Molecular Microbiology (2005) **56**(1), 8–27 doi:10.1111/j.1365-2958.2005.04525.x

The *Campylobacter jejuni* stringent response controls specific stress survival and virulence-associated phenotypes

Erin C. Gaynor,[1*†] Derek H. Wells,[2] Joanna K. MacKichan[1] and Stanley Falkow[1]
[1]*Department of Microbiology and Immunology, Stanford University, Stanford, CA, USA.*
[2]*Department of Biological Sciences, Stanford University, Stanford, CA, USA.*

Summary

Campylobacter jejuni **is a highly prevalent food-borne pathogen that causes diarrhoeal disease in humans. A natural zoonotic, it must overcome significant stresses both *in vivo* and during transmission despite the absence of several traditional stress response genes. Although relatively little is understood about its mechanisms of pathogenesis, its ability to interact with and invade human intestinal epithelial cells closely correlates with virulence. A *C. jejuni* microarray-based screen revealed that several known virulence genes and several uncharacterized genes, including *spoT*, were rapidly upregulated during infection of human epithelial cells. *spoT* and its homologue *relA* have been shown in other bacteria to regulate the stringent response, an important stress response that to date had not been demonstrated for *C. jejuni* or any other epsilon-proteobacteria. We have found that *C. jejuni* mounts a stringent response that is regulated by *spoT*. Detailed analyses of a *C. jejuni* Δ*spoT* mutant revealed that the stringent response is required for several specific stress, transmission and antibiotic resistance-related phenotypes. These include stationary phase survival, growth and survival under low CO_2/high O_2 conditions, and rifampicin resistance. A secondary suppressor strain that specifically rescues the low CO_2 growth defect of the Δ*spoT* mutant was also isolated. The stringent response additionally proved to be required for the virulence-related phenotypes of adherence, invasion, and intracellular survival in two human epithelial cell culture models of infection; *spoT* is the first *C. jejuni* gene shown to participate in longer term survival in epithelial cells. Microarray analyses comparing wild-type to the Δ*spoT* mutant also revealed a strong correlation between gene expression profiles and phenotype differences observed. Together, these data demonstrate a critical role for the *C. jejuni* stringent response in multiple aspects of *C. jejuni* biology and pathogenesis and, further, may lend novel insight into unexplored features of the stringent response in other prokaryotic organisms.**

Accepted 13 December, 2004. *For correspondence. E-mail egaynor@interchange.ubc.ca; Tel. (+1) 604 822 2710; Fax (+1) 604 822 6041. †Present address: Department of Microbiology and Immunology, University of British Columbia, Vancouver, BC, Canada.

© 2005 Blackwell Publishing Ltd

Introduction

The intestinal Gram-negative pathogen *Campylobacter jejuni* (*C. jejuni*) is the leading cause of bacterial gastroenteritis and food poisoning in developed countries, affecting more people than *Salmonella* and *Shigella* spp. combined (Blaser *et al*., 1983; Tauxe, 1992; CPHLS, 2000; WHO, 2000; Friedman *et al*., 2001). In North America alone, *C. jejuni* infects over 3.5 million individuals each year, >1% of the total population. The incidence of *C. jejuni*-induced disease may be even higher in developing countries, where infection often goes unreported. Acute *C. jejuni* infection causes watery to bloody diarrhoea, with fever, nausea and vomiting, and can be fatal in highly susceptible (i.e. very old, very young, and immunocompromised) individuals (Butzler and Skirrow, 1979; Walker *et al*., 1986). Infection can also lead to more serious medical sequelae such as reactive arthritis, inflammatory bowel syndrome, and the debilitating ascending bilateral paralysis Guillain–Barré syndrome (GBS) (Skirrow and Blaser, 1992; Karlinger *et al*., 2000; Thornley *et al*., 2001; Nachamkin *et al*., 2002a). Infection with *C. jejuni* is generally self-limiting; however, antibiotic therapy is thought to be important for preventing development of sequelae like GBS and can be required to quell symptoms in acutely ill individuals. Most strains of *C. jejuni* are naturally resistant to antibiotics commonly effective against Gram-negative bacteria, including trimethoprim, rifampicin and the penicillin family. Of the two antibiotic families most commonly used to treat *C. jejuni* infection, fluoroquinolones and macrolides, resistance to fluoroquinolones is rapidly increasing, and up to 90% of *C. jejuni* isolates from Thailand and other countries are now com-

monly found to be fluoroquinolone-resistant (Nachamkin *et al*., 2002b).

Campylobacter jejuni resides primarily in the intestinal mucosa of many animal species. It is frequently seen deep within intestinal crypts, both close to the cell surface and inside intestinal cells (Babakhani *et al*., 1993; Babakhani and Joens, 1993; Russell *et al*., 1993). It can adhere to intestinal cells, translocate through the intestinal barrier, and be highly invasive both *in vivo* and *in vitro* (Everest *et al*., 1992; Konkel *et al*., 1992a,b; Ketley, 1997; Wooldridge and Ketley, 1997). Although not an obligate intracellular pathogen, the ability of a *C. jejuni* strain to invade host cells often correlates well with virulence *in vivo* (Everest *et al*., 1992; Bacon *et al*., 2000; 2001; Carvalho *et al*., 2001). Consistent with the interaction of *C. jejuni* with host cells playing an important role in pathogenesis, infection in humans and primates causes a marked inflammation of intestinal tissue and disruption of intestinal cellular architecture (Black *et al*., 1988; Russell *et al*., 1993). *C. jejuni* can also survive intracellularly for relatively long periods of time in both phagocytes and intestinal epithelial cells (Konkel *et al*., 1992a; Wassenaar *et al*., 1997; Day *et al*., 2000), causing some to classify it as a facultative intracellular pathogen (Konkel and Cieplak, 1992; Konkel *et al*., 1992c; Babakhani *et al*., 1993; Day *et al*., 2000). As with other facultative intracellular pathogens (Martin and Mohr, 2000), intracellular survival may enhance its ability to evade the host immune system, cause relapse of the acute infection, and establish long-term persistent infections (Lastovica, 1996; Day *et al*., 2000). Intracellular survival also poses certain challenges (i.e. a nutrient-poor environment) that the bacterium must overcome to thrive or even survive. Several *C. jejuni* factors have been shown to be important for adherence and/or invasion (i.e. Bacon *et al*., 2000; 2001; Monteville *et al*., 2003), and two genes, *katA* encoding catalase and *sodB* encoding superoxide dismutase, are also known to participate in intramacrophage survival (Pesci *et al*., 1994; Day *et al*., 2000). To date, however, no *C. jejuni* genes or factors involved in survival inside host epithelial cells have been identified.

Although *C. jejuni* causes severe diarrhoea in humans, it is a commensal in most other animal species. It is most commonly transmitted to humans by consumption of contaminated poultry, cross-contamination of other food matter with raw poultry, and other sources such as contaminated water and milk products (Skirrow and Blaser, 1992; McQuigge, 2000; Friedman *et al*., 2001; Newell and Fearnley, 2003). Yet, *C. jejuni* has very fastidious *in vitro* growth and survival requirements: it is both microaerophilic and capnophilic (requiring lower O_2 and higher CO_2 than the ambient atmosphere), modestly thermophilic (requiring 37°C– 42°C growth), and requires a rich growth medium (Skirrow, 1990). Despite these requirements, *C. jejuni* must possess mechanisms for surviving a range of environmental stresses, both inside and outside of its natural zoonotic hosts. In contrast to many other bacteria, *C. jejuni* does not become more stress-resistant at later growth stages and has been described as lacking a 'classic' stationary phase (Kelly *et al*., 2001). It also lacks the stationary phase sigma factor *rpoS* (Parkhill *et al*., 2000), which modulates stress and stationary phase responses in many Gram-negative bacteria (Ishihama, 2000; Venturi, 2003).

The stringent response is a global stress response that alters gene expression pathways to allow bacterial survival under a multitude of unfavourable conditions. The stringent response is typically activated by environmental stress such as nutrient deprivation, which results in an abundance of uncharged tRNA molecules at the ribosomal acceptor site. Because of the lack of aminoacylated tRNAs during protein synthesis, the ribosome stalls. This is thought to cause ribosomal-bound RelA (or SpoT; see below) to catalyse synthesis of guanosine pentaphosphate (pppGpp). pppGpp is then hydrolysed to guanosine tetra-phosphate (ppGpp), which in turn is thought to bind RNA polymerase and alter gene expression by affecting promoter specificity, transcription initiation and elongation (Cashel *et al*., 1996; Chatterji and Ojha, 2001).

Previously, most Gram-negative bacteria were thought to have two genes, *relA* and *spoT*, which together control the stringent response (Cashel *et al*., 1996; Mittenhuber, 2001). In bacteria such as *Escherichia coli*, *Salmonella* spp. and *Legionella pneumophila*, RelA synthesizes pppGpp, while its close homologue SpoT degrades (p)ppGpp. SpoT in these bacteria also has a limited capacity to synthesize pppGpp; consequently, a double Δ*relA*Δ*spoT* strain is required to achieve a (p)ppGpp^0 phenotype. Gram-positive bacteria, on the other hand, typically contain a single *relA*/*spoT* homologue, termed *relA*, *spoT*, *rel*, or *rsh* (for *rel-spo*-homologue), which, when inactivated, completely abrogates (p)ppGpp production (Mittenhuber, 2001). It was recently demonstrated that stringent control in the Gram-negative plant symbiont *Sinorhizobium meliloti* is also regulated by a single *relA*/*spoT* homologue, calling into question the paradigm of two genes modulating stringent control in all Gram-negative bacteria (Wells and Long, 2002).

In a number of diverse bacterial species, the stringent response has been shown to affect a variety of important functions. Most bacteria require the stringent response for stationary phase survival (Cashel *et al*., 1996). In certain bacteria, the stringent response is also involved in resistance to specific stresses; for instance, osmotolerance in *Listeria monocytogenes* (Okada *et al*., 2002) and anaerobiosis in *Mycobacterium tuberculosis* (Primm *et al*., 2000). The stringent response is also critical for intracellular replication and virulence of several intracellular

pathogens (i.e. *L. pneumophila*, *L. monocytogenes* and *M. tuberculosis*), host adaptation (*Borrelia burgdorferei*) and symbiosis (*S. meliloti*) (Hammer and Swanson, 1999; Primm *et al*., 2000; Godfrey *et al*., 2002; Taylor *et al*., 2002; Wells and Long, 2002; Dahl *et al*., 2003).

Considering the numerous stresses encountered by *C. jejuni* and other related bacteria, it seems surprising that stringent control has never been demonstrated for any of the epsilon proteobacteria, including all *Campylobacter*, *Helicobacter*, *Wolinella*, *Arcobacter*, *Flexispira* and *Sulfospirillum* species. In fact, it was previously reported that *H. pylori*, with which *C. jejuni* shares many genetic and phenotypic traits, lacks a stringent response (Scoarughi *et al*., 1999). However, both sequenced strains of *H. pylori* contain a *relA*/*spoT* homologue (Tomb *et al*., 1997; Alm *et al*., 1999), and in the aforementioned study, ppGpp production was not assayed under total nutrient starvation conditions. The *C. jejuni* genome likewise contains a gene, annotated as *spoT*, which exhibits strong homology to *relA* and *spoT* genes in other bacteria. Therefore, as with *S. meliloti*, if *C. jejuni* does mount a stringent response, its single *spoT* gene is likely to play a critical role in this process.

To identify bacterial genes that might be involved in the pathogen–host cell interaction, we performed a microarray-based screen that revealed a cluster of *C. jejuni* genes that were immediately and strongly upregulated during infection of a human epithelial tissue culture cell line. Many of these genes are contained on a plasmid (pVIR) found in a subset of highly invasive and virulent *C. jejuni* strains; several of the upregulated genes encode putative type IV secretion machinery that have been shown experimentally to be important for invasion and virulence (Bacon *et al*., 2000; 2002). Several chromosomal genes, including *spoT*, were also found in this cluster. To investigate the biological relevance of this observation and to explore the role of the stringent response in *C. jejuni*, Δ*spoT* deletion and reconstituted *spoT* wild-type strains were generated, and extensive phenotype and gene expression analyses were undertaken. These studies revealed that *C. jejuni* mounts a *spoT*-dependent stringent response, the first demonstration of such for any epsilon-proteobacterium, and that this phenomenon plays several important roles in this pathogen. Some of these, such as an involvement in capnophilic growth and aerotolerance, are not ubiquitous to all bacteria and are, to date, unique to *C. jejuni*. Others, such as stationary phase survival, are interesting in the context of understanding how *C. jejuni* survives certain stresses in the absence of *rpoS*. Additional stringent response functions in rifampicin resistance and intraepithelial cell survival represent novel observations for *C. jejuni*. Finally, our findings are consistent with the goal of the initial screen, in that we have identified a new factor involved in the pathogen–host cell interaction that is also the first *C. jejuni* gene shown to be important for survival inside epithelial cells.

Results

The Campylobacter jejuni spoT *gene and several pVIR subclones are upregulated during infection of human epithelial tissue culture cells*

A *C. jejuni* DNA microarray was used to screen for genes exhibiting expression changes during bacterial infection of a human epithelial tissue culture cell line (INT407) commonly used in studies of *C. jejuni* adherence and invasion [e.g. (Konkel and Cieplak, 1992; Bacon *et al*., 2000; Guerry *et al*., 2002)]. The invasive *C. jejuni* strain 81-176 was allowed to infect semiconfluent monloayers of INT407 cells that were either alive ('live') or, as a control, that had been fixed with paraformaldehyde ('fixed') immediately prior to infection. At 30 min, 2 h, 6 h, and 10 h post infection, bacteria were harvested from both the 'media fraction', to collect bacteria swimming above the cells, and the 'cell-associated fraction', to collect bacteria that had adhered to and/or invaded the cells. In separate experiments, we have found that after 30 min of infection, ~5–10% of the bacteria are cell-associated, and after 2–3 h, ~10–15% are cell-associated. These numbers increase marginally over time, with total bacterial viability remaining constant out to 10 h (data not shown). RNA was isolated from each sample and processed for microarray analyses. As a control, we also performed quadruplicate hybridizations with RNA from live, mock-infected INT407 cells treated identically to infected cell samples (see *Experimental procedures*). Data analyses were performed to identify genes whose expression was statistically different between the 'live' and 'fixed' cell infections; among these, we further identified genes with similar expression patterns that differed between the two conditions. These experiments were performed twice with similar results; for simplicity, data from only one biological replicate are shown.

This screen revealed a cluster of genes that were strongly and rapidly upregulated in both the media and cell-associated populations of *C. jejuni* infecting live versus fixed INT407 cells (Fig. 1). Interestingly, this cluster contained multiple spot replicates of three subclones from the pVIR plasmid found in certain highly invasive *C. jejuni* strains, including 81-176. Each of these subclones harbours genes that have been shown to be important for invasion of host cells, and several of these genes encode putative type IV secretion machinery proteins (Bacon *et al*., 2000; 2002). Coexpressed with these pVIR subclones were several uncharacterized chromosomal *C. jejuni* genes, including both array spots corresponding to Cj1272c.

