

The Stringent Response Is Required for *Helicobacter pylori* Survival of Stationary Phase, Exposure to Acid, and Aerobic Shock

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The gastric pathogen *Helicobacter pylori* must adapt to fluctuating conditions in the harsh environment of the human stomach with the use of a minimal number of transcriptional regulators. We investigated whether *H. pylori* utilizes the stringent response, involving signaling through the alarmone (p)ppGpp, as a survival strategy during environmental stresses. We show that the *H. pylori* homologue of the bifunctional (p)ppGpp synthetase and hydrolase SpoT is responsible for all cellular (p)ppGpp production in response to starvation conditions. Furthermore, the *H. pylori* *spoT* gene complements the growth defect of *Escherichia coli* mutants lacking (p)ppGpp. An *H. pylori* *spoT* deletion mutant is impaired for stationary-phase survival and undergoes a premature transformation to a coccoid morphology. In addition, the *spoT* deletion mutant is unable to survive specific environmental stresses, including aerobic shock and acid exposure, which are likely to be encountered by this bacterium during infection and transmission.

Helicobacter pylori is an ϵ -proteobacterium that infects over half the world's population and is associated with a spectrum of gastric diseases (5). This bacterium, for which no environmental reservoir has been demonstrated, inhabits the harsh environment of human and primate stomachs. Within the environment of the stomach, as well as during the as-yet-ill-defined transmission process, *H. pylori* must endure rapid fluctuations in pH, oxygen tension, and chemical insults from the host immune system. The regulatory mechanisms of *H. pylori*'s survival strategies in the onslaught of these environmental stresses are of interest, in part because the bacterium's small genome encodes few transcriptional regulators, including just three sigma factors (29). Insight into *H. pylori*'s stress response programs will abet the design of better antibiotics to treat infections, since resistance to traditional therapies is increasing among *H. pylori* clinical isolates (19).

A well-characterized strategy used by *Escherichia coli* to survive stresses, such as starvation, is termed the stringent response (10). The stringent response involves the global regulation of transcription characterized by the repression of ribosomal genes and derepression of specific stress response genes (16). The molecular signal or "alarmone" for this response is the small hyperphosphorylated guanosine nucleotide ppGpp that binds directly to RNA polymerase and alters its promoter specificity and transcription rate (17). In *E. coli*, ppGpp and its precursor pppGpp are produced by the ribosome-associated synthetase, RelA, when it senses the presence of uncharged tRNAs. In addition, a bifunctional protein, SpoT, encodes both (p)ppGpp synthetase and hydrolase activities and is crucial for maintaining appropriate levels of (p)ppGpp in the cell. Mutants lacking both of these enzymes produce no detectable (p)ppGpp and exhibit a range of phenotypes, including an inability to survive in minimal media and during stationary phase.

Many other bacteria possess a single bifunctional (p)ppGpp synthetase and hydrolase (23). The distribution of these single bifunctional enzymes was previously thought to be restricted to gram-positive bacteria, but recent work has demonstrated that multiple gram-negative bacteria, particularly those of the α - and ϵ -proteobacterial families, also harbor single bifunctional synthetase/hydrolase enzymes (14, 18, 35). The ability to regulate transcription through production of the small signaling molecule (p)ppGpp is emerging as an important trait for multiple pathogens to survive environments specific to the infection and transmission processes (7). For example, a *spoT* mutant of the related ϵ -proteobacterium *Campylobacter jejuni* survives poorly in a low-CO₂/high-O₂ environment and is impaired in adhesion, invasion, and intracellular survival in cultured epithelial cells (14).

H. pylori has been reported to lack a stringent response (30); however, recent experiments have contradicted this claim and demonstrated that *H. pylori* produces (p)ppGpp under starvation and low-pH conditions (34). The *H. pylori* genome contains a homologue of the *C. jejuni* *spoT* gene, HP0775 or JHP0712 in the 26695 and J99 genomes, respectively (1, 32). Other *H. pylori* genes predicted to be involved in (p)ppGpp metabolism include *gppA* (HP0278, JHP0263), a guanosine-5-triphosphate-3-diphosphate pyrophosphatase, and *ndk* (HP0198, JHP0184), a nucleoside diphosphate kinase (Fig. 1A).

Here we report that the *H. pylori* *spoT* homologue encodes a functional (p)ppGpp synthetase that is responsible for (p)ppGpp production in *H. pylori* and can complement an *E. coli* mutant lacking *relA* and *spoT*. We further demonstrate that SpoT function is required for *H. pylori* to survive stationary phase, aerobiosis, and acid shock, all conditions that the bacterium is likely to experience during infection and transmission.

