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Polyphosphate Binds to the Principal Sigma Factor of RNA Polymerase during Starvation Response in *Helicobacter pylori*

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Summary

Helicobacter pylori persists deep in the human gastric mucus layer in a harsh, nutrient-poor environment. Survival under these conditions depends on the ability of this human pathogen to invoke starvation/stress responses when needed. Unlike many bacteria, *H. pylori* lacks starvation/stress-responding alternative sigma factors, suggesting an additional mechanism might have evolved in this bacterium. *H. pylori* produces polyphosphate, however, the role and target of polyphosphate during starvation/stress have not been identified. Here we show that polyphosphate accumulated during nutrient starvation directly targets transcriptional machinery by binding to the principal sigma factor in *H. pylori*, uncovering a novel mechanism in microbial stress response. A positively-charged-Lys-rich region at the NTD of the major sigma factor is identified as the binding region for polyphosphate (region P) *in vivo* and *in vitro*, revealing a new element in sigma 70 family proteins. This interaction is biologically significant because mutant strains defective in the interaction undergo premature cell death during starvation. We suggested that polyphosphate is a second messenger employed by *H. pylori* to mediate gene expression during starvation/stress. The putative “region P” is present in sigma factors of other human pathogens, suggesting that the uncovered interaction might be a general strategy employed by other pathogens.

Keywords

Helicobacter pylori; Polyphosphate; Sigma factor; Stress responses; Bacterial gene regulation

Introduction

About one half of the world population is infected with *Helicobacter pylori*, the causative agent of human gastritis and ulcers; and the infection is associated with gastric cancer (Marshall & Warren, 1984, Peek & Blaser, 2002, Fox & Wang, 2007). *H. pylori* colonizes the human gastric mucosa, where it encounters a constantly changing environment with poor nutrients and challenges from the host innate immune response (Schreiber *et al.*, 2004, Monack *et al.*, 2004, Allen, 2007). Thus, in order for *H. pylori* to persist in the host, it is critical for the bacterium to invoke a stress response when needed.

In eubacteria, typically, most transcription under regular growth conditions is initiated by RNA polymerase holoenzyme carrying a principal or housekeeping σ factor encoded by the *rpoD* gene. Multiple alternative σ factors control specialized regulons that are activated

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during different stress conditions (Gruber & Gross, 2003, Hengge, 2009, Paget & Helmann, 2003). Due to its small genome size, the *H. pylori* transcription machinery is simpler (Berg *et al.*, 1997) compared to that of other bacteria such as *E. coli*. For example, *H. pylori* has only three σ factors, the primary sigma factor σ_{80} and two alternative σ factors, σ_{28} and σ_{54} . The fact that *H. pylori* lacks the three starvation and stress-responding alternative sigma factors σ^S , σ^H and σ^E suggests that an additional stress-response mechanism might have evolved in this pathogen. Interestingly, *H. pylori* σ_{80} (Beier *et al.*, 1998), a member of the σ_{70} family proteins, has ~70 extra amino acid residues before conserved “region 1” at the N-terminal domain (NTD) compared to *E. coli* σ_{70} . The role of this extra region at the NTD of σ_{80} is unknown.

Two small alarmone molecules, pppGpp and ppGpp [(p)ppGpp], which are associated with the stringent response (Cashel *et al.*, 1996), are required for *H. pylori* survival in the stationary phase, during exposure to low pH, aerobic shock and macrophages (Mouery *et al.*, 2006, Wells & Gaynor, 2006, Zhou *et al.*, 2008). In *H. pylori* there is no RelA, and the SpoT protein is the only stringent response factor responsible for the synthesis and degradation of (p)ppGpp. Recently, we found that SpoT mediates a serum starvation response, which restricts cellular metabolic activity and limits cell growth while maintaining cell vitality for a long duration *in vitro* (Zhou *et al.*, 2008). In contrast to wild type, the *spoT* mutant exhibits the “relaxed” growth phenotype, which confers the ability to grow to a higher density than wild type leading to premature cell death during serum starvation. Thus, *H. pylori* SpoT or (p)ppGpp regulates cell growth in response to nutrient starvation and stress.

