Expression of the *htrB* gene is essential for responsiveness of *Salmonella typhimurium* and *Campylobacter jejuni* to harsh environments

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In *Campylobacter jejuni*, an *htrB* homologous gene is located in the lipo-oligosaccharide synthesis gene cluster. This study examined the effects of *htrB* expression on the responsiveness of *Salmonella typhimurium* and *C. jejuni* to harsh environments. Complementation experiments showed that the *C. jejuni htrB* gene could restore the normal morphology of the *Salmonella htrB* mutant, and its ability to grow without inhibition under heat, acid and osmotic stresses, but not bile stress. This indicated that the *htrB* genes in *C. jejuni* and *S. typhimurium* exhibit similar pleiotropic effects. Moreover, quantitative real-time RT-PCR showed that expression of the *C. jejuni htrB* gene was upregulated under acid, heat, oxidative and osmotic stresses, but did not change under bile stress. This indicated that the *C. jejuni htrB* gene plays a role in regulating cell responses to various environmental changes. Furthermore, deletion mutation of the *htrB* gene in *C. jejuni* was lethal, indicating that the *htrB* gene is essential for *C. jejuni* survival. Therefore, these results showed that expression of the *htrB* gene is essential for the response of *S. typhimurium* and *C. jejuni* to environmental stresses.

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INTRODUCTION

Modulation of lipid A acylation in Gram-negative bacteria results in pleiotropic effects. In *Escherichia coli*, mutation of the *htrB* gene encoding a lipid A acyltransferase leads to inhibition of bacterial growth at high temperature (Karow & Georgopoulos, 1991; Karow *et al.*, 1991), morphological change from short to filamentous rods (Karow *et al.*, 1991), and unusually increased bile resistance (Karow & Georgopoulos, 1992). In *Salmonella typhimurium*, inactivation of the *htrB* homologous gene exhibits not only the same effects as those seen in *E. coli*, but also hyperflagellation and severely limited virulence (Jones *et al.*, 1997; Sunshine *et al.*, 1997). In *Haemophilus influenzae*, knockout of the *htrB* gene results in increased bile sensitivity (Lee *et al.*, 1995), increased sensitivity to human antimicrobial peptides (β -defensins) (Starner *et al.*, 2002), decreased colonization capacity (Swords *et al.*, 2002), decreased intracellular viability (Swords *et al.*, 2002), and decreased pro-inflammatory cytokine induction (Tong *et al.*, 2001), but it does not affect morphology (Lee *et al.*, 1995).

Campylobacter jejuni is an enteric bacterium causing human gastroenteritis worldwide (Coker *et al.*, 2002; O’Ryan *et al.*, 2005). The predominant symptoms are inflammatory diarrhoea, abdominal pain, and/or fever. Poultry products,

milk and water are frequently reported as the infectious sources. Infections are mainly observed in children under 5 years of age, and cause serious complications in immunocompromised hosts (Monselise *et al.*, 2004; Ramon Maestre *et al.*, 2001).

The lipo-oligosaccharide (LOS) of *C. jejuni* is a major surface molecule consisting of two parts, the core oligosaccharide and lipid A. The core region is involved in virulence (Fry *et al.*, 2000) and induction of Guillain-Barré syndrome, an autoimmune neuropathy of the peripheral nervous system (Yuki *et al.*, 2004). The lipid A of the LOS molecule possesses endotoxic properties (Naess & Hofstad, 1984). This crucial molecule is partly encoded by the *wlaII* gene cluster, which shows a high degree of variation among strains (Gilbert *et al.*, 2002; Parker *et al.*, 2005). In *C. jejuni* strain HB 93-13, it contains 13 consecutive genes: *waaC*, *htrB*, *wlaNC*, *wlaND*, *cgtA*, *cgtB*, *cstII*, *neuB*, *neuC*, *neuA*, *wlaVA*, *wlaQA* and *waaF* (GenBank accession no. AY297047). The *htrB* homologue found in this *wlaII* gene cluster is conserved in *C. jejuni*, and is similar to the *htrB* gene of *S. typhimurium*, *E. coli* and *H. influenzae*. Functionally, the *C. jejuni htrB* gene encodes a putative acyltransferase involved in lipid A synthesis (Gilbert *et al.*, 2000, 2002; Parkhill *et al.*, 2000). This study examined the effects of *htrB* expression on the responsiveness of *S. typhimurium* and *C. jejuni* to harsh environments, using complementation, gene expression and mutation experiments.

Abbreviations: C_T, cycle threshold; DOC, sodium deoxycholate; Km, kanamycin resistance cassette; LOS, lipo-oligosaccharide.