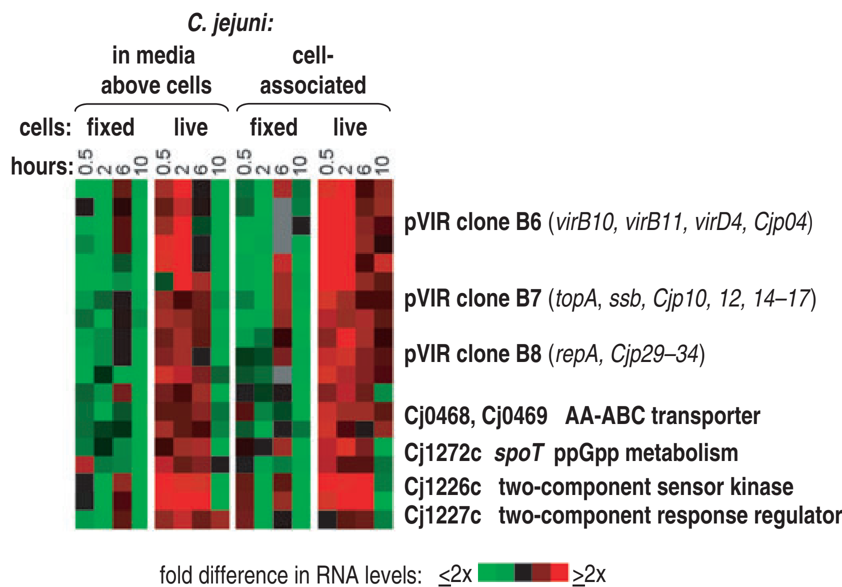


Fig. 1. An invasive *C. jejuni* strain (81-176) was used to infect semiconfluent INT407 monolayers that were alive ('live') or that had been fixed with paraformaldehyde ('fixed') prior to infection. At 0.5, 2, 6 and 10 h post infection, bacteria that were swimming in the media above the cells ('in media above cells') as well as adhered/invaded bacteria ('cell-associated') were harvested directly into stop solution to halt transcription and RNA degradation. RNA was prepared and hybridized to the *C. jejuni* microarray as described in *Experimental procedures*. Cluster, ORF order and SAM analyses were used to identify genes with significant expression differences between bacteria infecting live versus fixed cells. Shown is a cluster of genes with similar profiles that exhibited immediate upregulation in the presence of live versus fixed cells. Each horizontal row corresponds to a unique spot on the array. pVIR clones were spotted multiple times (six spots are shown for clone B6, three for clone B7, and three for clone B8). The chromosomal genes shown are represented by one or two spots each (both spots for *spoT* are shown). Genes are listed once for simplicity.

C. jejuni spoT *gene characteristics and generation of non-polar deletion (*ΔspoT*) and reconstituted wild-type (*spoT**) strains*

Cj1272c exhibits high sequence homology to *relA*/*spoT* genes from other bacteria (Parkhill *et al*., 2000) and was annotated as *spoT* because of its slightly higher similarity to *E. coli spoT* than to *E. coli relA*. When compared with other sequenced bacterial genomes, the predicted *C. jejuni* SpoT protein sequence is most similar to putative RelA/SpoT proteins found in *Wolinella succinogenes* (49%), *Helicobacter hepaticus* (45% identity), and *H. pylori* (40% identity in both sequenced strains). *C. jejuni* SpoT also exhibits >33% predicted amino acid identity to RelA/SpoT proteins from several fastidious Gram-negative bacteria (e.g. *Thermoanaerobacter tengcongensis*, *Aquifex aeolicus* and *Nitrosomonas europaea*) as well as several Gram-positive bacteria (e.g. *Streptococcus mutans*, *Streptococcus pneumoniae* and *Enterococcus faecalis*). According to genome sequence data, *C. jejuni* contains only one *relA*/*spoT* homologue; thus, if *C. jejuni* has a stringent response, it seemed likely that *spoT* might encode a dual-function enzyme responsible for both (p)ppGpp synthesis and hydrolysis.

To explore the roles of *spoT* and the stringent response in *C. jejuni*, we disrupted this gene in strain 81-176. *spoT* is located in a region of the *C. jejuni* genome (Fig. 2A) in which there are fewer than 12 bp between each gene, and many of these genes, such as *tyrS* (a putative tyrosyl t-RNA synthetase), Cj1270c, *amiA* (a putative N-acetylmuramoyl-L-alanine amidase) and Cj1268c, have overlapping predicted open reading frames (ORFs). Functional database searches suggested that one or more of the genes immediately downstream of *spoT* might be essential, and array data suggest that *spoT* and at least one downstream gene may be co-regulated (Fig. S1). Thus, we used a cassette containing a kanamycin resistance (kan^R) gene with the promoter and terminator regions deleted but which has ribosomal binding sites (rbs) up- and downstream of the kan^R coding region (Menard *et al*., 1993) to create a *spoT* deletion/disruption construct in which ~60% of the *spoT* gene was replaced with this 'non-polar' kan^R cassette (Fig. 2A). This kan^R cassette has been successfully used to generate non-polar gene deletions in several other bacteria (Trieu-Cuot *et al*., 1985; Menard *et al*., 1993; Allaoui *et al*., 1995; Skouloubris *et al*., 1998). Following natural transformation of 81–176 with the *spoT*::kan^R construct, many stable kan^R clones were isolated. Reverse transcription polymerase chain reaction (RT-PCR) (Fig. 2B) and microarray studies (Fig. S2) of one representative clone, henceforth named Δ*spoT*, demonstrated that the genes both up- and downstream of *spoT* were transcribed normally, indicating that the *spoT*::kan^R disruption is non-polar, and Southern analyses (Fig. 2C) demonstrated that there was a single kan^R insert in the *C. jejuni* chromosome, at the appropriate *spoT* locus.

To further confirm that the phenotypes observed for the Δ*spoT* mutant were attributed specifically to *spoT*, we also generated a reconstituted wild-type strain (*spoT**; see below). Prior to this, we attempted to generate an in frame *spoT* deletion using an established CAT-*rpsL* system (Hendrixson *et al*., 2001). However, an intermediate strain harbouring the CAT-*rpsL* cassette at the *spoT* locus was never achieved. This suggested that one or more of the genes downstream of *spoT* is essential, as the CAT-*rpsL*

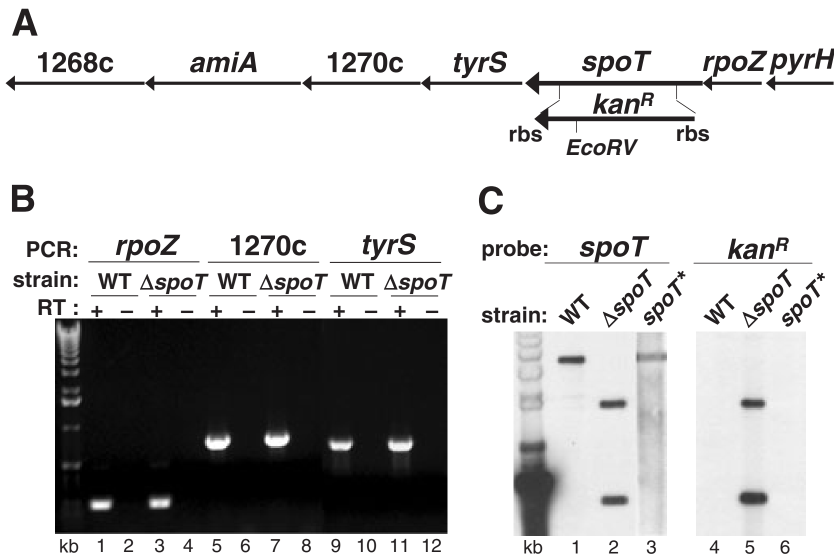


Fig. 2. Generation of a single insert, non-polar Δ*spoT* disruption strain (*spoT*::*kan*^{R}) and a reconstituted wild-type strain, *spoT** ([*spoT*::*kan*^{R}]::*spoT*).
A. Genomic location of *spoT*, putative operon organization, and site of insertion of a non-polar *kan*^{R} cassette containing ribosomal binding sites (rbs) at each end. All genetic manipulations were performed using the *C. jejuni* strain 81-176 background.
B. Reverse transcription, + (odd numbered lanes) or – (even numbered lanes) RT enzyme, was performed on WT and Δ*spoT* RNA followed by PCR amplification of up- and downstream genes.
C. Southern analyses were performed on WT, Δ*spoT* and *spoT** genomic DNA digested with *EcoRV* and probed with *spoT* or the *kan*^{R} cassette.

cassette is known to have polar effects (D. Hendrixson, pers. comm.). Plasmid-based complementation was also attempted using a conjugative plasmid [pRY112: (Yao *et al*., 1993)] and several different promoter-*spoT* constructs (see *Experimental procedures*). None of the plasmid-borne constructs yielded functional complementation, suggesting either that no construct generated a functional *spoT* transcript or, as copy number for pRY112 is not known, and consistent with observations in *E. coli* (Schreiber *et al*., 1991), that dose dependence for functional SpoT protein is important in *C. jejuni*. Therefore, we generated a reconstituted *spoT* wild-type strain by transforming the Δ*spoT* strain with a wild-type copy of *spoT*. Isolates in which the wild-type *spoT* gene replaced the *spoT*::*kan*^{R} construct via double cross-over homologous recombination at the *spoT* locus were selected by healthy 2 day single colony growth in a 5% CO_2 incubator (see Fig. 4 and *Experimental procedures*). The resultant strain, designated *spoT**, restored wild-type Southern blot profiles using either *spoT* or the *kan*^{R} cassette as a probe (Fig. 2C) and was used for further experiments described below.

Nutrient deprivation induces spoT-*dependent (p)ppGpp synthesis in* C. jejuni

The stringent response and (p)ppGpp production have not previously been described for *C. jejuni*. In fact, apart from one study, which concluded that *H. pylori* lacked the stringent response (Scoarughi *et al*., 1999), this phenomenon has not been described for any of the epsilon-proteobacteria. Nutrient deprivation has been shown to induce (p)ppGpp accumulation in *E. coli* and other bacteria (Cashel *et al*., 1996; Hammer and Swanson, 1999; Wells and Long, 2002). To test this in *C. jejuni*, early exponential phase cultures were shifted from rich [Mueller–Hinton (MH)] to minimal [MOPS-MGS; (Mendrygal and Gonzalez, 2000)] medium and labelled with ^{32}P. After 45 min, nucleotides were extracted and resolved by thin layer chromatography (TLC). Wild-type (WT) *C. jejuni* accumulated large amounts of both ppGpp and pppGpp following nutrient downshift (Fig. 3, lane 1). These nucleotides were absent from the Δ*spoT* mutant (Fig. 3, lane 2), indicating that it has a (p)ppGpp^0 phenotype. Both ppGpp and pppGpp were readily apparent in *spoT** (Fig. 3, lane 3), signifying restoration of WT *spoT* function. As a control, we also labelled WT *C. jejuni* in rich media and did not observe (p)ppGpp accumulation (Fig. 3, lane 4), demonstrating that (p)ppGpp production in WT bacteria occurred as a result of nutrient deprivation.

The C. jejuni ΔspoT *mutant is defective for growth and survival under low CO_2/high O_2 conditions; a spontaneous suppressor mutation rescues some of these defects*

In other bacteria, *relA*/*spoT* mutants, particularly those with a (p)ppGpp^0 phenotype, are defective for surviving a variety of stressful conditions (Cashel *et al*., 1996; Chatterji and Ojha, 2001; Godfrey *et al*., 2002). Initial tests of the *C. jejuni* Δ*spoT* mutant based on known and predicted environmental stresses revealed that the *C. jejuni* Δ*spoT* mutant was indistinguishable from WT for a variety of phenotypes (see supplementary material, Appendix S1, Table S1).

C. jejuni is naturally both microaerophilic and capnophilic, requiring lower O_2 and higher CO_2 concentrations than the ambient atmosphere. Ideal growth has been widely reported to occur at 5–10% O_2 and 5–15% CO_2, consistent with the 6% O_2, 12% CO_2 atmosphere generated by the Oxoid CampyGen system. However, *C. jejuni*

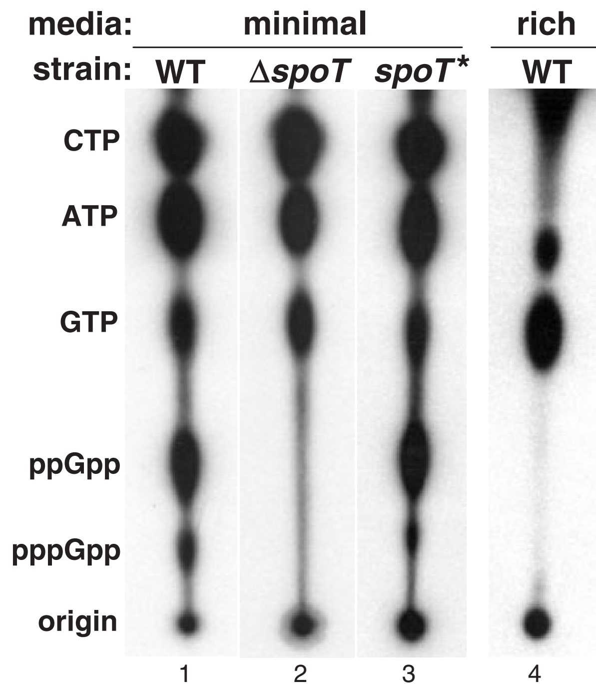


Fig. 3. The Δ*spoT* mutant fails to produce (p)ppGpp upon nutrient downshift. Lanes 1–3: strains were grown microaerobically in rich (MH) broth to early log phase (~0.3 OD_{600} ml^{-1}) then labelled in nutrient-poor minimal (MOPS-MGS) medium with ^{32}Pi for 45 min. Nucleotides were resolved by TLC. WT and reconstituted WT (*spoT**) strains (lanes 1, 3) generated high levels of (p)ppGpp, while the Δ*spoT* mutant was defective for (p)ppGpp production (lane 2). The WT strain labelled in rich media did not produce (p)ppGpp (lane 4).

can also grow fairly well in humidified CO_2 incubators ranging from 5% to 14% CO_2. Because most of the closest *C. jejuni spoT* homologues occur in fastidious microaerobic, anaerobic, and/or capnophilic bacteria, we next decided to test the Δ*spoT* mutant for growth in 5% and 14% CO_2 incubators.

All strains exhibited healthy growth after 2 days in a 14% CO_2 incubator (Fig. 4D). Likewise, in a 5% CO_2 incubator, the WT and *spoT** (reconstituted WT) strains grew well, with patches of bacteria and single colonies clearly visible after 2 days. In contrast, little to no growth was observed for the Δ*spoT* mutant after 2 days at 5% CO_2 (Fig. 4A). Prolonged incubation of the plates in a 5% CO_2 incubator resulted in the appearance of spontaneous suppressor mutations after 4 days (Fig. 4B); by 6 days, these colonies were large, robust and readily isolated (Fig. 4C, arrow). One representative suppressor, Δ*spoT* suppressor A (SSA), isolated from a previous set of plates, completely rescued the 5% CO_2 growth defect (Fig. 4A–C). When the SSA strain was subjected to nutrient downshift and labelled with ^{32}P as described above, no (p)ppGpp spots were observed (Fig. 4E), indicating that the suppressor mutation does not restore WT SpoT activity and is thus likely extragenic to *spoT*.