MATERIALS AND METHODS

Strains and growth conditions. *H. pylori* strain G27 (11) was used in these studies. *E. coli* wild-type MG1655 (CF1648) and isogenic mutant Δ *relA* (CF1652) and Δ *relA* Δ *spoT* (CF1693) strains (37) were obtained from Michael Cashel

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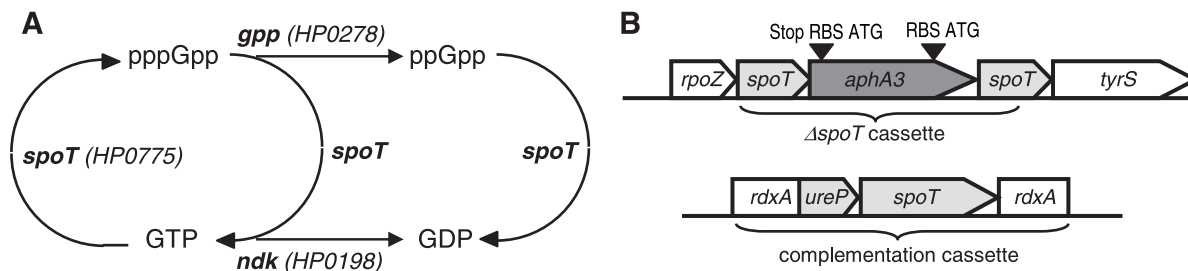


FIG. 1. (A) Putative (p)ppGpp metabolic pathway of *H. pylori* according to Cashel et al. (10). (B) Constructs used for the construction of the $\Delta spoT$ and $spoT^*$ strains.

(National Institutes of Health). *H. pylori* was grown on blood agar (BA) plates consisting of Columbia agar (Difco) supplemented with 5% defibrinated horse blood (Hemostat), 0.02 mg of β -cyclodextrin (Sigma) ml^{-1} , 20 μg of vancomycin (Sigma) ml^{-1} , and 8 μg of amphotericin B (Sigma) ml^{-1} . Selective plates were made with 10 μg of kanamycin (Fisher) ml^{-1} or 0.08 M sucrose. BA plates were grown in incubators at 37°C and 10% CO_2 . *H. pylori* cultures were passaged to fresh BA plates every 3 to 4 days and restarted from frozen stocks after three passages. Liquid media for *H. pylori* consisted of filter-sterilized brucella broth (Difco) supplemented with 10% fetal bovine serum (Gibco) and 20 μg of vancomycin ml^{-1} . Liquid cultures were grown in 50-ml conical tubes with loosened lids (BD Falcon) and shaking at 37°C in anaerobic jars (Oxoid) with CampyGen microaerobic sachets (Oxoid).

Construction of the $\Delta spoT$ and $spoT^*$ strains. The putative *H. pylori* *spoT*, HP0775, was PCR amplified from strain G27 genomic DNA using the primers SpoTXba1F forward (5'-GCTCTAGAGCTGAAGGGAAAATTGATATAGA C-3') and SpoTKpn1R reverse (5'-GCGGTACCCAATCCGCCTTATCTGTG G-3'). The HP0775 PCR product and pBSSK+ cloning vector (Stratagene) were then digested and ligated to generate pK220-1. The primers SpoTEcoRI (5'-CGA ATCCAGGGCGTTTTCAATTTTG-3') and SpoTBamHI (5'-GGGGATCCCC GTCGGTTTTAGCGGGTTTAT-3') were then used to PCR amplify outward from HP0775 and around pK220-1. The pK220-1 PCR product and pUC18 K-2, which contains a kanamycin-resistant nonpolar cassette with two internal ribosomal binding sites (20), were digested and ligated to generate pK220-2. pK220-2 was constructed so that the downstream ribosomal binding site is in frame with the downstream HP0775 fragment. The HP0775 chromosomal locus was disrupted via homologous recombination by transforming strain G27 with purified pK220-2. The resulting $\Delta spoT$ strain was then verified by PCR and sequencing of the genomic locus.

The selective *kan sacB* cassette was cloned into the vector pRdxA (31) and transformed into wild-type *H. pylori* to generate a *rdxA::kan sacB* strain with a kanamycin resistance (Kan^r) and sucrose sensitivity (Suc^s) phenotype. The primers SpoTXba1F forward and SpoTKpn1R reverse were modified to contain BamHI and EcoRI restriction sites, respectively, and HP0775 was PCR amplified and cloned into pRdxA::ureP::gfp (obtained from Nina Salama, Fred Hutchison Cancer Research Center) to generate the construct pRdxA::ureP::HP0775 in which HP0775 expression is regulated by promoter sequences encoded in the 750-bp region directly upstream of the *H. pylori* *ureA* (HP0073) gene. This construct was then used to transform the *rdxA::kan sacB* strain, and replace the *kan sacB* cassette with *ureP::HP0775* via homologous recombination. The appropriate *rdxA::ureP::HP0775* strain was selected and screened based on its Suc^r and Kan^s phenotype. The *rdxA::ureP::HP0775* strain was then transformed with pK220-2 to disrupt the native HP0775 allele via homologous recombination. The genotype of the complemented $spoT^*$ ($\Delta spoT$ *rdxA::ureP::HP0775*) transformants was verified by PCR and sequencing of the genomic loci.