METHODS

Bacterial strains and growth conditions. *C. jejuni* strains HB 93-13 (Ho *et al.*, 1995), O:4, O:41, O:36, 81116 (Palmer *et al.*, 1983), NCTC 11168, ATCC 43446 and OH 4382 (Aspinall *et al.*, 1994) were included in this study. *C. jejuni* was grown on Columbia agar plates supplemented with 5% (v/v) defibrinated horse blood under microaerobic conditions (5% O₂, 10% CO₂, 85% N₂) at 42 °C for 16 h, unless otherwise stated. *E. coli* DH5 α was grown in Luria-Bertani (LB) broth or agar at 37 °C for 16 h. *S. typhimurium* strains SL1344 (wild-type) and SL1344 *htrB1::Tn10* (*htrB* mutant, tetracycline resistance) were kindly provided by Dr B. D. Jones, Department of Microbiology, University of Iowa (Jones *et al.*, 1997; Sunshine *et al.*, 1997). *S. typhimurium* was grown in LB broth or on LB agar at 30 °C for 16 h, unless otherwise stated. Media were supplemented with 150 μ g ampicillin ml⁻¹, 15 or 50 μ g kanamycin ml⁻¹, 20 μ g tetracycline ml⁻¹, 2% (w/v) X-Gal in dimethyl formamide (40 μ l for each LB plate), and 100 mM IPTG (40 μ l for each LB plate), when appropriate.

Analysis of DNA and amino acid sequences. Clone manager version 6 (Scientific and Education Software) was used to design primers, plan cloning and analyse DNA and amino acid sequences. Primers were designed using the *wlaII*-LOS synthesis gene cluster of *C. jejuni* strain HB 93-13 (GenBank accession no. AY297047), unless otherwise stated.

DNA manipulation. Plasmid DNA was isolated using the mini-prep procedure described by Ausubel *et al.* (1995), and/or the QIAprep Spin Miniprep kit (Qiagen) according to the manufacturer's instructions. The DNA quantity was determined using spectrophotometry. Restriction enzymes, T4 DNA ligase and alkaline phosphatase were purchased from Promega, and used according to the manufacturer's instructions. Restriction mapping was performed to confirm the composition and size of the constructed plasmids by digestion with appropriate restriction enzymes. Transformation of *C. jejuni* with plasmid or genomic DNA was performed using electroporation (25 μ F, 1.25 kV and 600 Ω , gene pulser apparatus; Bio-Rad) and/or natural transformation (biphasic technique), as described by Wassenaar *et al.* (1993). Transformation of *E. coli* and *S. typhimurium* with plasmid DNA was performed using electroporation (25 μ F, 2.48 kV and 200 Ω , gene pulser apparatus; Bio-Rad). Competent cells for *E. coli* and *S. typhimurium* were prepared in cold 10% (v/v) glycerol as described by Sambrook & Russell (2000).

pfu-PCR. *pfu*-PCR was used to amplify a DNA fragment from purified chromosomal DNA. The reaction mixture was prepared in a 50 μ l total volume of 1 $\times$ *pfu* buffer containing 200 μ M each of dATP, dTTP, dCTP and dGTP, 100 ng of each primer, 100 ng DNA, and 5 U *pfu* polymerase (Roche). The PCR conditions were as follows: 94 °C for 3 min (initial denaturation); 35 cycles of 94 °C for 30 s (denaturation), 50 °C for 1 min (annealing), 72 °C for x min (extension); and 72 °C for 7 min (final elongation). x was calculated by dividing the length of the PCR product by 500 bp, as *pfu* polymerase synthesizes 500 bp min⁻¹.

Colony-PCR. Colony-PCR was used to screen transformants carrying new constructs. The reaction mixture was prepared in a 50 μ l total volume of 1 $\times$ *Taq* buffer containing 1.5 mM MgCl₂, 200 μ M each of dATP, dTTP, dCTP and dGTP, 100 ng of each primer, and 2.5 U *Taq* polymerase (ABI). A 200 μ l (maximum volume) tip was used to gently touch a colony on a culture plate, and the colony material was directly mixed into a PCR tube containing master reagent, which had been prepared beforehand. The PCR conditions were as follows: 94 °C for 10 min; 35 cycles of 94 °C for 30 s, 50 °C for 1 min, 72 °C for y min; and 72 °C for 7 min. y was calculated by dividing the length of the PCR product by 1000 bp, as *Taq* polymerase synthesizes 1000 bp min⁻¹.

Construction of a plasmid carrying the *C. jejuni* *htrB* gene in the *S. typhimurium* *htrB* mutant. A 917 bp DNA fragment containing 18 bp of the *waaC* gene (upstream adjacent gene), the entire *htrB* gene (888 bp) and 11 bp of the *wlaNC* gene (downstream adjacent gene) of *C. jejuni* strain HB 93-13 was amplified by *pfu*-PCR with primers *Bam*HI-*waaC*-F (5'-TTGCCAAAGGATCCCC-TTAATGAAAAATAGTGATAG-3') and *Clal*-*wlaNC*-R (5'-TTGT-TATCGATTTCATTTGCACCCTTGT-3'). A PCR product was cloned into the pBluescript plasmid in the same orientation as the ampicillin-resistance cassette, using the *Bam*HI and *Clal* sites, and the resultant construct was subsequently introduced into *E. coli* DH5 α by electroporation. Transformants carrying the *htrB* gene constructs were screened using colony-PCR with primers 172-pBlue-F (5'-GGTTCGATTTAGTGCTTA-3') and 825-pBu-R (5'-GAA-ACAGCTATGACCATGAT-3'). These primers were designed to amplify a 1516 bp plasmid fragment, which included a 917 bp inserted PCR product. The pBluescript carrying the *C. jejuni* *htrB* gene (named pBlue *htrB*+) was isolated from *E. coli*, and then introduced into the *S. typhimurium* *htrB* mutant by electroporation.