As a specific test for aerotolerance, liquid cultures were grown under CampyGen conditions to early exponential phase (OD_{600} ~0.1) and then removed to a 37°C air incubator and shaken upright at a 6:1 surface : volume ratio at 100 r.p.m. (moderate aeration; Fig. 4F) or put into a tube at a 30:1 surface : volume ratio and incubated in a roller drum spinning at top speed, ~56 r.p.m. (vigorous aeration; Fig. 4G). The Δ*spoT* mutant exhibited a clear defect in survival under atmospheric conditions, with vigorous aeration exacerbating the phenotype. The defect was partially suppressed in SSA during moderate but not vigorous aeration, while the reconstituted WT strain *spoT** was nearly identical to WT.

*The Δ*spoT *mutant is impaired for rifampicin resistance*

Many lines of evidence support a close relationship between ppGpp, rifampicin and RNA polymerase (RNAP). Both ppGpp and rifampicin act by binding RNAP (Zillig *et al.*, 1970; Reddy *et al.*, 1995; Toulokhonov *et al.*, 2001), and *E. coli* ppGpp^0 suppressor mutations have been identified in genes encoding RNAP subunits. Several of those in *rpoB* also confer rifampicin resistance (Little *et al.*, 1983; Hernandez and Cashel, 1995; Murphy and Cashel, 2003). Unlike most *E. coli* strains, nearly every environmental isolate of *C. jejuni* found to date is resistant to high concentrations of rifampicin. We therefore decided to test the Δ*spoT*, *spoT** and SSA strains for rifampicin sensitivity/resistance. Interestingly, both Δ*spoT* and SSA were significantly attenuated for rifampicin resistance compared with WT and *spoT** at all concentrations tested (Table 1). The most dramatic differences were observed between 100 and 200 μg ml^{-1} rifampicin, where WT and *spoT** grew essentially normally, while Δ*spoT* and SSA

Table 1. The Δ*spoT* mutant is significantly impaired for rifampicin resistance.

[Rif] (μg ml^{-1})	WT and *spoT** day 1	WT and *spoT** day 2	WT and *spoT** day 4	Δ*spoT* and SSA day 1	Δ*spoT* and SSA day 2	Δ*spoT* and SSA day 4
0	++	++	++	++	++	++
50	++	++	++	+/– –	+	+
100	++	++	++	– –	– –	+/– –
150	++	++	++	– –	– –	– –
200	+	++	++	– –	– –	– –
250	– –	+/– –	+/–	– –	– –	– –

Strains from fresh overnight plates were streaked onto MH plates containing various concentrations of rifampicin. Plates were incubated using the CampyGen system at 37°C for the indicated number of days, at which time growth was assayed described below. WT is wild-type, *spoT** is the reconstituted WT strain, Δ*spoT* is the Δ*spoT* deletion mutant, and SSA is the Δ*spoT* suppressor described in Fig. 4.
++, normal growth; +, slow growth, small single colonies; +/–, growth in thick part of streak only; +/– –,very marginal growth, only in thickest part of streak; – –, no growth.

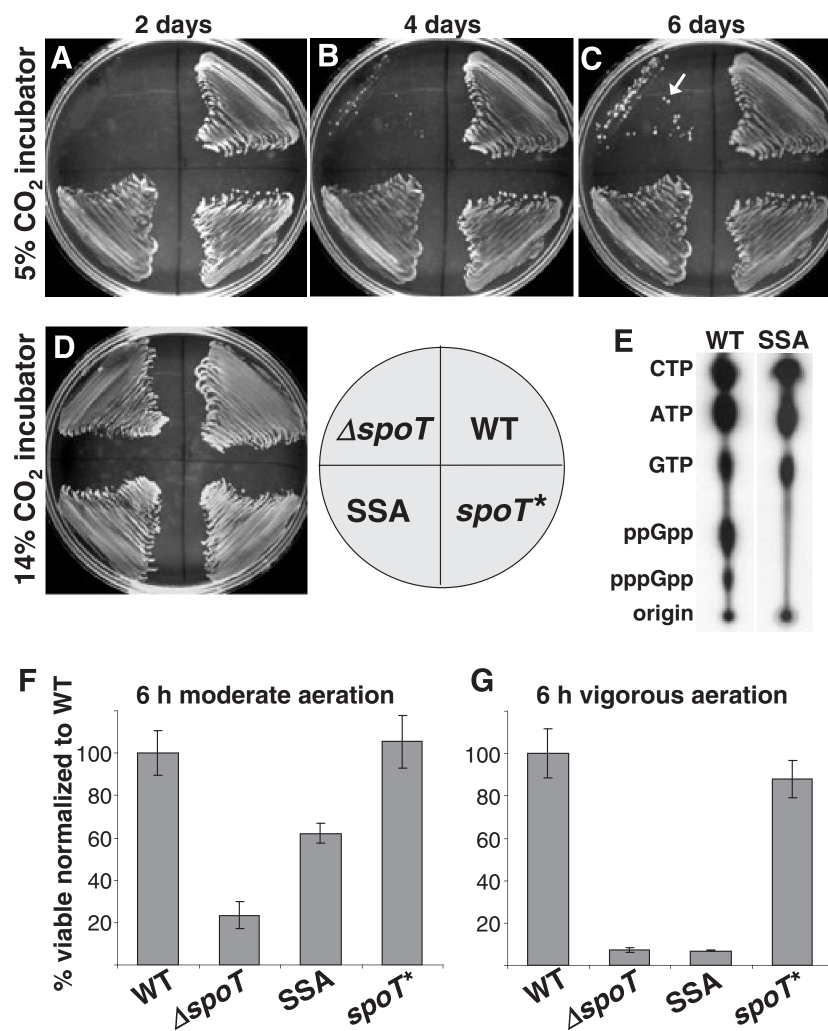


Fig. 4. The Δ*spoT* mutant is defective for plate growth under lower CO_2 conditions than ideal, and for survival during aeration under atmospheric conditions.
A–D. Strains [(plate key is shown next to D): WT is wild-type, Δ*spoT* is the Δ*spoT* mutant, *spoT** is the reconstituted WT strain, and SSA is a suppressor strain described below)] were streaked onto MH plates and incubated in a 5% CO_2 (A–C) or 14% CO_2 (D) incubator for 2 (A, D), 4 (B) and 6 (C) days. After 6 days, spontaneous suppressor mutants (i.e. Δ*spoT* suppressor A, 'SSA') rescuing the 5% CO_2 incubator plate growth defect were isolated (C, arrow).
E. WT and SSA strains were grown microaerobically in rich (MH) broth to early log phase then labeled in nutrient-poor minimal media (MOPS-MGS) with ^{32}P for 45 min. Nucleotides were resolved by TLC.
F and G. Early log cultures (~0.1 OD_{600} ml^{-1}) were removed from microaerobic growth conditions and inoculated in MH broth either into a flask at a 6:1 surface : volume ratio and shaken upright in a 37°C air incubator at 100 r.p.m. (F) or into a tube at a 30:1 surface : volume ratio and inserted sideways into the outermost slots of a roller drum spinning at top speed (~56 r.p.m) in a 37°C air incubator (G). cfu ml^{-1} were assayed after 6 h. WT survival was ≥100% for each condition; final viable cfu ml^{-1} of the other strains relative to their inputs were normalized to WT.

exhibited virtually no growth. Each strain also exhibited a partially adaptive response to rifampicin at sublethal concentrations over time, possibly indicative of additional suppressor mutations occurring under selective pressure or conformational adaptation of RNAP to a $ppGpp^0$ environment.

The C. jejuni ΔspoT *mutant has a stationary phase defect*

Work performed primarily in *E. coli* has shown that as bacteria enter stationary phase, the stringent response is invoked to promote survival under nutrient-poor conditions (Cashel *et al.*, 1996). In nearly all Gram-negative bacteria for which this phenomenon has been described, stationary-phase survival also involves the alternative sigma factor S (*rpoS*), which regulates genes involved in survival under stressful conditions and which itself can be regulated by ppGpp (Cashel *et al.*, 1996; Venturi, 2003). *C. jejuni* does not contain *rpoS* and has been described as lacking a 'classical' stationary phase (Kelly *et al.*, 2001), although the bacteria do undergo phenotypic changes as cultures age (Leach *et al.*, 1997).

To investigate the importance of the stringent response in *C. jejuni* growth and stationary phase survival, WT, Δ*spoT*, *spoT** and SSA cultures were grown using the CampyGen system in MH broth shaking at 200 r.p.m. to early log phase (OD_{600} ~0.2–0.3), back-diluted to OD_{600} = 0.1 and monitored for OD_{600}, cfu ml^{-1}, morphology, and motility. OD_{600} (Fig. 5A) and cfu ml^{-1} (Fig. 5B) trends were relatively similar for all of the strains through the 2, 6, and 10 h timepoints, although after 10 h, Δ*spoT* and SSA often yielded slightly (~0.1–0.2 units) higher OD_{600} values than WT and *spoT** (Fig. 5A). By 24 h, OD_{600} readings for Δ*spoT* and SSA strains were consistently 0.3–0.5 units higher than WT and *spoT** (Fig. 5A). However, cfu ml^{-1} enumeration at 24 h revealed an 8- to 12-fold decrease in viability for Δ*spoT* and SSA compared with WT and *spoT** (Fig. 5B). Light microscopy indicated that all strains exhibited rapid, darting motility at the 2 and 6 h timepoints (data not shown). At the 10 and 24 h time-

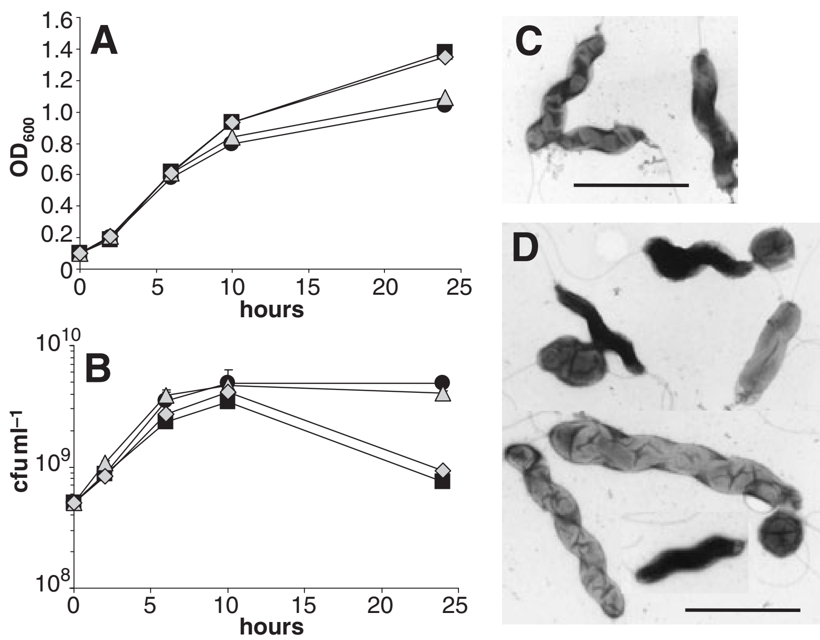


Fig. 5. The Δ*spoT* mutant is defective for stationary phase survival. Strains were grown in shaking (200 r.p.m) liquid culture microaerobically overnight to early log phase (~0.3 OD_{600} ml^{-1}) then back-diluted to 0.1 OD_{600} ml^{-1}. At 2, 6, 10 and 24 h after back-dilution, samples were harvested for OD_{600} readings (A) and colony-forming units (cfu) ml^{-1} (B). cfu ml^{-1} samples were also taken at t_0 and were plated in triplicate for all timepoints; error bars are very small and thus often obscured by the symbols. Symbols for strains: WT (wild-type) = black circles; *spoT** (reconstituted WT strain) = grey triangles; Δ*spoT* = black squares; SSA (Δ*spoT* suppressor A) = grey diamonds. At the 24 h timepoint, WT (C) and Δ*spoT* (D) strains were also harvested and prepared for negative staining transmission electron microscopy. *spoT** was identical to WT; SSA was identical to Δ*spoT* (not shown). The bar represents 5 μm.

points, WT and *spoT** still swam normally; however, Δ*spoT* and SSA were noticeably slower at 10 h, and by 24 h most of the bacteria were non-motile. Electron microscope analyses indicated that all strains exhibited a similar morphology through the 10 h timepoint (data not shown). At 24 h, WT (Fig. 5C) and *spoT** (data not shown) still appeared normal, with clear spiral morphology and typical staining density. However, Δ*spoT* (Fig. 5D) and SSA (data not shown) at 24 h exhibited several aberrant morphologies: some bacteria adopted a coccoid shape, others displayed markedly increased electron density and, most unusually, some appeared significantly enlarged and misshapen.

Transcriptional profiling reveals expression differences in several genes and operons in ΔspoT *and SSA versus WT; only two genes differ significantly in SSA versus* ΔspoT

We next employed the *C. jejuni* DNA microarray to investigate global gene expression changes incurred as a result of deleting *spoT* in *C. jejuni* and to explore possible gene expression differences between Δ*spoT* and the SSA suppressor. Strains were cultured as described in Fig. 5, and samples were collected for RNA at the 2, 6, 10, and 24 h timepoints. Data analyses were used to identify genes and putative operons (adjacent, likely co-regulated ORFs) that were statistically different between the strains and that, within a strain, exhibited similar gene expression patterns (see *Experimental procedures*). Whole-genome analyses indicated that some growth stage-specific genes exhibited identical profiles for all strains (i.e. most ribosomal genes, which were expressed much more highly at 2 h than any other timepoint), while others (i.e. *peb1*, *pebC* and several flagellar genes) differed somewhat in their expression levels between the strains. However, these genes either differed only at one timepoint or did not exhibit a high enough overall signal difference to be extracted using the criteria applied. Separate experiments also indicated that WT and *spoT** (reconstituted WT) strains exhibited nearly identical expression profiles over the entire genome (data not shown). These experiments were performed twice with nearly identical results; for simplicity, only one biological replicate is shown. Genes are presented in ORF order format to highlight the consistent operon expression profiles. Array data are shown in Fig. 6 in Red/Green format; exact numbers for these spots are in Table S2, with three representative genes also depicted graphically in Fig. S3.

Interestingly, we found that several pVIR plasmid clones were expressed at significantly lower levels in Δ*spoT* and SSA compared with WT over the entire timecourse (Fig. 6A). These clones harbour genes encoding several putative type IV secretion system components (*virB10*, *virB11*, *virD4*), a DNA topoisomerase (*topA*) that is most homologous to *topA* found within a *H. pylori* plasticity zone, and several uncharacterized *C. jejuni*-specific genes (i.e. *cjp15*) that, as with *virB10* and *virB11*, have been experimentally shown to be important for virulence and epithelial cell invasion (Bacon *et al.*, 2000; 2002).