(p)ppGpp assays. The production of (p)ppGpp in response to minimal media was assayed as previously described (14, 35). *H. pylori* strains were grown overnight in normal growth media to early exponential phase (optical density at 600 nm [OD₆₀₀] of ~0.4), diluted back to an OD₆₀₀ of 0.2, and incubated in normal growth media for an additional 2 h, at which point all strains had reached an OD₆₀₀ of ~0.3. For the nutrient deprivation treatment 0.25 OD₆₀₀ equivalents of each culture were removed, pelleted by centrifugation at 10,000 rpm for 5 min, and washed once with minimal medium consisting of 50 mM MOPS (morpholinepropanesulfonic acid; pH 7.4), 1 mM MgSO₄, 0.25 mM CaCl₂, 19 mM glutamic acid, and 0.004 mM biotin. Samples were resuspended in 250 μl of minimal medium, ³²P (Perkin Elmer) was added at 100 $\mu\text{Ci ml}^{-1}$, and cultures were labeled for 1 h at 37°C. After labeling, 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 $\times g$ in a microcentrifuge, and 3 μl of the supernatant was spotted directly onto polyethyleneimine (PEI) cellulose thin-layer chromatography plates (Sigma), dried, and developed in 1.5 M KH₂PO₄ for ~2.5 h. Nucleotides were visualized by autoradiography.

***E. coli* functional complementation assay.** *E. coli* *relA* and *spoT* were amplified from wild-type MG1655 genomic DNA using the primers RelAB glIIF.2 forward (5'-GGAAGATCTGCTGGATATGTTCCACACACG-3'), RelAXhoIR.2 reverse (5'-ATTCGCTCGAGCCCTTCTCAAACCGCTAT-3'), SpoTBglIIF.2 forward (5'-GGAAGATCTAGGAAGCCGCTGAATTACAA-3'), and SpoTXhoIR.2 reverse (5'-ATTCGCTCGAGATGAGGTTTGTGGACCTG CT-3'). *H. pylori* HP0775 was amplified from wild-type G27 genomic DNA with SpoTBglIIF Forward (5'-GGAAGATCTCCGCTGAAGGGAAAATTGATATAG AC-3') and SpoTKpn1R reverse. The PCR products were cloned into the IPTG (isopropyl- β -D-thiogalactopyranoside)-inducible pQE-30 expression vector (QIAGEN) in frame and transformed into the various *E. coli* strains. Transformants were maintained on standard LB agar media supplemented with 50 μg of ampicillin ml^{-1} . For the functional complementation assays, overnight cultures from LB plates were streaked onto M9 minimal medium plates supplemented with 50 μg of ampicillin ml^{-1} , 100 $\mu\text{g ml}^{-1}$ of each of the amino acids serine, methionine, and glycine (12), and ~140 μg of IPTG ml^{-1} . The plates were incubated overnight at 37°C and then photographed.

Growth curves and stress assays. Prior to all experiments, *H. pylori* cultures from BA plates were used to inoculate 2 ml of liquid medium and incubated overnight as described above. To assay viability at each time point, 100- μl samples of culture were serially diluted in 1 \times phosphate-buffered saline, and various dilutions were plated in duplicate on BA plates. Plates were incubated for 3 to 4 days, and then the CFU were counted. Each assay was replicated at least three times. For the growth curves, the overnight wild-type, $\Delta spoT$, and $spoT^*$ cultures were used to prepare 2-ml cultures with an OD₆₀₀ of 0.05. The cultures were grown in the same anaerobic jar shaking horizontally at 150 rpm. The viability of the cultures was assayed at 0, 24, and 50 h. For the aerobic stress experiments, the overnight wild-type, $\Delta spoT$, and $spoT^*$ cultures were used to prepare 2-ml cultures of 0.5 OD₆₀₀. The cultures were incubated in lidless anaerobic jars at 37°C with shaking at 150 rpm under normal atmospheric conditions. The viability of the cultures was assayed every 2 h over a 6-h time course. For the pH stress experiments, pH 4 acidic medium supplemented with 0.5 mM urea, which promotes *H. pylori* survival in acid (27), was prepared by adding 4.8 M HCl to standard growth media and filter sterilizing. The addition of acid to the growth media caused a precipitate to form, which was lost during the filtering process. To determine whether the components of the precipitate were critical for *H. pylori* survival, we adjusted the filtered acidic medium to pH 7 and assayed the viability of our strains. Loss of the precipitate components did not impair growth of the cultures during the course of these experiments (data not shown). The acidity of the media was monitored at each time point sampled and found to be unchanged during the course of the experiment. To ensure that the sensitivity of the $\Delta spoT$ mutant to acid shock was not due to impaired urease activity, urease activity assays were performed on the wild-type, $\Delta spoT$, and $spoT^*$ strains by growing them in Difco urea broth. All three strains exhibited the same amount of urease activity.

Transmission electron microscopy. Wild-type, $\Delta spoT$, and $spoT^*$ bacteria were grown for 48 h in liquid media from a starting OD₆₀₀ of 0.05. Samples (10 μl) of each strain were obtained at 12, 24, and 48 h and applied to copper grids (400 mesh) with Butvar support film coated with carbon, rinsed twice, and negatively stained for 1 min with 1% phosphotungstate (pH 7). Grids were visualized and photographed in a Philips CM12 TEM at an acceleration voltage of 80 kV.