Examination of bacterial growth and morphology. The wild-type, mutant and complemented *Salmonella* strains were grown on LB agar plates at 30, 37 and 42 °C for 24 h to OD₆₀₀ 0.3. The culture media were supplemented with appropriate antibiotics. Tetracycline was added to the growth medium used for the *S. typhimurium* mutant, while ampicillin and tetracycline were added to the growth medium used for the complemented strain. The ability of bacteria to grow at 30, 37 and 42 °C was observed. A Gram stain was performed and the morphology was observed under a light microscope.

Examination of bacterial sensitivity to acid and osmotic stresses. To test for acid sensitivity, the wild-type, mutant and complemented *Salmonella* strains (OD₆₀₀ 0.3) were grown at 30 °C in 0.1% (w/v) peptone water, pH 2.5–7.0, for 24 h. After incubation, cell density was measured at OD₆₀₀, and an equal volume of culture medium was distributed on LB agar plates. All culture media were supplemented with appropriate antibiotics as described above. The plates were incubated at 30 °C for another 24 h. To test for osmotic sensitivity, the bacteria were grown as described above, except that 0.1% peptone water (pH 7.0) was supplemented with NaCl (1–10%, w/v).

Examination of bacterial sensitivity to bile stress. To test for bile sensitivity, the wild-type, mutant and complemented *Salmonella* strains (OD₆₀₀ 0.3) were grown on LB agar plates containing sodium deoxycholate (DOC; 2, 4, 6, 8 and 10%). The culture media were supplemented with appropriate antibiotics as described above. The plates were incubated at 30 °C for 24 h.

Treatment of *C. jejuni* with stress environments, and RNA preparation. Prior to the treatment of *C. jejuni* with stress environments, the ability of *C. jejuni* HB 93-13 to grow in brucella broths under heat, acid, osmotic, oxidative or bile stresses was examined. A growth temperature of 44 °C was selected as heat stress, since *C. jejuni* was able to grow at 43 °C but not at 45 °C. pH 5.5 was selected as acid stress, since *C. jejuni* showed normal growth at pH 6.0, inhibited growth at pH 5.5, and no growth at pH 5.0. A NaCl concentration of 1.5% was selected as osmotic stress, since the bacteria showed normal growth at 1% NaCl, inhibited growth at 1.5% NaCl, and no growth at 2% NaCl. As *C. jejuni* was unable to grow in normal atmospheric conditions, these were used for the oxidative stress challenge. For bile stress, 500 μ g DOC ml⁻¹ was used (Lin *et al.*, 2005).

C. jejuni HB 93-13 was grown in 30 ml brucella broth with gentle shaking under microaerobic conditions at 37 °C for 19 h, and 100 μ l bacterial culture was aliquoted into six bottles of brucella broth (30 ml), and incubated for another 19 h. After incubation, 1 ml

brucella broth, which had been supplemented with concentrated HCl, NaCl or DOC, and pre-warmed at 37 °C, was added to the culture bottles to obtain a final pH of 5.5 (acid stress), a NaCl concentration of 1.5 % (osmotic stress), and a DOC concentration of 500 µg ml⁻¹ (bile stress). The fourth culture bottle, with 1 ml pre-warmed brucella broth added, was used as the calibrator (normal *htrB* expression level). The fifth culture bottle was immediately moved to the 44 °C incubator (heat stress). Incubation was performed at 37 °C, except for the fifth culture bottle, with gentle shaking under microaerobic conditions. The bacteria from the sixth culture bottle were poured onto culture plates, and incubated at 37 °C with gentle shaking under normal atmospheric conditions (oxidative stress). Culture samples were collected after incubation for 15 and 30 min, and transferred directly into a 1/10 volume of cold 10 × stop solution [5 % (v/v) phenol in 100 % ethanol] to halt transcription and RNA degradation. RNA samples were isolated using the RNeasy Total RNA Isolation system (Promega), and DNA decontamination was performed using the TURBO DNA-free kit (Ambion).

Quantitative real-time RT-PCR. Primers used for cDNA synthesis were as follows. The primers q-*htrB*-R (5'-TTGAGTGTATTGAG-GAAAAC-3'), q-16S rRNA-R (5'-GTATTCTTGGTGATATCTAC-3'; accession no. AL111168), q-*luxS*-R (5'-ATAAATCCTGCGAATAAATG-3'; accession no. AL111168) and q-*rpoA*-R (5'-ATTTGTCCATCAGTTGTTAC-3'; accession no. AL111168) were used for synthesis of cDNA for the *htrB*, 16S rRNA, *luxS* and *rpoA* genes, respectively.

cDNA synthesis was performed using the ImProm-II reverse transcriptase (Promega). One microgram of RNA and 50 ng anti-sense primer in a total volume of 5 µl were heated at 70 °C for 5 min, and immediately chilled on ice for at least 5 min. The master mix was prepared in a total volume of 15 µl, which consisted of 5 µl RNase-free water, 4 µl 5 × ImProm-II reaction buffer, 2 µl MgCl₂ (25 mM), 1 µl dNTP mix (10 mM each dNTP), 2 µl recombinant RNasin ribonuclease inhibitor (5 U µl⁻¹), and 1 µl ImProm-II reverse transcriptase (1 µl per reaction). The master mix was dispensed into the reaction tube containing the mixture of heated RNA and primer. The tube was gently mixed, followed by incubation at 25 °C for 5 min, 42 °C for 60 min, and 70 °C for 15 min. Reverse transcriptase was substituted by RNase-free water for the negative control. After cDNA synthesis, 10 µl RNase A solution (20 µg ml⁻¹) was added to the reaction mixture, incubated at 37 °C for 20 min, and 220 µl water was added. For each gene, a dilution series of newly synthesized cDNA was made and included in a quantitative PCR to examine the efficiency of PCR.