Several operons likely to be involved in redox balance, metabolism, and energy production and conversion were also expressed at significantly lower levels in Δ*spoT* and SSA compared with WT at all timepoints assayed (Fig. 6A). The Cj0073c operon contains a putative unchar-

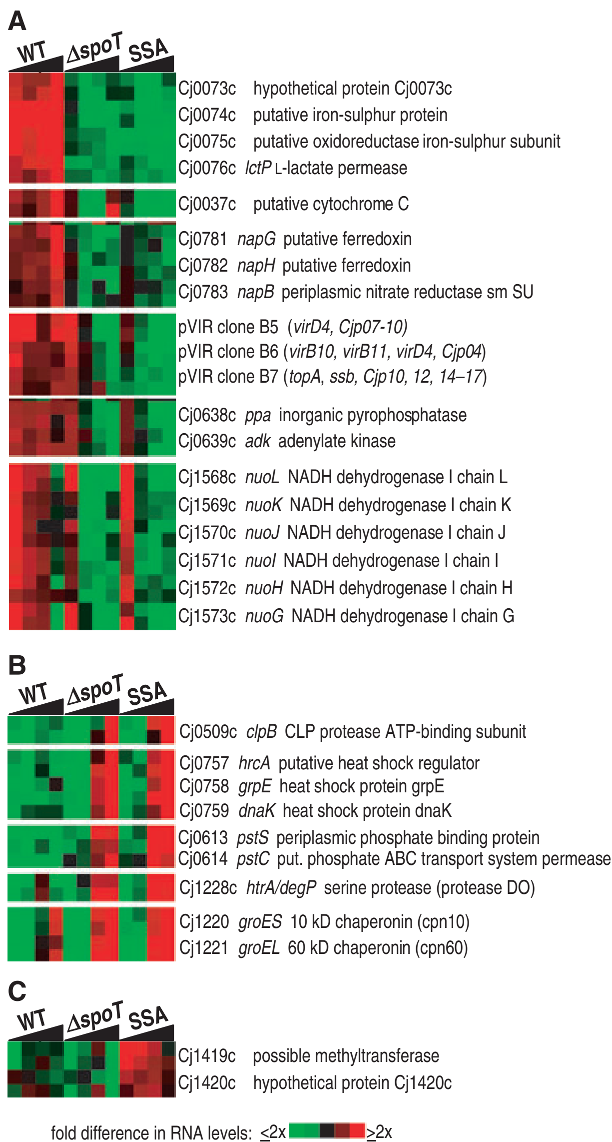


Fig. 6. Multiple operons exhibit gene expression differences between WT (wild-type) and Δ*spoT*; only two genes differ significantly between Δ*spoT* and SSA (Δ*spoT* suppressor A). Strains were grown as described for Fig. 5 and samples collected for RNA microarray analysis 2, 6, 10, and 24 h after back-dilution to $OD_{600} = 0.1$. Time is represented by the angled black triangle above the expression profiles. Cluster, ORF order, and two independent SAM analyses were performed to identify genes (A) with significantly higher expression in WT than Δ*spoT* (B) with significantly higher expression in Δ*spoT* than WT, and (C) with significant expression differences between Δ*spoT* and SSA. Separate experiments indicated that WT and *spoT** (reconstituted WT) strains exhibited nearly identical expression profiles over the entire genome (not shown). Genes are shown in ORF order, highlighting consistent operon expression profiles. Each horizontal row corresponds to a unique spot on the array. Where possible (in most cases), both spots per gene are shown, although gene names are listed once for simplicity.

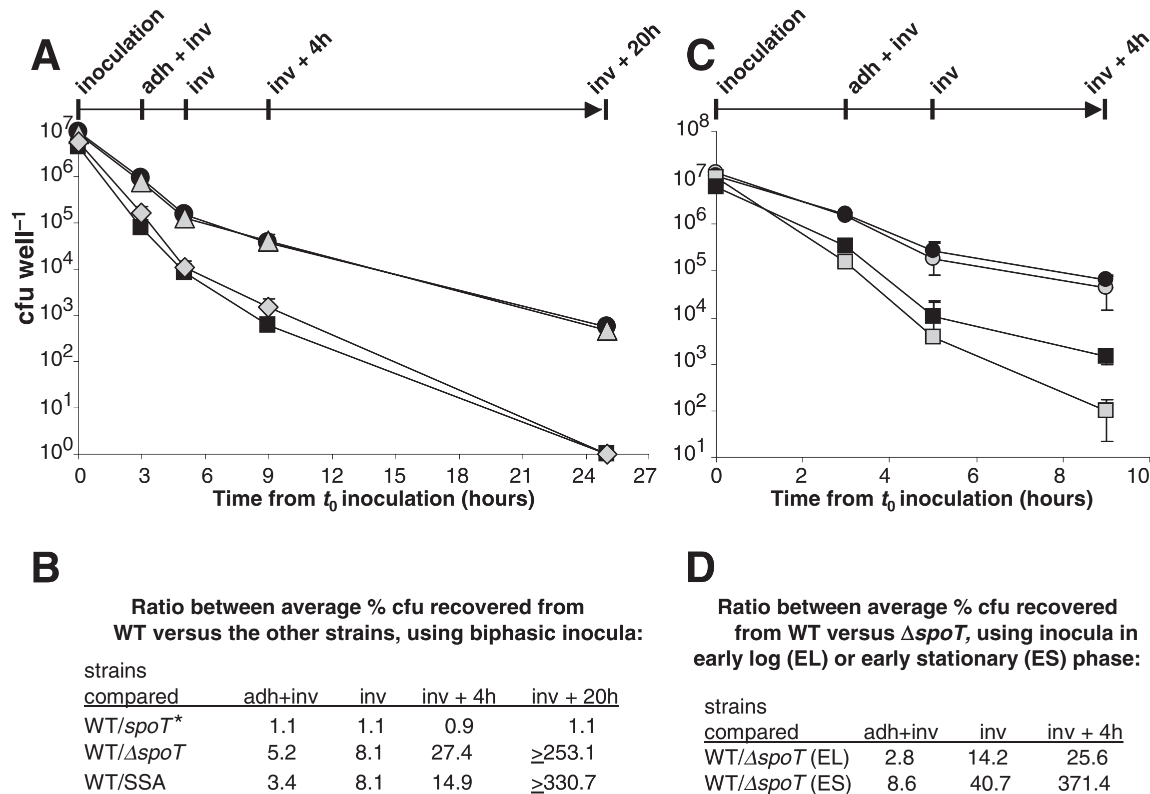
acterized iron-sulphur protein (Cj0074c) with motifs also found in proteins like fumarate reductase B, a likely cognate iron-sulphur oxidoreductase (Cj0075c), and a hypothetical protein (Cj0073c) harbouring a ferredoxin-like domain. The *nap* operon enodes a likely periplasmic nitrate reductase complex, and the *nuo* operon encodes the only NADH dehydrogenase (ndh-1) complex in *C. jejuni* and is likely to be an essential part of the aerobic respiratory chain. Cj0037c encodes an uncharacterized cytochrome C whose expression was recently found to be significantly higher in a laboratory strain adapted for survival in higher O_2 conditions compared with its parent clinical isolate (Gaynor *et al.*, 2004). Lastly, this group of genes includes a two-gene operon encoding a likely inorganic pyrophosphatase (*ppa*), which hydrolyses pyrophosphate to inorganic phosphate (PPi→Pi), and a likely adenylate kinase (*adk*), which interconverts AMP and ATP with two molecules of ADP ($AMP + ATP \leftrightarrows 2ADP$).

In contrast, several genes and operons were markedly upregulated at later timepoints in the Δ*spoT* and SSA strains compared with WT (Fig. 6B). Most of the affected genes encode proteins involved with protein folding, degradation, and repair and have been characterized primarily as heat-shock genes. GroES, GroEL and DnaK are chaperones that bind misfolded or aggregated proteins, HtrA/DegP is a multifunctional chaperone/protease, ClpB is involved in both degradation and refolding of damaged proteins, GrpE is a nucleotide exchange factor for DnaK, and HrcA regulates both the DnaK and GroESL chaperone systems. The *pst* operon was also upregulated in Δ*spoT* and SSA compared with WT at the later timepoints; these genes have been characterized as mediating high-affinity phosphate (Pi) transport in other bacteria (Wanner, 1993) but have not yet been studied in *C. jejuni*.

Finally, only two genes were identified as significantly different in SSA compared with Δ*spoT* or WT strains (Fig. 6C), exhibiting overall higher expression in SSA than the other two strains. These genes (Cj1419c/Cj1420c) fall into a region of the genome known to be involved in capsule biosynthesis (Parkhill *et al.*, 2000; St Michael *et al.*, 2002), but the precise function of these genes is not known. Cj1419c contains a domain conserved among several ubiquinone/menaquinone (coenzyme metabolism) methyltransferase proteins; Cj1420c is partially homologous to Cj1419c but contains no homologies to other known genes.

The ΔspoT *mutant is defective for adherence, invasion and intracellular survival*

As noted, the ability of *C. jejuni* to invade intestinal epithelial cells *in vitro* correlates well with virulence, and intracellular survival may likewise contribute both to viru-



strains compared	adh+inv	inv	inv + 4h	inv + 20h
WT/*spoT**	1.1	1.1	0.9	1.1
WT/Δ*spoT*	5.2	8.1	27.4	≥253.1
WT/SSA	3.4	8.1	14.9	≥330.7

strains compared	adh+inv	inv	inv + 4h
WT/Δ*spoT* (EL)	2.8	14.2	25.6
WT/Δ*spoT* (ES)	8.6	40.7	371.4

Fig. 7. The Δ*spoT* mutant is defective for adherence, invasion and intracellular survival; the intracellular survival defect is more pronounced when inocula are in early stationary phase.
A. WT (black circles), *spoT** (reconstituted WT, grey triangles), Δ*spoT* (black squares) and SSA (Δ*spoT* suppressor A, grey diamonds) strains were grown overnight in MH biphasic broth to ~0.3 OD_{600}. At t_0, semiconfluent monolayers of INT407 cells were inoculated with bacteria at an MOI ~100. After 3 h, the cells were washed, intracellular + adherent ('adh + inv') bacteria were harvested from some wells, and gentamicin (gent) was added to the remaining wells at 150 μg ml^{-1} to kill extracellular bacteria. After another 2 h, the gent was washed, intracellular bacteria were harvested for invasion ('inv') counts from some wells, and fresh media containing 1% FBS and 5 μg ml^{-1} gent was added to the remaining wells. After another 4 ('inv + 4h') or 20 ('inv + 20h') h, cells were washed, and surviving intracellular bacteria were harvested. The experiment was performed in triplicate, and cfu well^{-1} recovered are shown at each timepoint with standard error bars also shown.
B. Percentage recovered relative to input was calculated for each strain at each timepoint from A. Numbers shown are the average fold difference, or ratio, between WT and each of the other three strains. For instance, 'WT/Δ*spoT*' denotes the average percentage recovered for WT divided by the average percentage recovered for Δ*spoT*.
C. The experiment was performed as in A except that WT (circles) and Δ*spoT* (squares) were grown in shaking liquid culture to early log (EL; black) or early stationary (ES; grey) phase prior to infecting cells. The ES cultures were collected prior to the decrease in viability illustrated in Fig. 5. In C, cfu well^{-1} recovered are shown with standard error bars.
D. Percentage recovered relative to input was calculated for WT and Δ*spoT* at each timepoint from C. The numbers shown represent the average fold difference, or ratio, between WT and Δ*spoT* inoculated at early log (EL) or early stationary (ES) phase.

lence and cell damage as well as to persistence and immune evasion. To test the role of *spoT* and (p)ppGpp in these events, biphasic overnight cultures of WT, Δ*spoT*, *spoT** and SSA strains were used to infect semiconfluent INT407 monolayers at a multiplicity of infection (MOI) ~100. After 3 h, adhered + invaded bacteria (adh + inv) were collected from some of the wells. Gentamicin (150 μg ml^{-1}) was added to the other wells to kill extracellular bacteria. After 2 h of gentamicin treatment, the cells were washed, and invaded (inv) bacteria were collected from some wells. Fresh media containing 1% FBS and 5 μg ml^{-1} gentamicin (to suppress growth of bacteria from lysed cells) was added to the other wells. Surviving intracellular bacteria were collected 4 (inv + 4) and 20 (inv + 20) h later. Figure 7A shows the total cfu well^{-1} recovered; error bars from triplicate samples are shown but are in most cases too small to see. The numbers shown in Fig. 7B were calculated by dividing the average percentage cfu recovered for wild-type bacteria by the average percentage cfu recovered for each of the other three strains. It should be noted that (i) each strain survived equally well in the media on top of the cells during the 3 h invasion period and (ii) each strain exhibited similar gentamicin kill curves (data not shown).

As Fig. 7A and B illustrate, the WT and *spoT** (reconstituted WT) strains exhibited nearly indistinguishable

adherence, invasion, and intracellular survival profiles. In contrast, the Δ*spoT* mutant was defective for all events assayed, exhibiting a fivefold difference from WT in adherence + invasion prior to gentamicin addition, an eightfold difference in total invaded bacteria, a 27-fold difference in intracellular bacteria after an additional 4 h, and a >253-fold difference in 20 h intracellular surviving bacteria. The SSA strain was similar to Δ*spoT* but appeared slightly better able to survive intracellularly for short periods of time. Infecting at a lower MOI (MOI ~10) yielded identical trends as shown in Fig. 7A, as did using another human intestinal epithelial tissue culture cell line, Caco-2 (data not shown).

To test the effect of bacterial growth stage in adherence, invasion and intracellular survival, WT and Δ*spoT* bacteria were grown in shaking liquid culture to early log (EL) or early stationary (ES) phase, corresponding approximately to the 2 h (EL) and 10 h (ES) timepoints shown in Fig. 5 (ES cultures were collected prior to the decline in cfu ml^{-1} due to stationary phase defects), then used to infect INT407 cells (Fig. 7C and D). Adherence, invasion and intracellular survival assays were conducted identically to those described for Fig. 7A and B. The WT strain appeared unaffected by growth stage, and early log phase Δ*spoT* inocula yielded profiles similar to biphasic inocula. However, each defect, particularly the ability to invade and survive intracellularly, was much more pronounced for the Δ*spoT* mutant inoculated in early stationary phase: these bacteria exhibited an eightfold difference from WT for adherence/invasion and a 40-fold difference in invaded bacteria. Most strikingly, the early stationary phase inocula exhibited a 371-fold difference in intracellular surviving bacteria after only 4 h intracellular (Fig. 7D), comparable to the 20 h intracellular survival defect observed for biphasic inocula (Fig. 7B).

Discussion

A gene expression-based screen leads to the identification and characterization of the C. jejuni *stringent response, which is mediated by a single* relA/spoT *gene*

In an effort to identify *C. jejuni* factors involved in the pathogen–host cell interplay, we used a whole-genome *C. jejuni* microarray as a screening tool to uncover *C. jejuni* genes exhibiting expression changes during infection of human epithelial cells. One gene, *spoT* (Cj1272c), particularly piqued our interest because of its immediate upregulation in the presence of live epithelial cells, its coexpression with known virulence genes, and the fact that the stringent response had not previously been demonstrated for any epsilon-proteobacterial species. Subsequent biological characterization of the *C. jejuni* stringent response, including genetic, biochemical, molecular, phenotypic and gene expression analyses, identified crucial roles for the stringent response in growth and survival during several transmission- and antibiotic resistance-related stresses. Our studies also revealed a critical role for this phenomenon in the *C. jejuni*–host cell interaction, including the first identification of a *C. jejuni* factor involved in intra-epithelial cell survival.