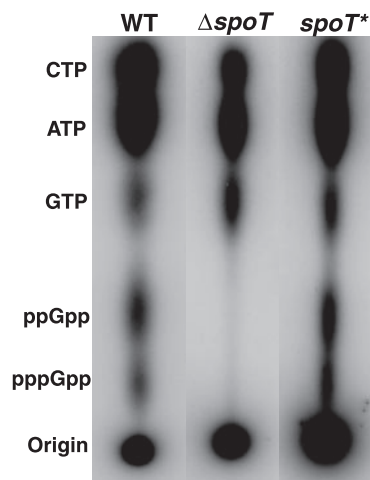


FIG. 2. The *H. pylori* $\Delta spoT$ mutant is deficient for (p)ppGpp production under nutrient deprivation conditions. WT, $\Delta spoT$, and $spoT^*$ cultures were incubated for 1 h in minimal medium (MOPS-MGS without mannitol or phosphate) in the presence of ^{32}P . Samples were resolved by thin-layer chromatography, and nucleotides were visualized with autoradiography. The WT and complemented strains produced abundant (p)ppGpp, whereas the $spoT$ mutant was defective for (p)ppGpp production.

Bacterial counts were conducted on three separate grids for each genotype at each time point with a total of more than 100 cells per genotype analyzed.

RESULTS

Construction of an HP0775 disruption mutation in *H. pylori*.

The *H. pylori* open reading frame (ORF) HP0775 is annotated as *spoT* and is 41% identical to the *C. jejuni spoT* gene. It is located in a putative operon with gene content similar to that of the *C. jejuni* gene, upstream of several genes that are likely to encode essential functions based on sequence and transposon mutagenesis studies (28). We engineered a nonpolar disruption of HP0775 by replacing nucleotides corresponding to amino acids 57 to 704 with a cassette encoding Kan^r flanked by ribosome-binding sites, lacking a transcriptional termination region, and containing a start codon in frame with the 3' end of the HP0775 ORF (Fig. 1B) (20). To verify that phenotypes observed with the HP0775::Kan^r strain (hereafter referred to as $\Delta spoT$) were due to loss of the HP0775 gene product, we engineered a complemented strain (hereafter referred to as $spoT^*$) in which the HP0775 ORF was introduced into the *rdxA* locus of the $\Delta rdxA::kan sacB$ strain by selecting and screening for a Suc^r and Kan^s phenotype. Because HP0775 lies in a large putative operon, the promoter sequence of which is not readily apparent, we expressed the copy of HP0775 inserted in the *rdx* gene with the urease promoter, which we had shown to be effective for transgene expression at this locus (6). The resultant $\Delta rdxA::ureP::HP0775$ strain was transformed with the HP0775::Kan^r construct to yield the $spoT^*$ strain.

The HP0775-deficient mutant fails to produce (p)ppGpp under nutrient deprivation conditions. To test whether the *H. pylori* HP0775 gene is required for production of (p)ppGpp, we subjected the $\Delta spoT$ mutant to nutrient deprivation, a condition which has been shown to induce (p)ppGpp in *H. pylori* (34). Wild-type G27 (WT), $\Delta spoT$, and $spoT^*$ strains were incubated in minimal media with ^{32}P for 1 h. As ex-

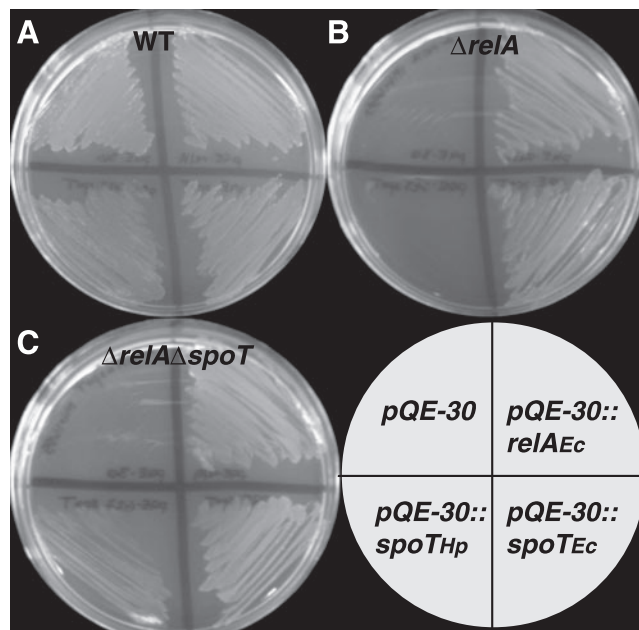


FIG. 3. The *H. pylori* $\Delta spoT$ gene complements the *E. coli* (p)ppGpp-null mutant. MG1655 *E. coli relA* and *spoT* and *H. pylori* HP0775 were cloned into the IPTG-inducible expression vector pQE-30. WT (A), $\Delta relA$ (B), and $\Delta relA \Delta spoT$ (C) *E. coli* strains were then transformed with the expression constructs shown in the key and plated onto M9 minimal medium plates supplemented with IPTG.

pected, the WT and $spoT^*$ strains accumulated significant amounts of (p)ppGpp upon exposure to this stress. In contrast, (p)ppGpp was absent from the $\Delta spoT$ mutant extracts (Fig. 2). Plate counts of parallel bacterial cultures before and after 1 h of incubation in minimal medium showed that all three strains remained viable during the experiment; thus, the absence of (p)ppGpp production in the $\Delta spoT$ strain was not due to bacterial death. These data suggest that HP0775 encodes the enzymatic activity responsible for (p)ppGpp production in *H. pylori*.