Inorganic polyphosphate (polyP), a polyanion “molecular fossil” present in all living cells, is a regulator of responses to microbial stresses including nutrient deprivation (Kornberg *et al.*, 1999, Rao *et al.*, 2009). For example, polyP promotes ribosomal protein degradation by the Lon protease to supply amino acids needed to respond to starvation in *E. coli* (Kuroda *et al.*, 2001). PolyP accumulation is regulated by (p)ppGpp, which inhibits the activity of exopolyphosphatase (PPX) in *E. coli* (Kuroda *et al.*, 1997). *H. pylori* is able to produce polyP; but its role in the starvation/stress response and its regulation is unknown. Here we show that polyP forms stable complexes with σ_{80} during serum starvation in wild type *H. pylori* but not in the *spoT* mutant, and that the binding site for polyP lies in the NTD of σ_{80} . Disruption of polyP synthesis or deletion of σ_{80} NTD similarly eliminates response to starvation in *H. pylori* leading to premature cell death. These findings reveal a novel strategy for *H. pylori* to respond to starvation through direct targeting of transcriptional machinery by ubiquitous polyP molecules.

Results and Discussion

σ_{80} is modified during serum starvation response

We recently showed that the sole stringent response factor SpoT in *H. pylori* mediates a serum-starvation response to protect cells from premature death (Zhou *et al.*, 2008). In this study, we searched for differences between wild type and the *spoT* mutant cells in the levels of transcriptional factors, such as σ_{80} , in response to serum starvation. *H. pylori* wild type strain G27 and its isogenic $\Delta spoT$ mutant were grown in a serum-free medium, and cultures were sampled at different time points. After cells were lysed by SDS, boiled cell lysates were analyzed by SDS-PAGE, followed by Western Blot with the σ_{80} -specific polyclonal antibodies. Intriguingly, the mobility of σ_{80} was notably different between wild type and the *spoT* mutant cells, whereas the intracellular levels of σ_{80} appeared to be not significantly changed during the growth of both strains (Fig. 1A). The mobility of σ_{80} from wild type cells was significantly retarded or “up-shifted” as smeared bands from one to three days growth but became normal after day four. Under this physiological condition, wild type cells were restricted in growth and their cellular metabolic activity was limited, but they

maintained viability after culturing for four days, while the $\Delta spoT$ mutant cells had a brief burst of growth for the first two days followed by rapid cell death afterward (Zhou et al., 2008). Although most of the $\Delta spoT$ mutant cells were non-cultivable at the later time points, comparable amount of $\sigma 80$ was detected during the course of the experiment, suggesting that those non-vital cells were still intact. The $\sigma 80$ up-shift required the SpoT function, as $\sigma 80$ was un-shifted in the $\Delta spoT$ mutant under the same growth conditions. The $\sigma 80$ up-shift was in response to serum starvation, as $\sigma 80$ mobility was unchanged when wild type cells were grown in a serum-supplemented medium (Fig. 1B). Thus, the $\sigma 80$ up-shift is a manifestation of the SpoT-mediated serum starvation response.

Binding of polyphosphate modifies $\sigma 80$

The $\sigma 80$ up-shift suggested a tight association of $\sigma 80$ with a putative agent, covalent modifications, or a major conformational change, resistant to SDS and boiling treatments in the protein. A cell lysate from which $\sigma 80$ was up-shifted was hereafter named a shifted lysate; and a cell lysate from which $\sigma 80$ was un-shifted an un-shifted lysate. The addition of increasing amounts of a shifted lysate to a fixed amount of an un-shifted lysate caused reduction of the mobility of $\sigma 80$ in the latter (Fig. 2A, lanes 3–6), gradually reaching the mobility of $\sigma 80$ in the shifted lysate (lane 1). These results demonstrated that a shifted lysate contained an agent that quantitatively modified $\sigma 80$ *in trans*. We purified the agent from shifted lysates by this assay.