Primers used for PCR were as follows. The primers q-*htrB*-F (5'-TTATGCCTGATTGTATCTTG-3') and q-*htrB*-R, as described above, were used to amplify a 125 bp fragment of the *htrB* gene-specific cDNA. The primers q-16S rRNA-F (5'-GTCTCTTGTGAAATCTAATG-3', accession no. CJ11168X3) and q-16S rRNA-R, as described above, were used to amplify a 123 bp fragment of 16S rRNA gene-specific cDNA. The primers q-*luxS*-F (5'-AAGTTATGAAAACACCTAAG-3', accession no. CJ11168X4) and q-*luxS*-R, as described above, were used to amplify a 124 bp fragment of *luxS* gene-specific cDNA. The primers q-*rpoA*-F (5'-GCTTTAGATGCTTCTTTAC-3', accession no. CJ11168X6) and q-*rpoA*-R, as described above, were used to amplify a 119 bp fragment of the *rpoA* gene-specific cDNA.

A quantitative PCR was performed on the MyiQ PCR detection system (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad), according to the manufacturer's instructions. The PCR reaction was performed in a total volume of 25 µl, which contained 12.5 µl iQ SYBR Green Supermix and 12.5 µl master mix, consisting of 1 µl forward primer (50 ng µl⁻¹), 1 µl reverse primer (50 ng µl⁻¹), 3 µl diluted cDNA, and 7.5 µl water. Each PCR was performed in duplicate. The same PCR

samples that showed variant values of the cycle threshold (C_T) of more than 1 were repeated. PCR conditions were as follows: cycle 1 (× 1), 95 °C for 5 min; cycle 2 (× 35), 95 °C for 30 s, 55 or 60 °C for 30 s (55 °C for *luxS*; 60 °C for *htrB*, 16S rRNA gene and *rpoA*), 72 °C for 30 s; cycle 3 (× 100), 95 °C for 10 s (decrease setpoint temperature after cycle 2 by 0.5 °C, enabling melt-curve data collection and analysis). Each specific amplicon was verified by the presence of both a single melting-temperature peak and a single band of expected size on a 3 % agarose gel after electrophoresis. C_T values were determined with the MyiQ software (Bio-Rad). The relative changes (x -fold) in gene expression between the induced and calibrator samples were calculated using the $2^{-\Delta\Delta C_T}$ method, as described by Livak and Schmittgen (2001). The 16S rRNA, *rpoA* and/or *luxS* genes were used as the internal controls. A relative expression value of more than twofold was considered as significant up- or down-regulation.

Construction of the *C. jejuni htrB* mutant. A pBluescript plasmid carrying the mutated *htrB* gene of *C. jejuni* HB 93-13 was constructed. Firstly, a 646 bp DNA fragment containing the partial *waaC* and *htrB* genes of *C. jejuni* HB 93-13 (nt 140–785; accession no. AY297047) was amplified by the *pfu*-PCR with primers *EcoRI*-*waaC*-F1 (5'-ATAGGAATTCATAGCGGTCCAACACA-3') and *Bam*HI-*htrB*-R1 (5'-AATCGGATCCTATTTAGCCGCATAAGC-3'). The PCR product was cloned into pBluescript in the forward direction via the *EcoRI* and *Bam*HI sites. The resultant construct was subsequently introduced into *E. coli* DH5 α by electroporation. A positive clone was selected on LB agar supplemented with ampicillin, X-Gal and IPTG, according to the blue and white phenotypes. This procedure identified the pBluescript carrying the partial *waaC* and *htrB* genes (named pBluA). Secondly, *pfu*-PCR with primers *Bam*HI-*htrB*-F2 (5'-TTACGGATCCAGACTGCGTAGAAAACGA-3') and *Xba*I-*wlaNC*-R2 (5'-CCTTTCTAGAGATTTTACGGCTAAGTG-3') was used to amplify a 678 bp DNA fragment containing the partial *htrB* and *wlaNC* genes (nt 934–1611; accession no. AY297047). This PCR product was cloned into pBluA in the forward orientation via the *Bam*HI and *Xba*I sites. Positive clones were identified by colony hybridization using the second PCR product, labelled with DIG using the DIG labelling kit (Roche), as a probe. The colony-DNA probe hybridization was performed at 65 °C overnight, and detection was performed using the alkaline phosphate-conjugated anti-DIG antibody and the nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolylphosphate *p*-toluidine salt (NBT/BCIP) substrate, as described in the user's guide handbook (Roche). Positive clones carrying the *htrB* gene with a 150 bp (nt 785–934) deletion were named pBluB. A 1494 bp kanamycin resistance cassette (Km) was cloned into pBluB using the *Bam*HI site. This resulted in a construct containing part of the *htrB* gene interrupted by the Km (named pBluC), in which the Km was flanked by a 646 bp upstream and 678 bp downstream DNA fragments. The pBluC carrying the Km in the same direction as the *htrB* gene was named pBluCF, while the construct carrying the Km in the opposite direction to the *htrB* gene was designated pBluCR. Finally, these constructed plasmids were confirmed by sequencing using the ABI sequencing mix V3.1 (ABI), according to the manufacturer's instructions.