The *H. pylori* HP0775 gene functionally complements an *E. coli* (p)ppGpp-null mutant. In *E. coli*, (p)ppGpp metabolism is regulated by the RelA synthetase and the bifunctional synthetase/phosphohydrolase SpoT. Null mutations in *relA* give rise to strains that produce only basal levels of (p)ppGpp and are unable to grow on minimal medium plates (10). In *E. coli*, the *spoT* gene is essential, but a $\Delta relA \Delta spoT$ double mutant is viable, indicating the importance of regulating (p)ppGpp levels in the cell (37). The double mutant produces no detectable (p)ppGpp and is unable to grow on minimal medium plates.

To test whether the *H. pylori* HP0775 gene could functionally complement either of the (p)ppGpp biosynthetic genes in *E. coli*, we transformed *E. coli* strain MG1655 (WT) and isogenic mutant strains $\Delta relA$ and $\Delta relA \Delta spoT$ with IPTG-inducible expression constructs encoding *E. coli relA*, *E. coli spoT*, and *H. pylori* HP0775. The transformed *E. coli* strains were then plated on minimal medium plates in the presence of IPTG and assayed for growth. The growth of WT *E. coli* strains on minimal medium was not adversely affected by the presence of either *H. pylori* HP0775 or extra copies of *E. coli relA* or *spoT* (Fig. 3). The extrachromo-

somal copies of both *E. coli* genes were able to functionally complement both *E. coli* mutant strains, as was expected, except for the case of the complementation of the double mutant by *relA* (37), which we discuss below. Significantly, the *H. pylori* HP0775 gene was able to restore partial growth of the $\Delta relA \Delta spoT$ double mutant strain on minimal medium; however, it was unable to complement the $\Delta relA$ single mutant. We interpret this to mean that the *H. pylori* HP0775 gene at least partially complements the *E. coli relA/spoT* genes in vivo. This result supports the idea that *H. pylori* SpoT functions in vivo in (p)ppGpp metabolism and validates the annotation of HP0755 as *spoT*.

The *H. pylori* $\Delta spoT$ mutant has a stationary-phase survival defect. The importance of the stringent response in stationary-phase survival in *E. coli* and other gram-negative bacteria has been well documented. This phenomenon was thought to depend upon the alternative sigma factor *rpoS*; however, SpoT has recently been shown to be required for stationary-stage survival in *C. jejuni*, a bacterium that, similar to *H. pylori*, lacks *rpoS* (14). *H. pylori* strain G27 exhibits a characteristic growth curve in liquid culture with rapid growth over the first 24 h, followed by stationary phase and a gradual decline in viable counts over the following day (4). To test whether the *spoT* gene is required for any aspect of *H. pylori* growth, we assayed the viability of WT, $\Delta spoT$, and *spoT** liquid cultures over 50 h under standard laboratory growth conditions. All strains exhibited similar population dynamics during log and early stationary phases (Fig. 4 and data not shown). However, during late stationary phase, at approximately 50 h, the $\Delta spoT$ cultures exhibited significantly lower viability than the WT and *spoT** cultures (Fig. 4).

The *H. pylori* $\Delta spoT$ mutant initiates the coccoid transition prematurely. To further characterize the loss of viability of the $\Delta spoT$ cultures, we examined the cellular morphology of cultures grown over 48 h. Viable *H. pylori* organisms have a characteristic helical shape. Older cultures have been described to adopt a different cellular morphology, termed the coccoid state, in which the cells become rounded up and can no longer be cultured (15). We documented this transformation to the coccoid state over the 48-h growth curve of a wild-type culture (Fig. 5). We were able to identify several distinct morphologies along this trajectory (Fig. 5A to D). Early in the transition cells adopted a U-shaped morphology. Subsequently, the inner and then the outer membranes fused the two arms of the U into a single rounded cell, which often still contained flagella. The final coccoid state was distinct in that it lacked flagella, and its membrane appeared to be less electron dense. We classified the morphologies of cell populations of WT, $\Delta spoT$, and *spoT** liquid cultures at 12, 24, and 48 h for more than 100 cells for each strain at each time points. At the 12-h time point the three strains appeared similar and were largely helical. At 24 h, a striking difference was seen in the $\Delta spoT$ mutant culture, in which the majority of the cells had initiated the coccoid transition and adopted the U-shaped morphology, in contrast to the wild-type and complemented populations that were still largely helical in morphology. By 48 h the majority of the cells in all three populations were nonhelical, but the $\Delta spoT$ culture contained more coccoid cells than the WT and *spoT** cultures.

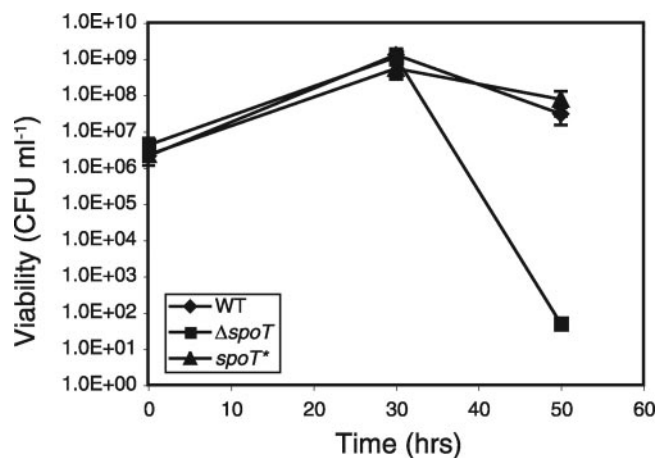


FIG. 4. The $\Delta spoT$ mutant has a stationary-phase survival defect. WT, $\Delta spoT$, and *spoT** *H. pylori* strains were grown by shaking under microaerophilic conditions at 37°C, and numbers of CFU ml⁻¹ were determined at the time points indicated. A representative assay of at least three trials is shown. Error bars indicate the standard deviations of replicate plating. The limit of detection of the assay was 10 CFU ml⁻¹.