We identified this agent as polyphosphate (polyP) based on several analyses. 1) The purified agent was positively stained pink with Toluidine blue O, a dye used for staining polyP (Kumble & Kornberg, 1995, Tijssen *et al.*, 1981) as seen with synthetic polyP₆₆ (Fig. 2B). Both the agent and polyP₆₆ appeared heterogeneous in size, characteristic of polyP molecules (Clark & Wood, 1987), which could explain why the up-shifted $\sigma 80$ appeared as smeared bands (Fig. 1A). The agent migrated slower than polyP₆₆ in the gel, suggesting that it had a longer average chain length than polyP₆₆. 2) Upon binding the purified agent, the fluorescent probe 4',6-diamidino-2-phenylindole (DAPI) shifted its peak emission from 475 nm to ~ 510 nm, a known property for polyP (Allan & Miller, 1980) as seen with polyP₆₆ (Fig. 2C). 3) The $\sigma 80$ up-shifting activity of the agent was sensitive to calf intestinal alkaline phosphatase (CIAP) and *E. coli* exo-polyphosphatase (PPX) treatments, two enzymes known to degrade polyP *in vitro* (Lorenz & Schroder, 2001, Akiyama *et al.*, 1993), whereas it was resistant to both DNase and RNase treatments (Fig. 2D). 4) Finally, addition of polyP₆₆ to an un-shifted lysate caused the $\sigma 80$ up-shift (Fig. 2E). These data positively identified the $\sigma 80$ up-shifting agent as polyP molecule that tightly binds to $\sigma 80$ during serum starvation.

To examine whether some other property of the *H. pylori* cells would be required to facilitate the interaction between polyP and $\sigma 80$, we determined the behavior of *H. pylori* $\sigma 80$ expressed in *E. coli* cells containing the pQE80-*rpoD* (Hp). The cells were grown in LB and then down-shifted to MOPS medium with a low concentration of Pi (0.1 mM) and without amino acids. While the polyP level is minimal when *E. coli* cells are grown in LB, cells accumulate large amounts of polyP₇₅₀ (polyP molecules with an average chain length of ~ 750 Pi residues) after the nutrient downshift (Ault-Riche *et al.*, 1998). Before the nutrient downshift, the mobility of $\sigma 80$ produced in *E. coli* was essentially unchanged (at time 0); however, all $\sigma 80$ proteins were up-shifted after the nutrient downshift (at 1.5 to 3 hours) (Fig. 2F). In contrast, the mobility of *E. coli* $\sigma 70$ was unchanged under the same growth conditions. These results indicated that no other element unique to *H. pylori* cells is required for the interaction between polyP and $\sigma 80$, and that the strong interaction between polyP and the primary sigma factor is specific for *H. pylori* as no such interaction is detected with $\sigma 70$ in *E. coli*.

The amino-terminal domain of $\sigma 80$ is the binding site for polyP

Compared to *E. coli* $\sigma 70$ and other $\sigma 70$ family proteins, there are ~70 additional amino acid residues at the amino-terminal domain (NTD) of $\sigma 80$ in the *H. pylori* G27 strain (Fig. 3A), which account for the difference in molecular weight of the two principal σ factors. Notably, this extra region of $\sigma 80$ is highly enriched with Lys (~33%) and has twice as many positively charged residues (~34%) as those that are negatively charged (~17%). The intact $\sigma 80$ protein is a basic protein with an estimated pI of 8.4. Without this extra region, the truncated $\sigma 80$ would have a theoretical pI value of 5.5, thus resembling an acidic $\sigma 70$ protein from *E. coli*.

To test if the NTD is a target for polyP, we engineered a deletion of 56 amino acid residues of the NTD of $\sigma 80$ including 19 Lys and 1 Arg residues ($\sigma 80\Delta 56$). Recombinant wild type and the mutant $\sigma 80\Delta 56$ proteins were purified and their ability to bind polyP₆₆ *in vitro* was examined. PolyP and wild type $\sigma 80$ protein formed stable complexes in the purified system (Fig. 3C, lane 2), demonstrating polyP is necessary and sufficient to cause $\sigma 80$ up-shift in the cell lysates. In contrast, neither the recombinant $\sigma 80\Delta 56$, nor the *E. coli* $\sigma 70$ protein bound polyP under the same conditions, as no changes in the mobility of the two proteins were detected in the presence of polyP.

When the same samples were analyzed using a non-denaturing gel in the absence of SDS, however, the $\sigma 80$ protein did not migrate into the gel (data not shown). Thus, we performed coimmunoprecipitation assay using purified components to determine the interaction between polyP and the NTD of $\sigma 80$ under native conditions. PolyP and wild type $\sigma 80$ protein formed stable complexes as they were coimmunoprecipitated (Fig. 3D, lane 2), demonstrating that the uncovered interaction between polyP and $\sigma 80$ is biologically significant. No such complex was detected with the $\sigma 80\Delta 56$ protein under the same condition *in vitro* (Fig. 3D, lane 4). As described below, the $\sigma 80\Delta 56$ protein in the *H. pylori* mutant containing a chromosomal *rpoD*($\sigma 80\Delta 56$) deletion was un-shifted during serum starvation. The data unambiguously identified the NTD of $\sigma 80$, which we named “region P” as a positively-charged-Lys-rich region and the binding site for polyP, a new element in $\sigma 70$ family proteins.