Natural transformation and electroporation were used to introduce each recombinant plasmid (pBluCF and pBluCR) into *C. jejuni* strains HB 93-13, O:4, O:41, O:36, 81116, 11168, ATCC 43446 and OH 4382. pBluescript plasmids carrying the Km within the *wlaVA* gene (pBlu11KR) or the *waaF* gene (pBlu13KF) were used as the positive controls. pBluescript alone was used as the negative control. Transformants were screened on 5 % blood agar plates supplemented with kanamycin (15 µg ml⁻¹). Culture media were incubated under microaerobic conditions at 30, 37 and 42 °C for 5 days.

RESULTS AND DISCUSSION

Characteristics of the *C. jejuni* htrB gene

The *htrB* homologue of *C. jejuni* HB 93-13 is located in the *wlaII*–LOS synthesis gene cluster. DNA sequence analysis of the *C. jejuni* *htrB* gene showed an ORF of 888 bp, starting with a methionine when translated. Under microaerobic conditions, the *htrB* gene and the other LOS synthesis genes were transcribed as part of several operons using multiple transcriptional start sites with promoters upstream of the start codons of the *Cj1132c*, *waaC*, *cgtA*, *cgtB*, *cstII*, *wlaQA* and *waaF* genes (V. Phongsisay, unpublished data). Multiple sequence alignments showed that the *C. jejuni* HB 93-13 HtrB protein was similar to *E. coli* HtrB (20%; accession no. NC_004431), *S. typhimurium* HtrB (20%; accession no. NC_003197) and *H. influenzae* HtrB (20%; accession no. NC_000907) proteins. Among other bacteria, *S. typhimurium* HtrB showed 78 % similarity to *E. coli* HtrB and 54 % similarity to *H. influenzae* HtrB. These results showed that the *S. typhimurium*, *E. coli* and *H. influenzae* HtrB proteins were more closely related to each other than to *C. jejuni* HtrB protein. In *S. typhimurium*, mutation of the *htrB* gene, which encodes an acyltransferase enzyme involved in lipid A synthesis, results in pleiotropic effects in both the pathology and physiology of *S. typhimurium*. These effects include morphological changes from short to filamentous rods, hyperflagellation, inability to grow at high temperatures, increased bile resistance, and reduced virulence (Jones *et al.*, 1997; Sunshine *et al.*, 1997). In this study, the *S. typhimurium* *htrB* mutant was used as a model for studying the pleiotropic effects resulting from expression of the *C. jejuni* *htrB* gene. The *S. typhimurium* *htrB* mutant was complemented with the *htrB* gene from *C. jejuni* HB 93-13. The wild-type, mutant and complemented *Salmonella* strains were characterized.

Role of the *C. jejuni* htrB gene in *S. typhimurium* morphology

To examine whether expression of the *C. jejuni* *htrB* gene affected the morphology of the *S. typhimurium* *htrB* mutant, the wild-type, mutant and complemented *Salmonella* strains were grown on LB agar plates at 30, 37 and 42 °C for 24 h. The culture media were supplemented with appropriate antibiotics. A Gram stain was performed and the morphology was observed under a light microscope. The morphology of the complemented and wild-type strains was similar, showing Gram-negative short rods at 30, 37 and 42 °C, while the morphology of the mutant displayed Gram-negative, filamentous, bulging, short rods at all temperatures tested (Fig. 1a). These results show that the *C. jejuni* *htrB* gene could restore the wild-type morphology of the *S. typhimurium* *htrB* mutant, and hence its expression affected the bacterial morphology.

Role of the *C. jejuni* htrB gene in growth of *S. typhimurium* at high temperature

To examine whether expression of the *C. jejuni* *htrB* gene affected the capacity of bacteria to grow at high temperature,

the wild-type, mutant and complemented *Salmonella* strains were grown on LB agar plates, as previously described. The results showed that the *C. jejuni* *htrB* gene could restore the ability of the *S. typhimurium* *htrB* mutant to grow at high temperatures, as the complemented and wild-type strains grew normally at 30, 37 and 42 °C, while the mutant was temperature-sensitive and showed inhibited growth at 37 and 42 °C (Fig. 1b). This shows that the *C. jejuni* *htrB* gene is essential for the *S. typhimurium* *htrB* mutant to grow properly at high temperature. A previous study has shown that the *S. typhimurium* *htrB* mutant is unable to grow at 37 °C (Sunshine *et al.*, 1997), while in this study, this bacterial strain showed inhibited growth at 37 °C (Fig. 1b). These inconsistent results may be explained by a loss of temperature sensitivity of the *S. typhimurium* *htrB* mutant after a few passages at 30 °C, which has also been observed for the *H. influenzae* *htrB* mutant (Lee *et al.*, 1995).