Unlike most other Gram-negative bacteria for which the stringent response has been characterized (Mittenhuber, 2001), the *C. jejuni* genome contains a single *relA*/*spoT* gene annotated as *spoT* (Parkhill *et al.*, 2000). Deletion of this gene led to a (p)ppGpp^0 phenotype (Fig. 3), indicating that *C. jejuni spoT* likely encodes a bifunctional (p)ppGpp synthetase/hydrolase akin to those described for Gram-positive bacteria. Two Gram-negative alpha-proteobacteria, *S. meliloti* and *Rhodobacter capsulatus*, likewise harbour a single *relA*/*spoT* gene whose deletion leads to a (p)ppGpp^0 phenotype (Wells and Long, 2002; Masuda and Bauer, 2004). The published genome sequences of other alpha- (i.e. *Caulobacter crescentus*, *Mesorhizobium loti*) and epsilon- (i.e. *H. pylori*, *H. hepaticus*, *W. succiogenes*) proteobacteria also contain only one *relA*/*spoT* gene (Tomb *et al.*, 1997; Alm *et al.*, 1999; Nierman *et al.*, 2001; Kaneko *et al.*, 2002; Baar *et al.*, 2003; Suerbaum *et al.*, 2003). We have recently found that *H. pylori* has the ability to initiate the stringent response (D.H. Wells and E.C. Gaynor, manuscript in preparation), supporting the hypothesis that it too exerts stringent control via its single *spoT* gene despite an earlier report to the contrary (Scoarughi *et al.*, 1999).

Many phenotypes connected with stringent control in gamma-proteobacteria like *E. coli* and *L. pneumophila* are controlled by the stationary phase sigma factor *rpoS*, whose transcription is induced by ppGpp and which has been characterized as an essential component of stringent control in Gram-negative bacteria. However, another common trait shared by *C. jejuni* and the other epsilon- and alpha-proteobacteria described above is that all sequenced members of these families lack *rpoS*. Consequently, two paradigms of stringent control in Gram-negative bacteria (i) that two *relA*/*spoT* genes are utilized and (ii) that RpoS is an integral component of this phenomenon, may in fact be the exception rather than the rule, and restricted to specific subspecies such as the well-studied gamma-proteobacteria in which stringent control was first explored.

The C. jejuni *stringent response is important for surviving both general (i.e. stationary phase) and niche-specific (i.e. high O_2/low CO_2) stresses*

The stringent response allows for survival of a variety of stresses in distinct bacterial species (Cashel *et al.*, 1996;

Chatterji and Ojha, 2001; Godfrey *et al*., 2002). Some of these, such as nutrient deprivation, are ubiquitous to all bacteria, including *C. jejuni* (Fig. 3). Other 'general' stresses in *C. jejuni*, such as high cell density in stationary phase, are somewhat distinct from those in many other bacteria. Many of the observed differences purportedly occur because *C. jejuni* lacks several hallmark stress response factors such as *rpoS*. For instance, as *C. jejuni* cultures reach stationary phase, they do not display increased resistance to stress; in contrast, they become significantly more susceptible to many environmental assaults (Kelly *et al*., 2001; Park, 2002).

Our data demonstrate a role for the stringent response in *C. jejuni* survival during this 'non-traditional' stationary phase (Fig. 5). Whether this is related to other longer-term *C. jejuni* stress survival strategies such as entry into a 'viable-but-nonculturable' (VBNC) state (Ng *et al*., 1985; Tholozan *et al*., 1999), a metabolic phenomenon for which the genetic basis is largely unknown, remains to be determined. However, certain gene expression differences between wild-type and Δ*spoT* strains (Fig. 6) correlate well with the early decline in viability of the Δ*spoT* mutant during culture (Fig. 5B) and may provide insight into how the stringent response affects later growth stages *C. jejuni*. For example, the *nuo* operon, which encodes the only NADH dehydrogenase complex in *C. jejuni*, was expressed more highly in wild-type than in the Δ*spoT* mutant. These genes are important for survival in later growth stages in *E. coli* (Pruss *et al*., 1994) and exhibit *rpoS*-independent yet growth phase-dependent expression (Wackwitz *et al*., 1999). We also observed that nearly every heat shock gene in the *C. jejuni* genome was dramatically upregulated in the Δ*spoT* mutant at later timepoints (Fig. 6B). Heat shock gene transcription has been shown in other bacteria to be stringent response-independent (Steiner and Malke, 2001; Dahl *et al*., 2003), and bacteria lacking the stringent response are known to accumulate large amounts of misfolded and damaged proteins (Cashel *et al*., 1996). This may in part account for the morphological aberrancies noted for Δ*spoT* at the 24 h timepoint (Fig. 5D), in particular the grossly enlarged bacteria that are likely responsible for the increased OD_{600} readings (Fig. 5A) and which may reflect a defect in cell division. These observations highlight the remarkable ability of *C. jejuni* and other bacteria to evolve multiple stress response pathways capable of functioning in the absence of each other and demonstrate that the stringent response is important for late growth stage survival in *C. jejuni*. However, as *C. jejuni* lacks *rpoS*, the precise mechanisms by which this occurs are likely to be distinct from those in *E. coli*.

The stringent response is also important for surviving highly species-specific stresses that likely reflect certain unfavourable environmental niches that bacteria have evolved to encounter and endure. *spoT* is important for at least one *C. jejuni*-specific stress: growth and survival in an environment containing lower CO_2 and/or higher O_2 concentrations than optimal (Fig. 4). The capnophilic and microaerophilic properties of *C. jejuni* are consistent with the fact that it is a zoonotic organism and only grows naturally inside an animal host; however, it must be able to survive in the atmosphere during the period of time the microbe is 'between' hosts. The stringent response is likely to participate in this by a mechanism distinct from those of two previously described oxidative stress response factors, superoxide dismutase (*sodB*) and catalase (*katA*): Δ*sodB* and Δ*katA* mutants are severely sensitive to hydrogen peroxide (Pesci *et al*., 1994; Day *et al*., 2000; Stead and Park, 2000) whereas the Δ*spoT* mutant was not, and transcription of neither *sodB* nor *katA* was altered in the Δ*spoT* mutant. It was recently found that *C. jejuni* can induce an adaptive tolerance response (ATR) for short-term survival of stresses such as aerobic challenge (Murphy *et al*., 2003a,b; Martinez-Rodriguez *et al*., 2004), and we and others have found that *C. jejuni* can undergo longer-term aerobic adaptation over time (Jones *et al*., 1993; Gaynor *et al*., 2004). Our CO_2/O_2 growth and survival data (Fig. 4) suggest that the stringent response may participate in both of these phenomena, and at least one gene, Cj0037c, an uncharacterized cytochrome C that was expressed at lower levels in Δ*spoT* compared with wild-type (Fig. 6A), was previously shown to be upregulated in an aerobic-adapted strain (Gaynor *et al*., 2004). The *C. jejuni* Δ*spoT* mutant was not defective for other stresses such as anaerobiosis and osmotolerance (Table S1), even though these phenotypes are observed for stringent response mutants in other bacteria [i.e. *M. tuberculosis* (Primm *et al*., 2000) and *L. monocytogenes* (Okada *et al*., 2002) respectively]. Thus, in *C. jejuni*, the stringent response is important for surviving both 'general' stress conditions such as stationary phase as well as stresses that are highly niche- and species-specific.

RNA polymerase, ppGpp, rifampicin and stringent response suppressors

Work performed primarily in *E. coli* has demonstrated a close connection between RNA polymerase (RNAP), ppGpp binding, and rifampicin. ppGpp is thought to exert stringent control by binding directly to RNAP either at the β (Reddy *et al*., 1995) or β′ (Toulokhonov *et al*., 2001) subunit and modifying RNAP confirmation in an allosteric manner (Toulokhonov *et al*., 2001). Rifampicin acts by binding the β subunit of RNAP (Zillig *et al*., 1970); one study estimated that this occurs only 27 Å away from the ppGpp binding site (Reddy *et al*., 1995). Additionally, *E. coli* $ppGpp^0$ suppressors have been identified in RNAP

subunits, with several in *rpoB* also conferring rifampicin resistance. These suppressors are generally global in phenotype suppression (Hernandez and Cashel, 1995; Gaal *et al*., 1997; Bartlett *et al*., 1998; Zhou and Jin, 1998; Murphy and Cashel, 2003; Wells and Long, 2003). Unlike *E. coli*, nearly every naturally occurring isolate of *C. jejuni* tested exhibits resistance to high rifampicin concentrations (Aleksandrova *et al*., 1990; Pozhalostina *et al*., 1992; Sicinschi, 1996), and rifampicin at ≥100 µg ml^{-1} is commonly used in Campylobacter selective medium.

The *C. jejuni* Δ*spoT* mutant exhibited a dramatic reduction in rifampicin resistance compared with wild-type (Table 1). This is epidemiologically significant in light of the fact that *C. jejuni* resistance to fluoroquinolones is rising dramatically worldwide (Nachamkin *et al*., 2002b), and the identification of a *C. jejuni* gene whose deletion increases antibiotic susceptibility may have implications for disease control. Previous studies have found that sublethal injury can increase *C. jejuni* rifampicin sensitivity (Humphrey and Cruickshank, 1985; Mason *et al*., 1999); thus, it is possible that other Δ*spoT* mutant phenotypes, such as those described above, may be responsible for the rifampicin resistance defect. However, it is also interesting to speculate that binding of ppGpp to *C. jejuni* RNAP may somehow exclude rifampicin from RNAP, either by inducing a conformational change in RNAP or by physically blocking the rifampicin binding site. This interpretation is supported by our observation that both mutant and wild-type strains exhibit some degree of 'adaptation' to rifampicin over time (Table 1), suggesting either the emergence of new suppressor mutations or conformational adaptation of RNAP to a $ppGpp^0$ environment.

In this study, we also identified one likely extragenic suppressor of the *C. jejuni* Δ*spoT* mutant (SSA) that, in contrast to previously described *relA* suppressors, appears to be highly phenotype-specific. This suppressor rescued the growth defect in a 5% CO_2 environment and partially rescued survival during acute aerobic stress (Fig. 4) but did not rescue any other Δ*spoT* defects identified, including rifampicin sensitivity. Only two genes exhibited significant gene expression differences in the suppressor compared with Δ*spoT* by the criteria applied (Fig. 6C), although other genes also exhibited expression differences at one or two timepoints (data not shown). The genes shown in Fig. 6C are part of the capsule biosynthetic locus and, apart from some homology of Cj1419c to genes involved in ubiquinone/coenzyme biosynthesis, share no homology with other predicted proteins. Future work is aimed towards identifying the site of mutation in this novel, phenotype-specific suppressor as well as isolating and characterizing additional specific and global suppressor mutations to explore this phenomenon further.

The C. jejuni *stringent response is required for the virulence-associated phenotypes of invasion and intra-epithelial cell survival*

The stringent response plays important roles in invasion and virulence in several intracellular bacterial pathogens, including *L. pneumophila* (Hammer and Swanson, 1999) and *L. monocytogenes* (Taylor *et al*., 2002), and has also been shown to participate in host adaptation in *B. burgdorferi* (Godfrey *et al*., 2002) and symbiosis in *S. meliloti* (Wells and Long, 2002). Although little is understood about the precise mechanisms by which *C. jejuni* causes disease, its ability to interact intimately with host cells both *in vivo* and *in vitro* closely correlates with virulence, as does the ability of a strain to invade cells *in vitro* (Everest *et al*., 1992; Ketley, 1997; Bacon *et al*., 2000; 2002; Carvalho *et al*., 2001). Our initial interest in *spoT* arose by virtue of its dramatic upregulation and coexpression with known virulence genes (pVIR) during host cell infections (Fig. 1), suggesting that it may participate in the pathogen–host cell interaction. Indeed, we found that the *C. jejuni* Δ*spoT* mutant was attenuated for invasion into at least two human epithelial cell lines, exhibiting an eightfold (biphasic inocula), 14-fold (log phase inocula), or 40-fold (early stationary phase incocula) defect compared with wild-type (Fig. 7). These numbers are equivalent to or greater than those for any *C. jejuni* mutant generated in 81-176 described to date, including Δ*virB11* (11-fold defect), Δ*kpsM* (9-fold defect), Δ*comB3* (3.5-fold defect) and Δ*sodB* (12-fold defect) (Pesci *et al*., 1994; Bacon *et al*., 2000; 2001). Each of these mutants, as with Δ*spoT*, are also reported as defective for adherence, but as it is impossible to distinguish between adhered and invaded bacteria prior to gentamicin treatment, that measure may in many cases largely reflect an invasion defect.

Several gene expression differences between wild-type and the Δ*spoT* mutant are also consistent with a role for the stringent response in *C. jejuni* virulence. Interestingly, three pVIR clones known to harbour virulence genes, including two clones that were coexpressed with *spoT* during the initial cell infection screen (Fig. 1), were downregulated in our Δ*spoT* mutant compared with wild-type (Fig. 6A). In *Vibrio cholerae*, links between virulence and the stringent response were also drawn following the observation that a *V. cholerae* Δ*relA* mutant exhibits downregulation of two known virulence factors, the toxin co-regulated pilus and cholera toxin (Haralalka *et al*., 2003). Other *C. jejuni* genes with altered expression profiles in the Δ*spoT* mutant are known to participate in virulence in other bacteria but are as yet uncharacterized in *C. jejuni*. Examples include adenylate kinase (*adk*) in *Yersinia pestis* and *Pseudomonas aereuginosa*, the *degP* protease in a variety of bacteria, and the *pst* phosphate transport operon in *Edwardsiella tarda* (Elzer *et al*., 1996; Li *et al*., 1996; Jones *et al*., 2001;

Markaryan *et al*., 2001; Munier-Lehmann *et al*., 2003; Srinivasa Rao *et al*., 2003); these genes provide excellent candidates for future work exploring *C. jejuni* virulence and other important biological properties.

Finally, one of the most intriguing phenotypes observed for the *C. jejuni* Δ*spoT* mutant was its striking defect in intra-epithelial cell survival. Using biphasic starting cultures, no Δ*spoT* mutant bacteria were recovered after 20 h inside cells, a >250-fold difference from wild-type. This defect was exacerbated with early stationary phase inocula, where an ~370-fold difference from wild-type was observed after just 4 h intracellular (Fig. 7). *spoT* is the first *C. jejuni* factor identified as participating in longer-term survival in epithelial cells. Intracellular survival has been proposed to be directly associated with a variety of pathogenesis-related functions, including persistence, immune evasion, increased virulence (via toxicity and cell death) and failure of antibiotic treatment to eradicate the acute infection. The intra-epithelial cell survival phenotype of the Δ*spoT* mutant could be attributed either to the general stationary phase defects described above or to specific genes. For instance, pyrophosphatase (*ppa*), which was downregulated in Δ*spoT* (Fig. 7), was shown in wild-type *L. pneumophila* to undergo an increase in expression during host–cell infection (Abu Kwaik, 1998), consistent with a predicted role for ATP and/or inorganic phosphate availability in intracellular survival. This phenotype may also be related to the O_2/CO_2 survival defect; however, if this is the case, it is likely to occur by a mechanism distinct from those of *katA* or *sodB* (Pesci *et al*., 1994; Day *et al*., 2000). Interestingly, a *M. tuberculosis* Δ*relA* mutant was found to be defective for persistence *in vivo* and also exhibited several similar gene expression changes as observed for the *C. jejuni* Δ*spoT* mutant, such as downregulation of nitrate reductase and certain virulence factors, and upregulation of *groEL* and *groES* (Dahl *et al*., 2003). Future work is planned to explore *in vivo* roles for the *C. jejuni* stringent response not only in virulence but also in less studied events such as persistence, relapse and immune evasion.