This unique feature (a positively-charged-Lys-rich region) of the NTD of $\sigma 80$ is conserved among different *H. pylori* strains whose genome sequences are available at this time, although there are some variations in the numbers of Lys residues within the region. For example, compared to the NTD of $\sigma 80$ protein from the G27 strain, there is an 11 aa deletion including four Lys residues in the counterpart from the *H. pylori* 26695 strain; however, the $\sigma 80$ protein in cell lysates of the 26695 strain was still up-shifted in the presence of polyP (data not shown). Our data indicated strongly that the interaction of polyP and $\sigma 80$ is likely a common mechanism conserved in *H. pylori*. The factors affecting the interaction remain to be determined. Bioinformatic analysis of the $\sigma 70$ family proteins from the database suggested that putative “region P” is present in primary σ factors of other human pathogens including *Bordetella pertussis* and *Coxiella burnetii* (Fig. 3B). It remains to be tested whether those σ factors also bind polyP during microbial starvation/stress.

PolyP- $\sigma 80$ complex is critical for bacterial persistence

Next, we evaluated the biological significance of the interaction between $\sigma 80$ and polyP during serum starvation by mutational analysis. We made an isogenic *H. pylori* strain carrying a chromosomal *rpoD* deletion ($\sigma 80\Delta 56$) missing the “region P” and another with a null ΔppK mutation rendering the cell incapable of synthesizing polyP (Tan *et al.*, 2005). Both mutants grew as well as the wild type, as the sizes of colonies of the two mutants were comparable to that of wild type on serum-supplemented plates after incubation at 37°C for

3–4 days (data not shown). This result indicated that $\sigma 80\Delta 56$ remained functional under regular growth conditions.

In contrast to wild type cells, $\sigma 80$ was un-shifted during serum starvation in the $\sigma 80\Delta 56$ and ΔppK mutant cells, just as in the $\Delta spoT$ mutant cells (Fig. 4A, see also Fig. 1A). These results confirmed that the region P of $\sigma 80$ is the target site for polyP *in vivo*. Importantly, the survival rates of the $\sigma 80\Delta 56$ and the ΔppK mutant cells grown in a serum-free medium were significantly reduced compared to wild type, as measured by colony forming unit (CFU) determination. The reduction was most pronounced at days 3–5 of the starvation approaching the lowest survival rate of the $\Delta spoT$ mutant cells (Fig. 4B). These data revealed a strong correlation between a lack of $\sigma 80$ -polyP complex in the starved cells and a premature death phenotype arguing that the interaction between $\sigma 80$ and polyP is critical for *H. pylori* survival during serum starvation, and they suggested a potential new target for drug design against the pathogen. The previously reported deficiency of other *ppK* mutants in colonization of mice might also derive from the inability of $\sigma 80$ to regulate the starvation response in the absence of polyP (Tan et al., 2005, Ayraud et al., 2005). If this is the case, one would expect the $\sigma 80\Delta 56$ and $\Delta spoT$ mutants to be defective in mouse colonization similarly to the *ppK* mutants because the interaction between $\sigma 80$ and polyP is prevented in all of the three mutants.

The $\sigma 80$ up-shift occurred in wild type cells grown in serum-free medium but not in the cells grown in serum-supplemented medium suggesting that polyP levels would be higher during nutrient starvation; and further, the absence of the $\sigma 80$ up-shift in the *spoT* mutant suggested that (p)ppGpp would positively regulate the accumulation of polyP in *H. pylori* (Fig. 1A). Indeed, the polyP levels were over five-fold higher in the wild type cells starved for serum for 48 hours than that in the cells without the starvation; and as expected, the polyP levels were very low in the *spoT* mutant cells under the same conditions (Fig. 5A).