Role of the *C. jejuni* htrB gene in acid sensitivity of *S. typhimurium*

To examine whether expression of the *C. jejuni* *htrB* gene affected acid sensitivity, the wild-type, mutant and complemented *Salmonella* strains were grown at 30 °C in 0.1 % (w/v) peptone water (pH 2.5–7.0) for 24 h. After incubation, the cell density was measured at OD₆₀₀, and an equal volume of culture medium was distributed on LB agar plates. All culture media were supplemented with appropriate antibiotics, as described above. The plates were incubated at 30 °C for another 24 h. The results showed that the mutant strain exhibited less growth on LB agar plates compared to the wild-type, and the complemented strain showed growth intermediate between those of the wild-type and mutant strains at lower pH (Fig. 1c, selected results of bacterial growth on LB plates). Therefore, expression of the *C. jejuni* *htrB* gene contributed to the acid tolerance of the *S. typhimurium* *htrB* mutant.

Role of the *C. jejuni* htrB gene in high osmotic sensitivity of *S. typhimurium*

To examine whether expression of the *C. jejuni* *htrB* gene affected osmotic sensitivity, the wild-type, mutant and complemented *Salmonella* strains were grown as described above in an acid-sensitivity assay, except that 0.1 % peptone water (pH 7.0) was supplemented with NaCl (1–10 %). The complemented and wild-type strains exhibited similar growth, while the mutant showed inhibited growth (Fig. 1d, selected result). Therefore, the results showed that expression of the *C. jejuni* *htrB* gene contributed to the osmotic resistance of the *S. typhimurium* *htrB* mutant.

Role of the *C. jejuni* htrB gene in bile sensitivity of *S. typhimurium*

To examine whether expression of the *C. jejuni* *htrB* gene affected bile sensitivity, the wild-type, mutant and complemented *Salmonella* strains were grown on LB agar plates supplemented with 2, 4, 6, 8 and 10 % DOC. The results

Fig. 1. Complementation experiments. WT, wild-type *S. typhimurium* strain SL1344; M, *S. typhimurium htrB* mutant; C, *S. typhimurium htrB* mutant carrying the functional *htrB* gene from *C. jejuni* HB 93-13; Na, NaCl concentration (%). (a) Morphology at different temperatures; (b) ability of bacteria to grow at high temperatures; (c) ability of bacteria to grow in high acidity; (d) ability of bacteria to grow in high osmolality.

showed that the *C. jejuni htrB* gene could not complement the bile sensitivity of the *S. typhimurium htrB* mutant, as the mutant and complemented strains were able to grow in all DOC concentrations tested, while the wild-type strain was able to grow in DOC up to 8 %. Similarly, a bile-resistant phenotype of the *S. typhimurium htrB* mutant has also been observed by Sunshine *et al.* (1997). However, the study of bile sensitivity in other bacteria has shown inconsistent results. The *H. influenzae htrB* mutant is more bile sensitive than the wild-type strain (Lee *et al.*, 1995), while the *E. coli htrB* mutant is more bile resistant than the wild-type strain (Karow & Georgopoulos, 1992; Sunshine *et al.*, 1997). Since

expression of the *C. jejuni htrB* gene did not affect the bile sensitivity of the *S. typhimurium htrB* mutant, the *C. jejuni htrB* gene might not have a role in bile sensitivity in *C. jejuni*.

Expression of the *htrB* gene in *C. jejuni* under stress environments

To examine whether stress environments, including heat, acid, osmotic, oxidative and bile stresses, affected expression of the *htrB* gene in *C. jejuni*, quantitative real-time RT-PCR was performed. Before the quantitative data were accepted, we required three criteria. First, no PCR product should be

detected from the negative control without reverse transcriptase, confirming the lack of DNA contamination in the RNA sample. Second, PCR efficiency for each gene should be $\geq 80\%$. This ensured that low amounts of PCR product were due to low expression levels of the genes of interest, and not the result of low PCR efficiency. Third, only one peak for each gene analysed should be observed in the melting curve, confirming the lack of non-specific PCR product. In this study, the *rpoA* gene encoding the alpha subunit of RNA polymerase, and the 16S rRNA gene (Parkhill *et al.*, 2000) were used as internal negative controls, and it was expected that their expression should not be affected by a change of environment. The *luxS* or *Cj1198* gene (Parkhill *et al.*, 2000), which produces the autoinducer-2 involved in quorum sensing (Elvers & Park, 2002), was used as an internal positive control, and its expression should be affected by a change of environment. The results showed that there was no ideal and universal internal positive/negative control. For example, after *C. jejuni* was exposed to stress environments for 15 min, expression of the *luxS* gene was not affected by heat and bile stresses but it was affected by other stresses. Expression of the 16S rRNA gene showed less variation under osmotic and oxidative stresses, but it showed higher variation under other environments when compared with that of other genes (Table 1). As a result, for each environment, expression of the control gene