The *H. pylori* $\Delta spoT$ strain is more sensitive to the environmental stresses of anaerobiosis and acidity. In numerous bacteria the stringent response has been identified as a key factor for survival during exposure to stressful environments. For *H. pylori*, the ability to survive stresses such as unfavorable atmospheric conditions and acidity are essential to its ability to infect and persist successfully within a human stomach. *H. pylori* is a microaerophilic and capnophilic and grows best at 3 to 7% O₂ and 5 to 10% CO₂, conditions similar to those in its gastric niche. A $\Delta spoT$ mutant of *C. jejuni*, which requires similar growth conditions, is extremely sensitive to ambient atmospheric conditions (14). We tested whether the stringent response might play a critical role in *H. pylori* survival during exposure to higher O₂ and lower CO₂ atmospheric conditions. We assayed viability while incubating WT, $\Delta spoT$, and *spoT** cultures in ambient atmospheric conditions for a 6-h time course. The WT and *spoT** cultures exhibited similar patterns of viability and declined gradually over the 6-h period. In four independent trials, the WT and *spoT** population sizes after 6 h of aerobic stress were within 2 orders of magnitude of each other, with one or the other showing a more rapid decline in different trials. In contrast, at between 4 and 6 h of exposure to ambient atmosphere, the $\Delta spoT$ cultures consistently exhibited a reduction in viability of 4 to 6 orders of magnitude relative to the WT and *spoT** strains (Fig. 6A).

The ability of *H. pylori* to survive in an acidic environment is critical to its ability to chronically infect the human stomach. Acid exposure has been shown to elicit (p)ppGpp production in WT *H. pylori* cultures (34). To test the role of the *H. pylori* stringent response in acid stress survival, WT, $\Delta spoT$, and *spoT** strains were grown in pH 4 growth medium supplemented with 0.5 mM urea for 3 h, during which time period the acidity of the media remained unchanged. During the first 2 h of acidic exposure all strains were relatively unaffected by the treatment. After 3 h, however, the $\Delta spoT$ strain consistently exhibited a significant decrease in viability relative to the WT and *spoT** strains (Fig. 6B). In three independent trials the