The determinants during starvation/stress response in *H. pylori* are summarized in Fig. 5B. PolyP accumulation is likely linked to the starvation/stress response, just as in *E. coli*. In *E. coli*, the level of polyP increases during amino acid starvation and by exposure to other stresses (Ault-Riche et al., 1998), mainly because accumulation of (p)ppGpp during stress response or the stringent response (Cashel et al., 1996) inhibits the activity of exopolyphosphatase (PPX) (Kuroda et al., 1997) that breaks down polyP. *H. pylori* lacks the gene for PPX but contains a homologous guanosine pentaphosphate phosphohydrolase gene for GppA that is also an exopolyphosphatase (Keasling et al., 1993) similar to PPX. In *H. pylori*, an increased level of (p)ppGpp, which is synthesized by the SpoT enzyme upon stress and nutrient downshift (Mouery et al., 2006, Wells & Gaynor, 2006), is likely to inhibit GppA activity, but not the activity of polyphosphate kinase (PPK), the enzyme for synthesis of polyP, causing accumulation of polyP in the cell. Therefore, a lack of (p)ppGpp synthesis in the $\Delta spoT$ mutant could account for a reduced polyP level causing no change in $\sigma 80$ mobility during serum starvation in the mutant strain cells (Fig. 1A). Moreover, studying the interaction of these determinants would provide a possible explanation why the mobility of $\sigma 80$ returned to normal in wild type cells after day 4 during serum starvation (Fig. 1A). The effects of other stresses on the polyP production and the regulation of synthesis and/or degradation of polyP in *H. pylori* are currently under investigation.

The mode of action of polyP in *H. pylori* differs, however, from that in *E. coli*. PolyP stimulates Lon protease activity to degrade ribosomal proteins supplying the amino acids needed to starved *E. coli* cells (Kuroda et al., 2001). In *H. pylori*, polyP targets the principal σ factor. PolyP unlikely mediates the degradation of $\sigma 80$ as levels of $\sigma 80$ appeared to be not significantly changed upon serum starvation in the wild type *H. pylori* cells. Our results support the hypothesis that in responding to nutrient starvation/stress, an elevated cellular

level of polyP acts as a second messenger to control gene regulation and pathogenesis by directly modulating the activity of transcriptional machinery in the cell. We are currently testing this working model experimentally.

Summary remarks

Our study revealed a novel mechanism exhibited by a human pathogen in responding to stress caused by starvation. During a SpoT mediated serum starvation response in *H. pylori*, polyP forms a strong association with $\sigma 80$. Such an interaction is critical for the bacterial persistence during nutrient depletion, a likely environment deep in human gastric mucus layer where the pathogen lives. A positively-charged-Lys-rich region at the NTD of $\sigma 80$ is identified as the binding region for polyP (region P) *in vivo* and *in vitro*, revealing a new element for $\sigma 70$ family proteins. Putative “region P” is present in primary sigma factors of other human pathogens including *Bordetella pertussis* and *Coxiella burnetii*, suggesting that the uncovered interaction might be a general strategy employed by other pathogens to cope with starvation/stress.

Experimental procedures

Bacterial strains and general methods

Bacterial techniques, and growth conditions for *H. pylori* either with a serum-free or serum-supplemented medium (BLBB) under microaerophilic environment (5% O₂, 10% CO₂ and 85% N₂) are as described (Zhou et al., 2008). Unless otherwise mentioned, all *H. pylori* strains used are G27 and its derivatives, and cells were grown serum-supplemented, either in broth or solid agar plate. *H. pylori* strains containing the $\Delta ppK1-cat$ mutation (Tan et al., 2005) were kindly provided by Douglas E. Berg, Washington University Medical School, St. Louis, Missouri. The $\Delta ppK1-cat$ mutation was introduced to the G27 strain by transformation using the mutant's genomic DNA, and chloramphenicol (8 μ g/ml) resistant recombinants were selected. The G27 strain containing the $\Delta ppK1-cat$ mutation was named ΔppK . The *rpoD* NTD deletion mutation ($\sigma 80\Delta$ NTD2-57 or $\sigma 80\Delta 56$) was constructed by allelic exchange after cells were transformed with a PCR DNA fragment (~ 2.2 kb), which contained 5'-DNA sequence (NC_011333.1:95106..94374) located immediately upstream of the *rpoD* gene, the nonpolar *aphA-3* gene, a ribosome binding site and ATG, and followed by the *rpoD* sequence encoding the 58th to 273rd amino acid residues of $\sigma 80$ (NC_011333.1:94202..93551), and kanamycin (15 μ g/ml) resistant recombinants were selected. The strategy of linking several overlapped PCR products generated with appropriate primers (sequences available on request) to make this 2.2 kb PCR DNA fragment is as described (Zhou et al., 2008). The *rpoD* NTD deletion mutation was verified by PCR with appropriate primers and DNA sequencing.