that showed lowest variation was selected as the internal control. Therefore, at 15 min, the *rpoA* gene was selected as the internal control for studying *htrB* expression under acid stress, the *luxS* gene was used as the control under heat and bile stresses, and the 16S rRNA gene as the control under oxidative and osmotic stresses. At 30 min, the *luxS* and *rpoA* genes were selected as the control genes under acid and heat stresses, respectively. The 16S rRNA gene was selected as the control gene under oxidative, bile and osmotic stresses. After the expression level of the *htrB* gene was subtracted from that of the internal control, it was found that, after *C. jejuni* was exposed to each artificial environment for 15 min, expression of the *htrB* gene was highly upregulated under acid stress, moderately upregulated under heat and oxidative stresses, slightly upregulated under osmotic stress, and did not change under bile stress. After 15 min of exposure, *htrB* expression gradually decreased with time to reach or approach the baseline level at 30 min. At this time point, expression of the *htrB* gene was moderately upregulated under acid stress, slightly upregulated under oxidative stress, and did not change under bile, osmotic or heat stresses (Table 1). These results show that the *C. jejuni htrB* gene is involved in regulating cell responses to various environmental changes. This is consistent with our previous results showing that the *C. jejuni htrB* gene is essential for the *S. typhimurium htrB* mutant to grow at high temperatures, acidity and osmolality.

Table 1. Expression of the *C. jejuni htrB* gene under stress environments

ND, Not determined. Bold type signifies a C_T value of the internal control genes that did not change or was slightly affected by change of environment; these genes were selected as the internal controls for particular environments. For example, at 15 min, the *rpoA* gene was used as the control gene under acid stress, as its expression (C_T) was not affected by acid stress (24.00 versus 24.24 under normal conditions).

Condition	C _T for:				ΔC _T calibrator*	ΔC _T htrB†	ΔΔC _T ‡	2 ^{-ΔΔC_T} §
	<i>htrB</i>	16S rRNA	<i>luxS</i>	<i>rpoA</i>				
At 15 min								
Normal	26.06	11.33	31.63	24.24	—	—	—	—
Acid	20.34	10.58	29.6	24.00	-1.82	3.66	-5.48	44.63
Bile	25.53	7.88	31.29	ND	5.57	5.76	-0.19	1.14
Osmotic	23.32	9.83	29.44	ND	-14.73	-13.49	-1.24	2.36
Heat	23.32	8.58	31.13	ND	5.57	7.81	-2.24	4.72
Oxidative	20.35	8.37	25.94	19.51	-14.73	-11.98	-2.75	6.73
At 30 min								
Normal	20.49	10.31	28.1	21.07	—	—	—	—
Acid	18.39	9.68	28.59	ND	7.61	10.20	-2.59	6.02
Bile	20.32	9.81	29.98	ND	-10.18	-10.51	0.33	-1.26
Osmotic	21.02	10.03	26.38	ND	-10.18	-10.99	0.81	-1.75
Heat	20.75	8.6	29.39	21.70	0.58	0.95	-0.37	1.29
Oxidative	19.03	10.12	26.03	20.81	-10.18	-8.91	-1.27	2.4

*The ΔC_T calibrator was calculated by subtracting the C_T value of the control gene under normal conditions from that of the *htrB* gene under normal conditions.

† ΔC_T htrB was calculated by subtracting the C_T value of the control gene under stress from that of *htrB* gene under the same stress.

‡ $\Delta \Delta C_T$ was calculated by subtracting the value of the ΔC_T calibrator from the value of ΔC_T htrB.

§ $2^{-\Delta \Delta C_T} > 2$, gene expression was upregulated; $2^{-\Delta \Delta C_T} < -2$, gene expression was down-regulated; $-2 \geq 2^{-\Delta \Delta C_T} \leq 2$, gene expression was not affected by change of environment.

The results of the real-time RT-PCR experiments showed that expression of the *C. jejuni htrB* gene was upregulated under harsh environments, including heat stress. In contrast, an earlier study using Northern blot analysis has shown that expression of the *E. coli htrB* gene is not affected by heat-shock (Karow & Georgopoulos, 1991). It is proposed that the *E. coli htrB* gene is a member of a new class of genes whose products are required for growth at high temperature, but are not heat-shock genes (Karow & Georgopoulos, 1992). Similarly, using microarray analysis, expression of the *htrB* gene of *C. jejuni* NCTC 11168 is not significantly up- or down-regulated after the growth temperature is shifted from 37 to 42 °C (Stintzi, 2003). This inconsistent result might be due to the sensitivity of the methods used. From our results and the fact that real-time RT-PCR is the most sensitive method for differential gene expression, we believe that expression of the *C. jejuni htrB* gene is affected by heat shock.