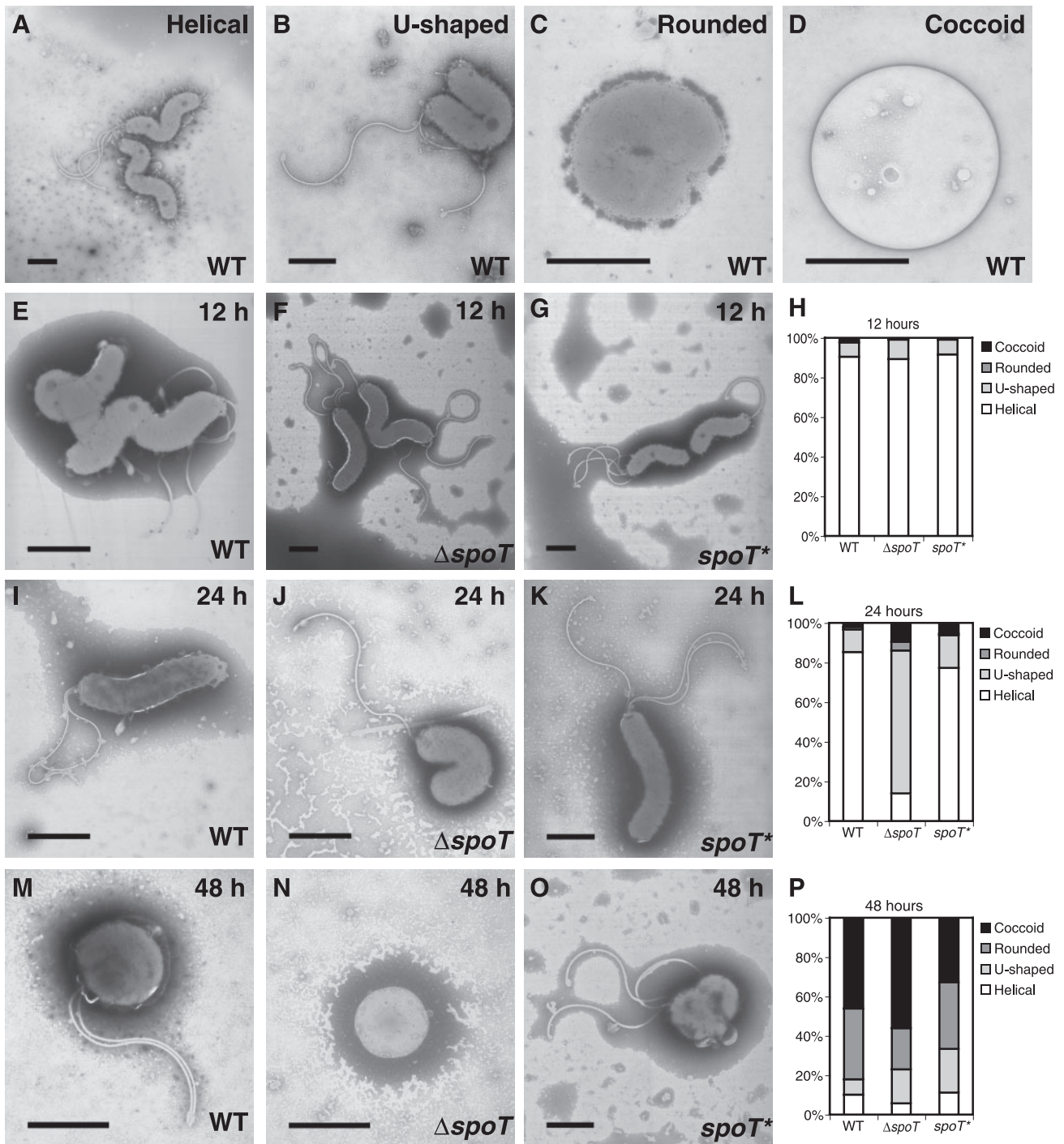


FIG. 5. The $\Delta spoT$ mutant initiates the coccoid transformation prematurely. Transmission electron micrographs of WT (A-D, E, I, and M), $\Delta spoT$ (F, J, and N), and $spoT^*$ (G, K, and O) cells during growth in liquid culture over 48 h. Typical stages of the helical-to-coccoid transformation (A to D). Representative cells for each culture at 12 (E to G), 24 (I to K), and 48 (M to O) hours are shown. The distributions of morphologies of more than 100 cells of each genotype for each time point are shown (H, L, and P). Bars, 1 μ m.

$spoT^*$ strain exhibited a slight but significant increase in viability relative to the WT strain at 3 h, which may have been due to the fact that the levels of the $spoT$ gene in the $spoT^*$ strain, expressed from the acid-inducible urease promoter (25), may have exceeded those in the WT strain and conferred extra protection against the acid stress. These data indicate that the

H. pylori stringent response is required for survival of specific environmental stresses.

DISCUSSION

H. pylori is one of the most widespread pathogens of humans, yet its survival strategies during infection and transmis-

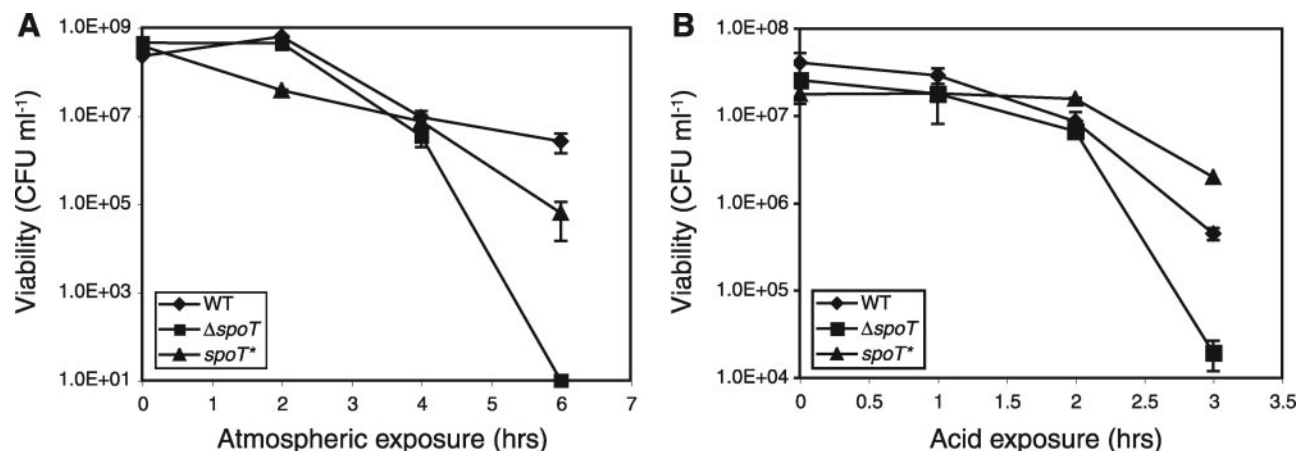


FIG. 6. The $\Delta spoT$ mutant has impaired survival in aerobic and acidic stress. (A) WT, $\Delta spoT$, and $spoT^*$ *H. pylori* strains were incubated under ambient atmospheric conditions at 37°C and shaken at 150 rpm. (B) WT, $\Delta spoT$, and $spoT^*$ *H. pylori* strains were incubated by shaking in pH 4 media with 0.5 mM urea, at 37°C. Representative assays of at least three trials are shown. Error bars indicate the standard deviations of replicate plating. The limits of detection of the assays were 10 CFU ml⁻¹ in panel A and 100 CFU ml⁻¹ in panel B.

sion between human hosts are not well understood. The compact *H. pylori* genome encodes few transcription factors, raising the question of how this organism adapts to the constantly changing environment of the human stomach. The experiments described here demonstrate that *H. pylori* has a functional homologue of the stringent response regulator SpoT responsible for (p)ppGpp synthesis. This small molecule alarmone is a global regulator of transcription in *E. coli* that functions through direct interaction with RNA polymerase (3). One consequence of ppGpp binding is to alter polymerase affinity for certain promoters, thus conferring a level of transcriptional regulation independent of the organism's complement of transcriptional activators and repressors (17). The global transcriptional regulation of the stringent response may be especially important for bacterial species such as *H. pylori* with limited repertoires of transcriptional regulators.