Cloning of the *H. pylori rpoD* gene and its derivatives, expression and purification of the recombinant $\sigma 80$ proteins in *E. coli*

Standard molecular biology and DNA techniques are as described (Sambrook *et al.*, 1989). DNA fragments containing the *H. pylori rpoD* gene and its derivatives flanked by BamHI and PstI sites from *H. pylori* DNA were generated by PCR with appropriate primers (sequences available on request) and were cloned into the BamHI and PstI sites of vector pQE80L (Qiagen). The resulting wild type recombinant *rpoD* construct and its derivatives encoded recombinant $\sigma 80$ proteins containing the additional sequence MRGSHHHHHHGS at their amino-terminus. These clones were transformed into *E. coli* K12 strains and their sequence was confirmed by DNA sequencing.

To overproduce the recombinant $\sigma 80$ proteins from cells harboring the pQE80-*rpoD* (Hp) constructs, mid-log cultures (LB containing 50 μ g/ml of ampicillin) were induced with IPTG

(1 mM) for about 2h before cells were harvested. Most of the overproduced proteins were soluble, and they were purified by Talon superflow metal affinity resin (Clontech, Palo Alto, CA) using a procedure essentially as described for the purification of the recombinant *E. coli* $\sigma 70$ protein (Zhi & Jin, 2003). Rabbit polyclonal antibodies against purified recombinant wild type $\sigma 80$ and $\sigma 70$ proteins were prepared for us by Spring Valley Laboratories, Inc. MD.

SDS-PAGE and Western Blots

After cultures were sampled (0.1–1 ml depending on optical density (OD) at a wavelength of 600 nm), cells were spun down. The cell pellets were resuspended with 0.1 ml of protein loading buffer containing SDS (Invitrogen) and heat boiled for 5–10 min to lyse cells. About 5–10 μ l of the resulted cell lysates (usually from cells of OD₆₀₀ 0.005–0.015 unit, which corresponded to 1/20 to 1/10 of the original cultures sampled) were analyzed by 4–12% SDS NuPAGE (Invitrogen), followed by Western Blot with $\sigma 80$ specific polyclonal antibodies. In each set of experiments, an equal amount of cell lysates was analyzed.

Nutrient downshift experiments in *E. coli*

To determine the behavior of $\sigma 70$ in *E. coli* cells accumulating large amounts of polyP, earlier stationary phase cultures (OD₆₀₀ ~ 0.9) in LB (as time 0) were nutrient downshifted to MOPS medium with a low concentration of Pi (0.1 mM) and without amino acids for three hours as described (Ault-Riche et al., 1998). Similarly, *E. coli* cells harboring the pQE80-*rpoD* (Hp) were grown in LB supplemented with ampiciline (50 μ g/ml) to late-log phase (OD₆₀₀ ~ 0.5), and then IPTG (1 mM) was added to induce the expression of $\sigma 80$ for 90 min. After samples were taken (as time 0), cultures were nutrient downshifted to the same minimal medium supplemented with ampiciline (50 μ g/ml) and IPTG (1 mM) for three hours. Samples were prepared and analyzed by SDS-PAGE and Western blots as described above, and the $\sigma 70$ protein was detected by $\sigma 70$ specific polyclonal antibodies.

Extraction and analyses of the agent that caused $\sigma 80$ up-shift, and polyP assays

The agent was extracted from shifted cell lysates (from ~15 ml cultures) using a procedure that was originally developed for isolation of plasmid DNA from *H. pylori* (De Ungria et al., 1998), and after a final step of isopropanol precipitation, the resulting precipitates were resuspended in ~50 μ l H₂O. As little as 0.5 μ l of the purified agent caused $\sigma 80$ up-shift from un-shifted lysates as measured by the $\sigma 80$ up-shift assay.