In summary, by starting with an expression-based screen to identify new *C. jejuni* factors involved in the pathogen–host cell interaction, we have uncovered a probable new *C. jejuni* virulence factor and provided new insight into stringent response-related attributes that are likely to be relevant for other bacteria. The importance of the *C. jejuni* stringent response not only in virulence-related events but also those potentially involved in *ex vivo* transmission and antibiotic resistance suggests that future work exploring stringent response-related phenomena will continue to shed light onto how this pathogen causes disease, spreads from host to host, and evolves to evade host and antimicrobial defences.

Experimental procedures

Bacterial strains and growth conditions

Campylobacter jejuni strain 81-176, originally isolated from a diarrhoeic patient (Korlath *et al*., 1985), was the WT strain used for these studies. Isogenic variants of this strain, generated as described below and in *Results*, are named as follows:

i. Δ*spoT*: *spoT* deletion strain; 81-176 *spoT*::*kan*^{R}
ii. *spoT**: reconstituted WT strain; [*spoT*::*Kan*^{R}]::*spoT*
iii. SSA: Δ*spoT* suppressor; Δ*spoT* harbouring a likely extragenic point mutation that rescues the CO_2/O_2 growth defects.

Routine culture of *C. jejuni* and culture of *C. jejuni* prior to most experimental procedures was performed at 37°C on MH agar or in MH broth with 5 μg ml^{-1} trimethoprim and 10 μg ml^{-1} vancomycin, using the Oxoid CampyGen system to generate a 6% O_2, 12% CO_2 growth atmosphere. Broth cultures were grown shaking at 200 r.p.m. Biphasic cultures consisted of 10 ml MH agar overlaid with 5 ml MH broth in T25 tissue culture flasks in which bacteria were grown standing at 37°C using the CampyGen system. Where indicated, kanamycin was used at 50 μg ml^{-1} and chloramphenicol at 30 μg ml^{-1}. *E. coli* strains DH10 and DH5α were grown on Luria–Bertani (LB) agar or in LB broth at 37°C under atmospheric conditions; LB was supplemented with kanamycin and chloramphenicol as above, or with carbenicillin at 100 μg ml^{-1}.

Generation of Δ*spoT* *mutant and reconstituted wild-type (*spoT**) strains*

spoT (Cj1272c) was PCR-amplified from 81-176 genomic DNA with a 9:1 pfu : taq (proofreading) polymerase mixture using primers *spoT*-fwd1 (gctgtggaaattgatgcagatatgg) and *spoT*-rev1 (gctgtaggatcaaaccctgcctttat), cloned into the TOPO-XL vector (Invitrogen), and then cloned into pUC18 using *EcoRI*. Fragments of 500 bp corresponding to the 5′-most and 3′-most regions of *spoT* were generated by proofreading PCR; these fragments also introduced new restriction sites at the ends of each fragment (*Eco*RI and *Kpn*I on the 5′ and 3′ ends of the '*spoT* 5′ fragment', and *Bam*HI and *Xba*I on the 5′ and 3′ ends of the '*spoT*-3′ fragment'). Primers for this were: *spoT*-5′-fwd-*Eco*RI (cggaattcaagctg ctaaggctttgctttt), *spoT*-5′-rev-*Kpn*I (cggggtaccggctattggggca tatacaact), *spoT*-3′-fwd-*Bam*HI (gcggatccgtcagacgtattagac aagtagcaaca) and *spoT*-3′-rev-*Xba*I (gatctagacgagattttaac ctatccttgacac). These PCR products were digested with the indicated enzymes, and the pUC18K2 plasmid (Menard *et al*., 1993) was digested with *Kpn*I and *Bam*HI. The three fragments were ligated together into pUC19 that had been digested with *Eco*RI and *Xba*I, resulting in a *spoT*::*kan*^{R} disruption cassette with the non-polar *kan*^{R} gene inserted in the same direction as the *spoT* gene. Clones were confirmed by restriction digestion and sequencing. pUC19 cannot replicate in *C. jejuni*; this *C. jejuni* suicide disruption plasmid was introduced to 81-176 by natural transformation (van Vliet *et al*., 1998). Transformants were recovered on MH plates supplemented with kanamycin and purified by 1 day regrowth on kanamycin-containing plates.

Double crossover homologous recombination of the *spoT*::*kan*^{R} construct into the *spoT* locus was first analysed by PCR on genomic DNA, prepared using the Wizard Genomic DNA kit (Promega) from 12 transformants. Each transformant appeared to harbour the *spoT*::*kan*^{R} insert at the correct locus. To confirm this, Southern analyses were performed on genomic DNA, prepared using the Qiagen Genomic Midi Kit, from four of the transformants. Wild-type 81-176 genomic DNA was prepared identically. One microgram of genomic DNA from each sample was digested for 16 h using *EcoRV* and resolved on a 1% agarose gel. The random prime labelling system, version II (Amersham) was used to label *spoT* in pUC18 and the *kan*^{R} gene in pUC18K2. Hybridization of these probes to the *EcoRV*-digested *C. jejuni* genomic DNA was visualized using the ECL detection system (Amersham). To confirm non-polar insertion of the *kan*^{R} gene, RNA was isolated from *spoT*::*kan*^{R} (Δ*spoT*) strains and wild-type as described below and subjected to RT reactions + or – RT enzyme. PCR reactions were performed using primers specific to genes indicated on Fig. 2.

Plasmids for Δ*spoT* complementation attempts were generated in pRY112 (*Cm*^{R}), a conjugative replicating plasmid in *C. jejuni* for which copy number is not known but, based on other work in our laboratory, is likely at least several plasmid copies per bacterial cell (E.C. Gaynor, J.K. MacKichan and S. Falkow, unpubl.). Constructs were generated which contained (a) the *C. jejuni spoT* gene plus three different length segments of upstream DNA [specifically (i) the region just upstream of *spoT*; (ii) two upstream genes with a potential promoter region and (iii) three upstream genes with a potential promoter region], (b) the *C. jejuni spoT* gene driven by two different *C. jejuni* promoters [23E5 (Wosten *et al*., 1998) and *pebC*] previously shown to yield high expression of the green fluorescent protein (E.C. Gaynor and S. Falkow, unpubl.), or (c) a *B. subtilis relA*/*spoT* promoter-gene construct previously shown to complement a *S. meliloti relA* mutant (Wells and Long, 2002). Plasmids were conjugated into the Δ*spoT* strain by triparental matings. For the matings, the *E. coli* donor and helper strains were DH5α harbouring (i) the plasmid to be conjugated and (ii) pRK600 (Figurski and Helinski, 1979) respectively. Approximately 10^8 of each *E. coli* strain in log phase were mixed with 10^9 log phase *C. jejuni* in 100 μl MH broth (no antibiotics), spotted onto MH plates (no antibiotics), and incubated upright 5 h under CampyGen conditions at 37°C. Transconjugants were selected by plating the bacterial mixture on MH plates containing chloramphenicol, kanamycin, vancomycin and trimethoprim at concentrations described above. Clones were purified by 1× passage on plates with the above antibiotics, loss of *E. coli* confirmed by lack of bacterial growth on L-agar in a 37°C air incubator, and plasmid transfer assayed by miniprep and restriction analysis. Functional complementation was tested by determining if the transconjugants could grow normally in a 5% CO_2 incubator (see below). Generation of reconstituted wild-type strains, one of which was subsequently designated *spoT**, were achieved by natural transformation of the four individual Δ*spoT* mutants (described above) with the wild-type *spoT* gene in pUC18. Serial 10-fold dilutions were spread onto MH plates without kanamycin and placed in a 5% CO_2 incubator at 37°C (see Fig. 4). Healthy colonies arising after 2 days were selected and purified by growth under CampyGen conditions. Multiple single colonies were retested for 5% CO_2 growth and kanamycin sensitivity. Two putative reconstituted wild-type strains from each transformation were tested by Southern analysis as above; each yielded a wild-type profile, indicating that the restoration event was robust and reproducible.

(p)ppGpp assays

(p)ppGpp production was assayed essentially as described (Wells and Long, 2002). Briefly, strains were cultured overnight in MH broth (rich medium) to early exponential phase (OD_{600} ~0.3), back-diluted to OD_{600} = 0.1, and cultured in MH broth for an additional 2 h, at which point all strains were OD_{600} ~0.25. For nutrient downshift experiments, 0.25 OD_{600}-equivalents of each culture were removed, pelleted by centrifugation at 10 000 r.p.m. for 5 min, and washed once in MOPS-MGS (Mendrygal and Gonzalez, 2000). Samples were resuspended in 250 μl MOPS-MGS, ^{32}P was added at 100 μCi ml^{-1}, and cultures were labelled for 1 h at 37°C. For rich media labelling experiments, 250 μl of the culture was removed and directly labelled with ^{32}P for 1 h at 37°C under CampyGen conditions. Following labelling, 50 μl samples were removed and added to an equal volume of 2 M formic acid and placed on ice for at least 15 min. Samples were spun for 5 min at 16 000 *g* in a microcentrifuge, and 3 μl of the supernatant was spotted directly onto PEI cellulose thin-layer chromatography (TLC) plates, dried, and developed in 1.5 M KH_2PO_4 for ~2.5 h. Nucleotides were visualized by autoradiography.

Tests for low CO_2/high O_2 growth and survival and rifampicin resistance

To assay growth ability under low [CO_2] growth conditions, fresh (14 h) overnight plates or exponential broth-grown bacteria were used to prepare MH plates that were incubated for the indicated number of days (Fig. 4) in 5% or 14% CO_2 incubators. In these incubators, a [CO_2] of 3–6% results in an [O_2] of ~19%, and a [CO_2] of 10–15% results in an [O_2] of ~17% (NuAire, pers. comm. and Boyle's law). For direct tests of aerotolerance, early log cultures (~0.1 OD_{600}) were removed from microaerobic growth conditions and inoculated in MH broth either into a flask at a 6:1 surface : volume ratio and shaken upright in a 37°C air incubator at 100 r.p.m. (moderate aeration) or into a tube at a 30:1 surface : volume ratio and inserted sideways into the outermost slots of an 18 inch roller drum spinning in an air incubator at 37°C at a speed of ~56 r.p.m. (vigorous aeration). cfu ml^{-1} were assayed after 6 h. To test rifampicin resistance, MH plates containing rifampicin (rif) were prepared to final rif concentrations as described in *Results* and in Table 1. Rif plates were stored and incubated in the dark. Qualitative assessment of growth was performed at the days indicated.

INT407 cell infections for identifying C. jejuni *genes exhibiting expression changes during cell infection*

INT407 cells were seeded into 18 × 150 mm dishes at semi-confluency (~5×10^7 cells $dish^{-1}$) and allowed to grow for 16 h prior to infection. Eight plates of cells were washed twice

with PBS and fixed with 2% paraformaldehyde for 15 min then washed again twice with PBS and once with MEM prior to infection. Untreated cells (10 plates) were washed 1× with MEM prior to infection. *C. jejuni* 81-176 from an 8 h plate was used to inoculate biphasic cultures at 0.001 OD_{600}, which were grown overnight to an OD_{600} of ~0.3. Bacteria were washed 1× in MEM and resuspended at 0.08 OD_{600} in MEM. Twenty-five millilitres of bacteria was added to the eight fixed cell and eight of the untreated (live) cell dishes, for a final MOI of ~200. Two dishes of live cells were not infected and used as controls for cross-reactivity of INT407 RNA with the *C. jejuni* array. At the timepoints specified, bacteria were harvested from both the media fraction (bacteria swimming above the cells) and the cell-associated fraction (adhered and invaded bacteria) of two dishes of cells for both the live and fixed cell infections. Media fractions of bacteria were harvested directly into a 1/10 volume of 10× 'stop solution' (5% phenol in 100% ethanol) to halt transcription and RNA degradation. Cell-associated fractions were collected by adding 1× stop solution in water to the cell + bacteria layer to lyse the cells osmotically, followed by extensive syringe manipulation to disrupt cell membranes and solubilize INT407 RNA. Each sample was washed again with 1× stop solution, frozen in liquid nitrogen, and stored at −80°C until RNA preparation.

Growth curve analyses

Fresh overnight plate-grown bacteria were restreaked, grown for 8 h on MH plates, inoculated into MH broth cultures at 0.002 OD_{600}, and grown shaking overnight. After 14 h, all strains were at an OD_{600} of ~0.15–0.3. Cultures were back-diluted to $OD_{600} = 0.1$ and grown for timecourse analyses. At the indicated timpoints, bacteria were harvested for OD_{600}, cfu ml^{-1}, motility and electron microscope (EM) analyses. Negative staining of bacteria by EM was performed as described previously (Gaynor *et al*., 2001).

Microarray analyses

The *C. jejuni* microarray used for these studies is described in detail elsewhere (Leonard *et al*., 2003). In brief, the array consists primarily of PCR products corresponding to the largest unique, non-overlapping region of >99% of the ORFs in the NCTC11168 genome. Each PCR product was spotted twice, with different pins. The array also contains nine *Bgl*II-*Bcl*I subclones of the pVIR plasmid from strain 81-176, spotted 2–6× per array. RNA for all microarray analyses was prepared identically, as described previously (Gaynor *et al*., 2004). Briefly, frozen bacterial pellets were resuspended in 0.4 mg ml^{-1} lysozyme, incubated at room temperature for 5 min, and lysed in a 20× volume of Trizol reagent (Invitrogen). Following chloroform extraction, samples were mixed with an equal volume of 70% ethanol, loaded onto an RNeasy Mini Column (Qiagen), and processed according to manufacturer's procedures, including on-column DNAse treatment (Qiagen). RNA was confirmed as DNA-free by RT-PCR. For hybridizations, RT was performed with 0.6 pmol *C. jejuni* gene-specific primer mix (Sigma-Genosys) and 1.5 μg *C. jejuni* RNA per hybridization. cDNA labelling and array hybridizations were performed as described previously (Gaynor *et al*., 2004). Reference cDNA was generated by performing RT reactions on a pool of RNA comprised of equimolar amounts of RNA from each sample in the experiment. Probe generation and statistical significance [SAM: Significance Analysis of Microarrays (Tusher *et al*., 2001)] analyses of gene expression differences between strains were performed as described in the study by Gaynor *et al*. (2004), using the timepoints as technical replicates. Spots identified were confirmed as significant using ORF order analyses to identify operons with similar expression profiles and Cluster analyses to investigate coexpression of genes with similar expression profiles (Eisen *et al*., 1998). Data for each spot were mean-centred, and Cluster or ORF order outputs were visualized using TreeView (Eisen *et al*., 1998). Figure 1 shows a Cluster output; Fig. 6 shows an ORF order output. Previous work demonstrated that our microarray data correlate extremely well with real time RT-PCR data for every gene analysed (Gaynor *et al*., 2004). This, together with the facts that (i) both spots for each gene yielded nearly identical array signals and (ii) the remarkably consistent operon gene expression profiles in our experiments, provides a high degree of confidence in the array data shown in Figs 1 and 6, Figs S1–S3 and Table S2. For the experiments in Fig. 1, it should be noted that our osmotic lysis, washing, and RNA isolation procedures removed >90% of the INT407 RNA from the preparations while the *C. jejuni* cells remained intact (data not shown). Hybridization of residual RNA from uninfected INT407 cells processed identically to the infected samples yielded minor cross-reactivity with ~300 spots on the *C. jejuni* microarray. These spots were removed from subsequent analyses. Uninfected 'media fractions' did not contain detectable INT407 RNA.

