02 M, pH 7.2) with tea extract (25% v/v of 25% w/v boiled tea extract in PBS)

Exposure time (h)	Bacterial viable count (cfu/ml) in		
	PBS + tea	NB + tea	NB control*
0	2.9×10^5	2.6×10^5	3.0×10^5
3	5.0×10^4	9.0×10^4	5×10^7
6	30	2.0×10^4	7.6×10^9
9	0	3.0×10^3	5×10^9
24	0	0	8×10^9

**S. Typhimurium* DT 98 cells in NB without tea extract.

The effect of the addition of tea extract to blood agar on haemolysin production was inhibitory at concentrations of $\geq 6\%$ v/v, but stimulatory at lower concentrations (1.5–3% v/v). These findings corroborate the earlier report of antihemolysin activity of tea and coffee extracts against *Staphylococcus aureus* and *Vibrio parahaemolyticus* hemolysins [30]. To investigate the dual effect of boiled tea extract on hemolysin production, tea extract was evaluated for its antibacterial activity against *S. Typhimurium* DT 98. At the higher concentration of 25% v/v, the tea extract was found to be bactericidal for *S. Typhimurium* DT 98. The strain showed no hemolytic activity when cultured on blood agar plates supplemented with tea extract at concentrations of 6% or 12% v/v, and the colonies produced were smaller. The hemolysin expression was also greater in strains cultured under micro-aerobic conditions in growth medium containing low concentrations of tea extract. This stimulation of hemolytic activity may be related to altered hydrophobicity or an alteration in bacterial cell surface characteristics like the changes induced by Congo red binding. It is surprising that even the Congo red binding by *S. Typhimurium* DT 98 cells pre-treated with tea extract at a concentration of 6% or 12% v/v was negligible when compared with the Congo red bound to bacterial cells exposed to tea extract at concentrations of 1.5–3% v/v. Pre-treatment with low concentrations of tea extract appeared to up-regulate the receptors for Congo red binding and hemolysin adsorption/secretion, whereas higher concentrations probably masked such receptors completely. These activities have also been correlated with the adhesiveness of clinical isolates of shigellae to colonic epithelial cells [23, 31]. The results of the present study appear to confirm a direct correlation between Congo red binding and hemolysin expression by *S. Typhimurium* DT 98.

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