SpoT function is conserved between *H. pylori* and *E. coli*. Our data indicate that the *H. pylori* *spoT* gene not only is required for (p)ppGpp synthesis in *H. pylori* but that it can also functionally complement the minimal medium growth defect of an *E. coli* *relA* and *spoT* double mutant lacking all (p)ppGpp synthetase and hydrolase functions. Interestingly, the *H. pylori* *spoT* gene was not able to rescue an *E. coli* *relA* single mutant, possibly because its (p)ppGpp hydrolase activity prevents accumulation of sufficiently high (p)ppGpp levels for cell survival under starvation conditions. Unexpectedly, despite the fact that an *E. coli* *spoT*-null allele is lethal in the presence of a wild-type copy of *relA* (37), we were able to generate the $\Delta relA$ $\Delta spoT$ double mutant with an extrachromosomal copy of the *E. coli* *relA* gene. The viability of this strain is likely due to the fact that under the conditions of the strain construction (abundant nutrients and no IPTG), the *relA* transgene was not induced, thus mimicking the situation of a strong loss-of-function *relA* allele (*relA1*) in a *spoT*-null mutant background, which has been shown to be viable (37). The fact that induction of *relA* under starvation conditions was not toxic to the double mutant and could promote its growth on minimal media (lacking nutrients and containing IPTG) leads us to hypothesize that (p)ppGpp synthetase activity in the absence of hydrolase activity (the situation in a *relA*⁺ *spoT* cell) is toxic to the cell

only under conditions when levels of (p)ppGpp are normally low. Under nutrient starvation conditions, however, when (p)ppGpp is normally abundant, we speculate that cells can tolerate RelA activity that is not counteracted by SpoT activity. Our (p)ppGpp labeling and complementation data, together with conservation of key residues in the hydrolytic domain (23) and the absence of another putative (p)ppGpp hydrolase in the *H. pylori* genome, indicate that the *H. pylori* *spoT* gene encodes a bifunctional enzyme that is essential for the production and hydrolysis of the stringent response alarmone (p)ppGpp.

Loss of stationary-phase viability is preceded by a premature coccoid transition in the $\Delta spoT$ mutant. In several bacteria, including *E. coli* (37) and *C. jejuni* (14), mutants lacking (p)ppGpp production exhibit aberrant elongated morphologies. *H. pylori* $\Delta spoT$ mutants undergo a precipitous decline in viability by 50 h of growth in liquid culture. This decline in viability is preceded a day earlier by a striking change in cell morphology. Unlike *E. coli* and *C. jejuni*, however, the *H. pylori* $\Delta spoT$ mutant appears to enter prematurely into U-shaped and coccoid morphologies similar to those normally observed during later growth stages in wild-type *H. pylori* strains. The biological significance of the coccoid state has been greatly debated. Some have proposed that it represents a dormant, spore-like stage and is possibly the transmissible form of the organism (24), although analysis of the proteome content argues against this model (8). Our observation that more of the $\Delta spoT$ mutant cells appeared coccoid at 24 and 48 h than the wild-type population is consistent with a role for *spoT* in protection against stationary-phase stresses, but it cannot in itself account for the dramatic difference in viability between these populations at 50 h. The fact that *H. pylori* precociously adopts the coccoid form in the absence of *spoT* argues against the coccoid morphology being part of a developmental program of stress resistance that is regulated by the stringent response, as in *Bacillus subtilis* (26).

SpoT confers protection against specific environmental stresses. Besides being impaired in stationary-phase survival, the $\Delta spoT$ mutant is susceptible to specific environmental stresses, including atmospheric exposure (containing higher oxygen and lower carbon dioxide levels than are optimal for *H.*

pylori growth) and growth in acidified media. Both of these stresses are likely encountered by *H. pylori* during transmission between hosts and transit through the gastric lumen before the bacteria can situate themselves in the neutral pH environment of the gastric mucosa (27). Acid exposure has been shown to elicit numerous transcriptional changes in *H. pylori* with a great deal of variation depending on the specific acid exposure regimen (2, 9, 22, 36). In one study, the *gppA* component of the (p)ppGpp metabolic pathway was found to be upregulated in acidic media (22). Specific transcriptional regulators, including the metal-responsive transcription factors NikR and Fur (9, 13, 33) and the two-component ArsRS system (25), have been shown to control gene expression in response to acid. Acid shock was also recently found to induce (p)ppGpp production in *H. pylori* under nonstarvation (i.e., rich medium) conditions (34). Together, these data are consistent with a model in which global transcriptional regulation by (p)ppGpp signaling provides an additional level of gene expression control that protects *H. pylori* from the specific stresses of its environment.

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