INT407 and Caco-2 infections for assaying adherence, invasion and intracellular survival

INT407 or Caco-2 (not shown) cells were seeded into 24 wellplates at semiconfluency (~1×10^5) approximately 16 h prior to infection. For biphasic culture infections, *C. jejuni* strains were harvested from 8 h plates and inoculated into biphasic cultures at 0.001 OD_{600}; cultures were grown overnight to an OD_{600} of ~0.3. For EL versus ES phase infections, bacteria from 8 h plates were used to infect shaking MH broth cultures at 0.002 OD_{600}. Cultures were grown overnight (~14 h), back-diluted to 0.1 OD_{600}, and harvested at timepoints approximating the 2 h and 10 h timepoints shown in Fig. 5. For all infections, bacteria were washed once in MEM and resuspended at 0.002 OD_{600} in MEM. One millilitre of the bacterial suspensions were used to infect the tissue culture cells (MOI ~100), which had been washed once in MEM prior to infection. Other experiments (not shown) utilized bacteria at 0.0002 OD_{600} (MOI ~10). Three wells per strain per timepoint were allocated for each experiment, and the experiments were performed ≥3×; for simplicity, data from one experiment per bacterial growth condition are shown. After 3 h of infection, adhered + invaded (adh + inv) bacteria were harvested by washing the cells 2× with MEM, adding 1 ml dH_2O, and using a 27G syringe to disrupt cell membranes. At this time, wells for assaying invasion and intracellular survival were washed 2× with MEM, and 150 μg ml^{-1} gentamicin

was added in MEM. After 2 h, invaded (inv) bacteria were harvested as above. Samples for assaying intracellular survival were washed 2× with MEM, and 5 μg ml^{-1} gentamicin was added to halt growth of bacteria from lysed cells. After 4 and 16 h, intracellular bacteria were harvested as above. Although data are not shown for these controls, several points should be noted: (i) all strains exhibited identical gentamicin susceptibility/resistance both in the presence and absence of cells; (ii) all strains were resistant to the H_2O/syringe lysis procedures and (iii) all strains survived equally well in the media above the cells during the 3 h infection. All strains also survived equally well for 3 h in tissue culture medium alone. Bacterial enumeration was by serial 10-fold dilution and plating on MH plates.

Acknowledgements

The authors would like to thank past and current members of the Falkow and Long labs for scientific input and helpful discussions throughout the course of this work. We would especially like to thank Dr Sharon Long for financial and scientific support for the (p)ppGpp labelling experiments, Drs Denise Monack and Igor Brodsky for assistance with the cell infection screen experiments, Inna Bilis for technical assistance, Dr Scotty Merrell for the pUC18K2 plasmid (originally obtained from Dr Agnes Labigne via Dr Jay Solnick) and Drs Karen Guillemin and Elizabeth Joyce for critical input to the manuscript. E.C.G. was funded by the Life Sciences Research Foundation and by a Career Development Award from the Burroughs Wellcome Fund. D.H.W. was supported by Grant NIH GM30962 to Dr Sharon Long. J.K.M. and S.F. were supported by Grant NIH AI38459.

Supplementary material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/mmi/mmi4525/mmi4525sm.htm

Appendix S1. Phenotype and stress testing of strains

Fig. S1. A. Numeric values for data shown in Fig. 1.

B. Array data for genes up- and downstream of *spoT* in the cell infection screen described in the first *Results* section and in Fig. 1.

C. Numeric values for data shown in B above.

Fig. S2. Array data for the two genes immediately downstream of *spoT* in the experiment comparing global expression profiles in WT, Δ*spoT* and SSA strains.

Fig. S3. Graphical numeric display of array values for three representative genes shown in Fig. 6 and listed in Table S2, from the experiment comparing global expression profiles in WT, Δ*spoT* and SSA strains.

Table S1. Phenotypes that are similar in WT and Δ*spoT* strains.

Table S2. Numeric values for array data shown in Fig. 6.

References

Abu Kwaik, Y. (1998) Induced expression of the *Legionella pneumophila* gene encoding a 20-kilodalton protein during intracellular infection. *Infect Immun* **66:** 203–212.

Aleksandrova, N.Z., Minaev, V.I., and Gorelov, A.V. (1990) [Antibiotic resistance of Campylobacter and its epidemiologic significance]. *Antibiot Khimioter* **35:** 34–36.

Allaoui, A., Schulte, R., and Cornelis, G.R. (1995) Mutational analysis of the *Yersinia enterocolitica* virC operon: characterization of yscE, F, G, I, J, K required for Yop secretion and yscH encoding YopR. *Mol Microbiol* **18:** 343–355.

Alm, R.A., Ling, L.S., Moir, D.T., King, B.L., Brown, E.D., Doig, P.C., *et al.* (1999) Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. *Nature* **397:** 176–180.

Baar, C., Eppinger, M., Raddatz, G., Simon, J., Lanz, C., Klimmek, O., *et al.* (2003) Complete genome sequence and analysis of *Wolinella succinogenes*. *Proc Natl Acad Sci USA* **100:** 11690–11695.

Babakhani, F.K., and Joens, L.A. (1993) Primary swine intestinal cells as a model for studying *Campylobacter jejuni* invasiveness. *Infect Immun* **61:** 2723–2726.

Babakhani, F.K., Bradley, G.A., and Joens, L.A. (1993) Newborn piglet model for campylobacteriosis. *Infect Immun* **61:** 3466–3475.

Bacon, D.J., Alm, R.A., Burr, D.H., Hu, L., Kopecko, D.J., Ewing, C.P., *et al.* (2000) Involvement of a plasmid in virulence of *Campylobacter jejuni* 81–176. *Infect Immun* **68:** 4384–4390.

Bacon, D.J., Szymanski, C.M., Burr, D.H., Silver, R.P., Alm, R.A., and Guerry, P. (2001) A phase-variable capsule is involved in virulence of *Campylobacter jejuni* 81–176. *Mol Microbiol* **40:** 769–777.

Bacon, D.J., Alm, R.A., Hu, L., Hickey, T.E., Ewing, C.P., Batchelor, R.A., *et al.* (2002) DNA sequence and mutational analyses of the pVir plasmid of *Campylobacter jejuni* 81–176. *Infect Immun* **70:** 6242–6250.

Bartlett, M.S., Gaal, T., Ross, W., and Gourse, R.L. (1998) RNA polymerase mutants that destabilize RNA polymerase-promoter complexes alter NTP-sensing by rrn P1 promoters. *J Mol Biol* **279:** 331–345.

Black, R.E., Levine, M.M., Clements, M.L., Hughes, T.P., and Blaser, M.J. (1988) Experimental *Campylobacter jejuni* infection in humans. *J Infect Dis* **157:** 472–479.

Blaser, M.J., Wells, J.G., Feldman, R.A., Pollard, R.A., and Allen, J.R. (1983) *Campylobacter enteritis* in the United States. A multicenter study. *Ann Intern Med* **98:** 360–365.

Butzler, J.P., and Skirrow, M.B. (1979) Campylobacter enteritis. *Clin Gastroenterol* **8:** 737–765.

Carvalho, A.C., Ruiz-Palacios, G.M., Ramos-Cervantes, P., Cervantes, L.E., Jiang, X., and Pickering, L.K. (2001) Molecular characterization of invasive and noninvasive *Campylobacter jejuni* and *Campylobacter coli* isolates. *J Clin Microbiol* **39:** 1353–1359.

Cashel, M., Gentry, D.R., Hernandez, V.J., and Vinella, D. (1996) The stringent response. In *Escherichia coli and Salmonella; Cellular and Molecular Biology*. Washington, DC: American Society for Microbiology.

Chatterji, D., and Ojha, A.K. (2001) Revisiting the stringent response, ppGpp and starvation signaling. *Curr Opin Microbiol* **4:** 160–165.

CPHLS (2000) Common gastrointestinal infections, England and Wales. *Commun Dis Rep* **10:** 9–12.

Dahl, J.L., Kraus, C.N., Boshoff, H.I., Doan, B., Foley, K.,

Avarbock, D., *et al.* (2003) The role of RelMtb-mediated adaptation to stationary phase in long-term persistence of *Mycobacterium tuberculosis* in mice. *Proc Natl Acad Sci USA* **100:** 10026–10031.

Day, W.A., Jr, Sajecki, J.L., Pitts, T.M., and Joens, L.A. (2000) Role of catalase in *Campylobacter jejuni* intracellular survival. *Infect Immun* **68:** 6337–6345.

Eisen, M.B., Spellman, P.T., Brown, P.O., and Botstein, D. (1998) Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci USA* **95:** 14863–14868.

Elzer, P.H., Phillips, R.W., Robertson, G.T., and Roop, R.M., II (1996) The HtrA stress response protease contributes to resistance of *Brucella abortus* to killing by murine phagocytes. *Infect Immun* **64:** 4838–4841.

Everest, P.H., Goossens, H., Butzler, J.P., Lloyd, D., Knutton, S., Ketley, J.M., and Williams, P.H. (1992) Differentiated Caco-2 cells as a model for enteric invasion by *Campylobacter jejuni* and *C. coli*. *J Med Microbiol* **37:** 319–325.

Figurski, D.H., and Helinski, D.R. (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc Natl Acad Sci USA* **76:** 1648–1652.

Friedman, C.R., Neimann, J., Wegener, H.C., and Tauxe, R. (2001) Epidemiology of Campylobacter jejuni infection in the United States and other industrialized nations. In *Campylobacter*. Nachamkin, I., and Blaser, M.J. (eds). Washington, DC: American Society for Microbiology Press, pp. 121–138.

Gaal, T., Bartlett, M.S., Ross, W., Turnbough, C.L., Jr, and Gourse, R.L. (1997) Transcription regulation by initiating NTP concentration: rRNA synthesis in bacteria. *Science* **278:** 2092–2097.

Gaynor, E.C., Ghori, N., and Falkow, S. (2001) Bile-induced 'pili' in *Campylobacter jejuni* are bacteria-independent artifacts of the culture medium. *Mol Microbiol* **39:** 1546–1549.

Gaynor, E.C., Cawthraw, S., Manning, G., MacKichan, J.K., Falkow, S., and Newell, D.G. (2004) The genome-sequenced variant of *Campylobacter jejuni* NCTC 11168 and the original clonal clinical isolate differ markedly in colonization, gene expression, and virulence-associated phenotypes. *J Bacteriol* **186:** 503–517.

Godfrey, H.P., Bugrysheva, J.V., and Cabello, F.C. (2002) The role of the stringent response in the pathogenesis of bacterial infections. *Trends Microbiol* **10:** 349–351.

Guerry, P., Szymanski, C.M., Prendergast, M.M., Hickey, T.E., Ewing, C.P., Pattarini, D.L., and Moran, A.P. (2002) Phase variation of *Campylobacter jejuni* 81–176 lipooligosaccharide affects ganglioside mimicry and invasiveness *in vitro*. *Infect Immun* **70:** 787–793.

Hammer, B.K., and Swanson, M.S. (1999) Co-ordination of legionella pneumophila virulence with entry into stationary phase by ppGpp. *Mol Microbiol* **33:** 721–731.

Haralalka, S., Nandi, S., and Bhadra, R.K. (2003) Mutation in the relA gene of *Vibrio cholerae* affects *in vitro* and *in vivo* expression of virulence factors. *J Bacteriol* **185:** 4672–4682.

Hendrixson, D.R., Akerley, B.J., and DiRita, V.J. (2001) Transposon mutagenesis of *Campylobacter jejuni* identifies a bipartite energy taxis system required for motility. *Mol Microbiol* **40:** 214–224.

Hernandez, V.J., and Cashel, M. (1995) Changes in conserved region 3 of *Escherichia coli* sigma 70 mediate ppGpp-dependent functions *in vivo*. *J Mol Biol* **252:** 536–549.

Humphrey, T.J., and Cruickshank, J.G. (1985) Antibiotic and deoxycholate resistance in *Campylobacter jejuni* following freezing or heating. *J Appl Bacteriol* **59:** 65–71.

Ishihama, A. (2000) Functional modulation of *Escherichia coli* RNA polymerase. *Annu Rev Microbiol* **54:** 499–518.

Jones, D.M., Sutcliffe, E.M., Rios, R., Fox, A.J., and Curry, A. (1993) *Campylobacter jejuni* adapts to aerobic metabolism in the environment. *J Med Microbiol* **38:** 145–150.

Jones, C.H., Bolken, T.C., Jones, K.F., Zeller, G.O., and Hruby, D.E. (2001) Conserved DegP protease in Gram-positive bacteria is essential for thermal and oxidative tolerance and full virulence in *Streptococcus pyogenes*. *Infect Immun* **69:** 5538–5545.

Kaneko, T., Nakamura, Y., Sato, S., Minamisawa, K., Uchiumi, T., Sasamoto, S., *et al.* (2002) Complete genomic sequence of nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* USDA110. *DNA Res* **9:** 189–197.

Karlinger, K., Gyorke, T., Mako, E., Mester, A., and Tarjan, Z. (2000) The epidemiology and the pathogenesis of inflammatory bowel disease. *Eur J Radiol* **35:** 154–167.

Kelly, A.F., Park, S.F., Bovill, R., and Mackey, B.M. (2001) Survival of *Campylobacter jejuni* during stationary phase: evidence for the absence of a phenotypic stationary-phase response. *Appl Environ Microbiol* **67:** 2248–2254.

Ketley, J.M. (1997) Pathogenesis of enteric infection by *Campylobacter*. *Microbiology* **143:** 5–21.

Konkel, M.E., and Cieplak, W., Jr (1992) Altered synthetic response of *Campylobacter jejuni* to cocultivation with human epithelial cells is associated with enhanced internalization. *Infect Immun* **60:** 4945–4949.

Konkel, M.E., Hayes, S.F., Joens, L.A., and Cieplak, W., Jr (1992a) Characteristics of the internalization and intracellular survival of *Campylobacter jejuni* in human epithelial cell cultures. *Microb Pathog* **13:** 357–370.

Konkel, M.E., Mead, D.J., Hayes, S.F., and Cieplak, W., Jr (1992b) Translocation of *Campylobacter jejuni* across human polarized epithelial cell monolayer cultures. *J Infect Dis* **166:** 308–315.

Konkel, M.E., Corwin, M.D., Joens, L.A., and Cieplak, W. (1992c) Factors that influence the interaction of *Campylobacter jejuni* with cultured mammalian cells. *J Med Microbiol* **37:** 30–37.

Korlath, J.A., Osterholm, M.T., Judy, L.A., Forfang, J.C., and Robinson, R.A. (1985) A point-source outbreak of campylobacteriosis associated with consumption of raw milk. *J Infect Dis* **152:** 592–596.