Effect of modulation of lipid A acylation in *C. jejuni*

To examine the effect of modification of lipid A acylation in *C. jejuni*, the acyltransferase-encoding *htrB* homologous gene was inactivated using mutagenesis. Firstly, two recombinant pBluescript plasmids were constructed. The first construct carried the Km within the *htrB* gene in the same orientation (pBluCF). The second construct carried the Km in the reverse orientation to the *htrB* gene (pBluCR). Secondly, natural transformation and electro-transformation were used to transform various *C. jejuni* strains with these constructs. Plasmids carrying a Km within the *wlaVA* gene (pBlu11KR) and within the *waaF* gene (pBlu13KF) were used as positive controls. The pBluescript without an insert was used as a negative control. Transformants were screened on a selective medium that was supplemented with a low concentration of kanamycin (15 µg ml⁻¹), since the *H. influenzae htrB* mutant has been shown elsewhere to be hypersensitive to kanamycin (Lee *et al.*, 1995). The culture media were incubated at 30, 37 and 42 °C for 5 days, since an earlier study has shown that the *E. coli htrB* mutants cannot initially grow on rich media at temperatures above 33 °C (Karow *et al.*, 1991). As expected, a number of *C. jejuni waaF* and *wlaVA* mutants were obtained from the positive controls, and no transformants were recovered from the negative control. This shows that the possibility that different restriction-modification systems between *C. jejuni* and *E. coli* are a barrier for interstrain plasmid transfer can be ruled out. No transformants carrying the mutated *htrB* gene as a result of homologous recombination via a double crossover event were recovered on the selective medium. However, a few transformants carrying both an intact and a mutated *htrB* gene resulting from a single crossover were observed. After several passages of these transformants, and after transformant-derived genomic DNA was introduced into the parental *C. jejuni* strain HB 93-13, individual progeny still carried both mutated and intact *htrB* genes (data not shown). These results indicate

that the *htrB* gene is essential for *C. jejuni* survival, and hence gene deletion in *C. jejuni* causes loss of cell viability.

A previous study has shown that the plasmid transformation frequency via homologous recombination directly correlates with the size of the flanking regions (Wassenaar *et al.*, 1993). DNA recombination between the inserts present in suicide vectors and the genome occurs with as little as 200 homologous base pairs being present (Wassenaar *et al.*, 1993). pBluCF and pBluCR carried the Km flanked by a 646 bp upstream region consisting of the partial *waaC* and *htrB* genes, and a 678 bp downstream region consisting of the partial *htrB* and *wlaNC* genes. Therefore, the sizes of these flanking regions should have been sufficient to initiate homologous recombination in *C. jejuni*.

The possible explanations as to why the *htrB* gene could be mutated in other bacteria but not in *C. jejuni* are as follows. First, unlike most other bacteria, *C. jejuni* is a fragile enteric bacterium. For example, we found that growth of *C. jejuni* was not observed in brucella broth with NaCl > 1.5 %, DOC > 2 % or pH < 5.5. In addition, *C. jejuni* is hypersensitive to normal atmospheric conditions. In contrast, the growth of *S. typhimurium* was still observed in medium consisting of 10 % NaCl, 10 % DOC or pH 3.0, and it was able to grow under normal atmospheric conditions. Second, the results of the complementation and gene expression experiments presented here indicate that the *C. jejuni htrB* gene is involved in morphology (cell wall formation), and is essential for growth under stress environments, including acid, heat, osmotic and oxidative stresses. Third, accomplishment of inactivation of the *htrB* genes might depend on mutagenesis-based techniques. In this study, deletion mutation was employed to inactivate the *htrB* gene in *C. jejuni* (see Methods). Several attempts were made without success. In contrast, insertional mutagenesis of the *htrB* genes using transposon-based techniques is successful in *S. typhimurium* (Sunshine *et al.*, 1997), *E. coli* (Karow *et al.*, 1991) and *H. influenzae* (Lee *et al.*, 1995). Similarly, deletion mutation of the *waaC* gene, which is located upstream of the *htrB* gene, results in loss of viability of *C. jejuni* strain 81116 (B. N. Fry, unpublished data), while insertion mutation of the *waaC* homologous gene in *C. jejuni* strain 81-176 is successful (Kanipes *et al.*, 2006). Therefore, deletion mutation of the *C. jejuni htrB* gene might result in cell wall dysfunction, such as loss of membrane permeability, leading to bacterial cell death.

The role of the *C. jejuni htrB* gene in lipid A synthesis is controversial. A number of previous studies have proposed that the *C. jejuni htrB* gene encodes a putative acyltransferase involved in lipid A synthesis (Gilbert *et al.*, 2002; Parkhill *et al.*, 2000). Since bile is responsible for digesting fats by disaggregating the lipid bilayer of the cellular membrane, inactivation of the lipid A synthesis gene should increase the bile sensitivity of bacterial cells. Generally, if the *C. jejuni htrB* gene has a role in lipid A synthesis, its expression should be affected under bile stress. This study indicates that the *htrB* gene might not play a role in lipid A synthesis, since its

expression did not change when *C. jejuni* was exposed to bile stress. Additionally, it could not complement the bile sensitivity of the *S. typhimurium* *htrB* mutant. As the role of the *S. typhimurium* *htrB* gene in lipid A synthesis could be demonstrated by MS of the crude lipid A fraction, this technology could also be used to analyse the *C. jejuni* *htrB* gene and its role in lipid A synthesis.

In conclusion, this study shows that: (i) expression of the *C. jejuni* *htrB* gene is essential for the response of *S. typhimurium* to stress environments; (ii) the *C. jejuni* *htrB* gene is involved in regulating cell responses to environmental changes; and (iii) it is likely that the *htrB* gene is essential for *C. jejuni* survival.

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