Lastovica, A.J. (1996) *Campylobacter*/*Helicobacter* bacteraemia in Cape Town, South Africa, 1977–95. In *Campylobacters, Helicobacters and Related Organisms*. Newell, D.G., Ketley, J.M., and Feldman, R.A. (eds). New York: Plenum Press, pp. 475–479.

Leach, S., Harvey, P., and Wali, R. (1997) Changes with growth rate in the membrane lipid composition of and amino acid utilization by continuous cultures of *Campylobacter jejuni*. *J Appl Microbiol* **82:** 631–640.

Leonard, E.E., II, Takata, T., Blaser, M.J., Falkow, S., Tompkins, L.S., and Gaynor, E.C. (2003) Use of an open-reading

frame-specific *Campylobacter jejuni* DNA microarray as a new genotyping tool for studying epidemiologically related isolates. *J Infect Dis* **187:** 691–694.

Li, S.R., Dorrell, N., Everest, P.H., Dougan, G., and Wren, B.W. (1996) Construction and characterization of a *Yersinia enterocolitica* O:8 high-temperature requirement (htrA) isogenic mutant. *Infect Immun* **64:** 2088–2094.

Little, R., Ryals, J., and Bremer, H. (1983) rpoB mutation in *Escherichia coli* alters control of ribosome synthesis by guanosine tetraphosphate. *J Bacteriol* **154:** 787–792.

McQuigge, M. (2000) The investigative report on the Walkerton outbreak of waterborne gastroenteritis: May–June 2000. In *Bruce-Grey-Owen Sound Health Unit*, Vol. 2000.

Markaryan, A., Zaborina, O., Punj, V., and Chakrabarty, A.M. (2001) Adenylate kinase as a virulence factor of *Pseudomonas aeruginosa*. *J Bacteriol* **183:** 3345–3352.

Martin, D.W., and Mohr, C.D. (2000) Invasion and intracellular survival of *Burkholderia cepacia*. *Infect Immun* **68:** 24–29.

Martinez-Rodriguez, A., Kelly, A.F., Park, S.F., and Mackey, B.M. (2004) Emergence of variants with altered survival properties in stationary phase cultures of *Campylobacter jejuni*. *Int J Food Microbiol* **90:** 321–329.

Mason, M.J., Humphrey, T.J., and Martin, K.W. (1999) Isolation of sublethally injured campylobacters from poultry and water sources. *Br J Biomed Sci* **56:** 2–5.

Masuda, S., and Bauer, C.E. (2004) Null mutation of HvrA compensates for loss of an essential relA/spoT-like gene in *Rhodobacter capsulatus*. *J Bacteriol* **186:** 235–239.

Menard, R., Sansonetti, P.J., and Parsot, C. (1993) Nonpolar mutagenesis of the ipa genes defines IpaB, IpaC, and IpaD as effectors of *Shigella flexneri* entry into epithelial cells. *J Bacteriol* **175:** 5899–5906.

Mendrygal, K.E., and Gonzalez, J.E. (2000) Environmental regulation of exopolysaccharide production in *Sinorhizobium meliloti*. *J Bacteriol* **182:** 599–606.

Mittenhuber, G. (2001) Comparative genomics and evolution of genes encoding bacterial (p)ppGpp synthetases/hydrolases (the Rel, RelA and SpoT proteins). *J Mol Microbiol Biotechnol* **3:** 585–600.

Monteville, M.R., Yoon, J.E., and Konkel, M.E. (2003) Maximal adherence and invasion of INT 407 cells by *Campylobacter jejuni* requires the CadF outer-membrane protein and microfilament reorganization. *Microbiology* **149:** 153–165.

Munier-Lehmann, H., Chenal-Francisque, V., Ionescu, M., Chrisova, P., Foulon, J., Carniel, E., and Barzu, O. (2003) Relationship between bacterial virulence and nucleotide metabolism: a mutation in the adenylate kinase gene renders *Yersinia pestis* avirulent. *Biochem J* **373:** 515–522.

Murphy, H., and Cashel, M. (2003) Isolation of RNA polymerase suppressors of a (p)ppGpp deficiency. *Meth Enzymol* **371:** 596–601.

Murphy, C., Carroll, C., and Jordan, K.N. (2003a) Identification of a novel stress resistance mechanism in *Campylobacter jejuni*. *J Appl Microbiol* **95:** 704–708.

Murphy, C., Carroll, C., and Jordan, K.N. (2003b) Induction of an adaptive tolerance response in the foodborne pathogen, *Campylobacter jejuni*. *FEMS Microbiol Lett* **223:** 89–93.

Nachamkin, I., Liu, J., Li, M., Ung, H., Moran, A.P., Prendergast, M.M., and Sheikh, K. (2002a) *Campylobacter jejuni* from patients with Guillain–Barre syndrome preferentially expresses a GD(1a)-like epitope. *Infect Immun* **70:** 5299–5303.

Nachamkin, I., Ung, H., and Li, M. (2002b) Increasing fluoroquinolone resistance in *Campylobacter jejuni*, Pennsylvania, USA, 1982–2001. *Emerg Infect Dis* **8:** 1501–1503.

Newell, D.G., and Fearnley, C. (2003) Sources of Campylobacter colonization in broiler chickens. *Appl Environ Microbiol* **69:** 4343–4351.

Ng, L.K., Sherburne, R., Taylor, D.E., and Stiles, M.E. (1985) Morphological forms and viability of *Campylobacter* species studied by electron microscopy. *J Bacteriol* **164:** 338–343.

Nierman, W.C., Feldblyum, T.V., Laub, M.T., Paulsen, I.T., Nelson, K.E., Eisen, J.A., *et al.* (2001) Complete genome sequence of *Caulobacter crescentus*. *Proc Natl Acad Sci USA* **98:** 4136–4141.

Okada, Y., Makino, S., Tobe, T., Okada, N., and Yamazaki, S. (2002) Cloning of rel from *Listeria monocytogenes* as an osmotolerance involvement gene. *Appl Environ Microbiol* **68:** 1541–1547.

Park, S.F. (2002) The physiology of *Campylobacter* species and its relevance to their role as foodborne pathogens. *Int J Food Microbiol* **74:** 177–188.

Parkhill, J., Wren, B.W., Mungall, K., Ketley, J.M., Churcher, C., Basham, D., *et al.* (2000) The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature* **403:** 665–668.

Pesci, E.C., Cottle, D.L., and Pickett, C.L. (1994) Genetic, enzymatic, and pathogenic studies of the iron superoxide dismutase of *Campylobacter jejuni*. *Infect Immun* **62:** 2687–2694.

Pozhalostina, L.V., Solodovnikova, A.V., and Avakov, A.A. (1992) [Sensitivity of the pathogen of *Campylobacter infection* to various antibiotics]. *Antibiot Khimioter* **37:** 34–35.

Primm, T.P., Anderson, S.J., Mizrahi, V., Avarbock, D., Rubin, H., and Barry, C.E., III (2000) The stringent response of *Mycobacterium tuberculosis* is required for long-term survival. *J Bacteriol* **182:** 4889–4898.

Pruss, B.M., Nelms, J.M., Park, C., and Wolfe, A.J. (1994) Mutations in NADH: ubiquinone oxidoreductase of *Escherichia coli* affect growth on mixed amino acids. *J Bacteriol* **176:** 2143–2150.

Reddy, P.S., Raghavan, A., and Chatterji, D. (1995) Evidence for a ppGpp-binding site on *Escherichia coli* RNA polymerase: proximity relationship with the rifampicin-binding domain. *Mol Microbiol* **15:** 255–265.

Russell, R.G., O'Donnoghue, M., Blake, D.C., Jr, Zulty, J., and DeTolla, L.J. (1993) Early colonic damage and invasion of *Campylobacter jejuni* in experimentally challenged infant *Macaca mulatta*. *J Infect Dis* **168:** 210–215.

Schreiber, G., Metzger, S., Aizenman, E., Roza, S., Cashel, M., and Glaser, G. (1991) Overexpression of the relA gene in *Escherichia coli*. *J Biol Chem* **266:** 3760–3767.

Scoarughi, G.L., Cimmino, C., and Donini, P. (1999) *Helicobacter pylori*: a eubacterium lacking the stringent response. *J Bacteriol* **181:** 552–555.

Sicinschi, L. (1996) [The antibiotic susceptibility of *Campylobacter* strains isolated in *Moldova*. The possibilities for estimation and the results]. *Bacteriol Virusol Parazitol Epidemiol* **41:** 123–129.

Skirrow, M.B. (1990) *Campylobacter*, *Helicobacter and* other mobile curved Gram-negative rods. In *Topley and Wilson's Principles of Bacteriology, Virology and Immunity*. Parker, M.T., and Collier, L.H. (eds). London: Edward Arnold.

Skirrow, M.B., and Blaser, M.J. (1992) Clinical and epidemiological considerations. In *Campylobacter Jejuni: Current Status and Future Trends*. Nachamkin, I., Blaser, M.J., and Tompkins, L.S. (eds). Washington, DC: American Society for Microbiology, pp. 3–8.

Skouloubris, S., Thiberge, J.M., Labigne, A., and De Reuse, H. (1998) The *Helicobacter pylori* UreI protein is not involved in urease activity but is essential for bacterial survival *in vivo*. *Infect Immun* **66:** 4517–4521.

Srinivasa Rao, P.S., Lim, T.M., and Leung, K.Y. (2003) Functional genomics approach to the identification of virulence genes involved in *Edwardsiella tarda* pathogenesis. *Infect Immun* **71:** 1343–1351.

St Michael, F., Szymanski, C.M., Li, J., Chan, K.H., Khieu, N.H., Larocque, S., *et al.* (2002) The structures of the lipooligosaccharide and capsule polysaccharide of *Campylobacter jejuni* genome sequenced strain NCTC 11168. *Eur J Biochem* **269:** 5119–5136.

Stead, D., and Park, S.F. (2000) Roles of Fe superoxide dismutase and catalase in resistance of *Campylobacter coli* to freeze-thaw stress. *Appl Environ Microbiol* **66:** 3110–3112.

Steiner, K., and Malke, H. (2001) relA-Independent amino acid starvation response network of *Streptococcus pyogenes*. *J Bacteriol* **183:** 7354–7364.

Suerbaum, S., Josenhans, C., Sterzenbach, T., Drescher, B., Brandt, P., Bell, M., *et al.* (2003) The complete genome sequence of the carcinogenic bacterium *Helicobacter hepaticus*. *Proc Natl Acad Sci USA* **100:** 7901–7906.

Tauxe, R.V. (1992) Epidemiology of *Campylobacter jejuni* infections in the United States and other industrialized nations. In *Campylobacter jejuni: Current Status and Future Trends*. Nachamkin, I., Blaser, M.J., and Tompkins, L.S. (eds). Washington, DC: American Society for Microbiology, pp. 9–20.

Taylor, C.M., Beresford, M., Epton, H.A., Sigee, D.C., Shama, G., Andrew, P.W., and Roberts, I.S. (2002) *Listeria monocytogenes* relA and hpt mutants are impaired in surface-attached growth and virulence. *J Bacteriol* **184:** 621–628.

Tholozan, J.L., Cappelier, J.M., Tissier, J.P., Delattre, G., and Federighi, M. (1999) Physiological characterization of viable-but-nonculturable *Campylobacter jejuni* cells. *Appl Environ Microbiol* **65:** 1110–1116.

Thornley, J.P., Jenkins, D., Neal, K., Wright, T., Brough, J., and Spiller, R.C. (2001) Relationship of *Campylobacter* toxigenicity *in vitro* to the development of postinfectious irritable bowel syndrome. *J Infect Dis* **184:** 606–609.

Tomb, J.F., White, O., Kerlavage, A.R., Clayton, R.A., Sutton, G.G., Fleischmann, R.D., *et al.* (1997) The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* **388:** 539–547.

Toulokhonov, I.I., Shulgina, I., and Hernandez, V.J. (2001) Binding of the transcription effector ppGpp to *Escherichia coli* RNA polymerase is allosteric, modular, and occurs near the N terminus of the beta′-subunit. *J Biol Chem* **276:** 1220–1225.

Trieu-Cuot, P., Gerbaud, G., Lambert, T., and Courvalin, P. (1985) *In vivo* transfer of genetic information between Gram-positive and Gram-negative bacteria. *EMBO J* **4:** 3583–3587.

Tusher, V.G., Tibshirani, R., and Chu, G. (2001) Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci USA* **98:** 5116–5121.

Venturi, V. (2003) Control of rpoS transcription in *Escherichia coli* and *Pseudomonas*: why so different? *Mol Microbiol* **49:** 1–9.

van Vliet, A.H., Wooldridge, K.G., and Ketley, J.M. (1998) Iron-responsive gene regulation in a *Campylobacter jejuni* fur mutant. *J Bacteriol* **180:** 5291–5298.

Wackwitz, B., Bongaerts, J., Goodman, S.D., and Unden, G. (1999) Growth phase-dependent regulation of nuoA-N expression in *Escherichia coli* K-12 by the Fis protein: upstream binding sites and bioenergetic significance. *Mol General Genet* **262:** 876–883.

Walker, R.I., Caldwell, M.B., Lee, E.C., Guerry, P., Trust, T.J., and Ruiz-Palacios, G.M. (1986) Pathophysiology of *Campylobacter enteritis*. *Microbiol Rev* **50:** 81–94.

Wanner, B.L. (1993) Gene regulation by phosphate in enteric bacteria. *J Cell Biochem* **51:** 47–54.

Wassenaar, T.M., Engelskirchen, M., Park, S., and Lastovica, A. (1997) Differential uptake and killing potential of *Campylobacter jejuni* by human peripheral monocytes/macrophages. *Med Microbiol Immunol (Berl)* **186:** 139–144.

Wells, D.H., and Long, S.R. (2002) The *Sinorhizobium meliloti* stringent response affects multiple aspects of symbiosis. *Mol Microbiol* **43:** 1115–1127.

Wells, D.H., and Long, S.R. (2003) Mutations in rpoBC suppress the defects of a *Sinorhizobium meliloti* relA mutant. *J Bacteriol* **185:** 5602–5610.

WHO (2000) *The Increasing Incidence of Human Campylobacteriosis.* Reports and proceedings of a WHO consulation of experts. Denmark: Copenhagen, p. 137.

Wooldridge, K.G., and Ketley, J.M. (1997) *Campylobacter*–host cell interactions. *Trends Microbiol* **5:** 96–102.

Wosten, M.M., Boeve, M., Koot, M.G., van Nuene, A.C., and van der Zeijst, B.A. (1998) Identification of *Campylobacter jejuni* promoter sequences. *J Bacteriol* **180:** 594–599.

Yao, R., Alm, R.A., Trust, T.J., and Guerry, P. (1993) Construction of new *Campylobacter* cloning vectors and a new mutational cat cassette. *Gene* **130:** 127–130.

Zhou, Y.N., and Jin, D.J. (1998) The rpoB mutants destabilizing initiation complexes at stringently controlled promoters behave like 'stringent' RNA polymerases in *Escherichia coli*. *Proc Natl Acad Sci USA* **95:** 2908–2913.

Zillig, W., Zechel, K., Rabussay, D., Schachner, M., Sethi, V.S., Palm, P., *et al.* (1970) On the role of different subunits of DNA-dependent RNA polymerase from *E. coli* in the transcription process. *Cold Spring Harb Symp Quant Biol* **35:** 47–58.