The purified agent (~10 μ l) and polyP₆₆ (~ 2 μ g) [heterogeneous molecules with an average chain length of ~ 66 inorganic phosphate (P_i) residues, purchased from Sigma] in 10 mM Tris-HCl (pH 8.0) buffer were analyzed by 6% urea-PAGE until the dye bromophenol blue reached 3 cm from the bottom of the gel, followed by staining with Toluidine Blue O (Sigma), as described (Kumble & Kornberg, 1995). DAPI fluorescence in the presence of the purified agent (5 μ l) or polyP₆₆ (0.2 mg/ml) was analyzed essentially as described (Allan & Miller, 1980), except that a SpectraMax Gemini EM Fluorescence microplate reader (Molecular Devices) was used. Samples were prepared in 25 mM Tris-HCl buffer (pH. 7.0), 1 M glycerol, either with or without DAPI (20 μ g/ml). Samples were excited at 372 nm and fluorescence emission spectra were recorded. To test the sensitivity of the purified agent toward different enzymes, 1 μ l of the agent was treated with an indicated enzyme in a 10 μ l reaction first, and then the reaction mixtures were precipitated with isopropanol. Each of the precipitates was resuspended in 8 μ l of H₂O and 4 μ l were used for the $\sigma 80$ up-shift assays. For the control without treatment, 0.5 μ l of the agent was used. Calf intestinal alkaline phosphatase (New England Biolabs), DNase and RNase (Invitrogen) were used according to the manufacturer's instructions. *E. coli* recombinant exo-polyphosphatase (PPX) was

purified from *E. coli* cells harboring pQE80-*ppX* (Ec) essentially as described above for $\sigma 80$ proteins purification, and it was used as described (Akiyama et al., 1993).

PolyP was extracted from cultures of *H. pylori* wild type and the *spoT* mutant cells grown either serum-free or serum-supplemented for 48 hours using Glassmilk (MP Biomedicals) as described (Ault-Riche et al., 1998). PolyP was quantified in terms of Pi residues by a two-enzyme assay as described (Ault-Riche et al., 1998). PolyP was first converted to ATP in the presence of ADP by *E. coli* polyphosphate kinase (PPK) (a gift from Wenqing Xu, University of Washington, Seattle, WA), and then ATP was measured by a luciferase reaction (CellTiter-Glo, Promega) as described (Zhou et al., 2008). A standard curve for ATP (1 nM to 1 μ M) was used for calibration. Protein concentration in cell lysates was determined using a DC Protein Assay Reagent (Bio-Rad) using a BSA standard.

The $\sigma 80$ up-shift assay, determination of the polyP-binding region of $\sigma 80$ and coimmunoprecipitation assay

For the $\sigma 80$ up-shift assay, unless otherwise indicated, an un-shifted lysate (typically from cells of OD₆₀₀ ~0.015) was mixed with either a shifted lysate, the purified agent (0.5 μ l), or polyP₆₆ (0.25 μ g) for 10 min at 37 °C. The mixtures were analyzed by SDS-PAGE and Western Blot as described above.

To detect the formation of $\sigma 80$ -polyP complexes and determine the polyP-binding region of $\sigma 80$ using purified components, each of the purified proteins (~ 0.4 μ g) was used alone or mixed with polyP₆₆ (3 μ g) in a 10 μ l buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl and 1 mM MgCl) for 10 min at 37 °C. They were analyzed by 4–12% SDS NuPAGE, and the gels were stained by QuickBlue Stain (Bostonbiologicals) and dried using Gel-Dry drying solution (Invitrogen).

Coimmunoprecipitation assay was used to detect the formation of $\sigma 80$ -polyP complexes under non-denaturing conditions. Each of the purified recombinant $\sigma 80$ proteins (~ 2.4 μ g) were mixed with (~ 2.5 μ g) of polyP₇₅₀, which was synthesized by *E. coli* PPK as described (Ault-Riche et al., 1998), in 120 μ l of buffer B (50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 0.1 mM EDTA and 0.1 mg/ml BSA), and samples were rotated gently at room temperature (25°C) for 5 min. Then 3 μ l of mouse anti-polyHistidine antibody (Sigma) was added and the mixtures were rotated in a cold room (4°C) for 1 hour, followed by the addition of 20 μ l of protein A/G agarose (Santa Cruz). After incubation in the cold room overnight with rotation, the mixtures were spun down. The immunoprecipitates were washed with the buffer B (800 μ l) twice before resuspended in the buffer (40 μ l). Samples (4 μ l) were analyzed by SDS-PAGE, followed by Western Blot with $\sigma 80$ specific polyclonal antibodies as described above.

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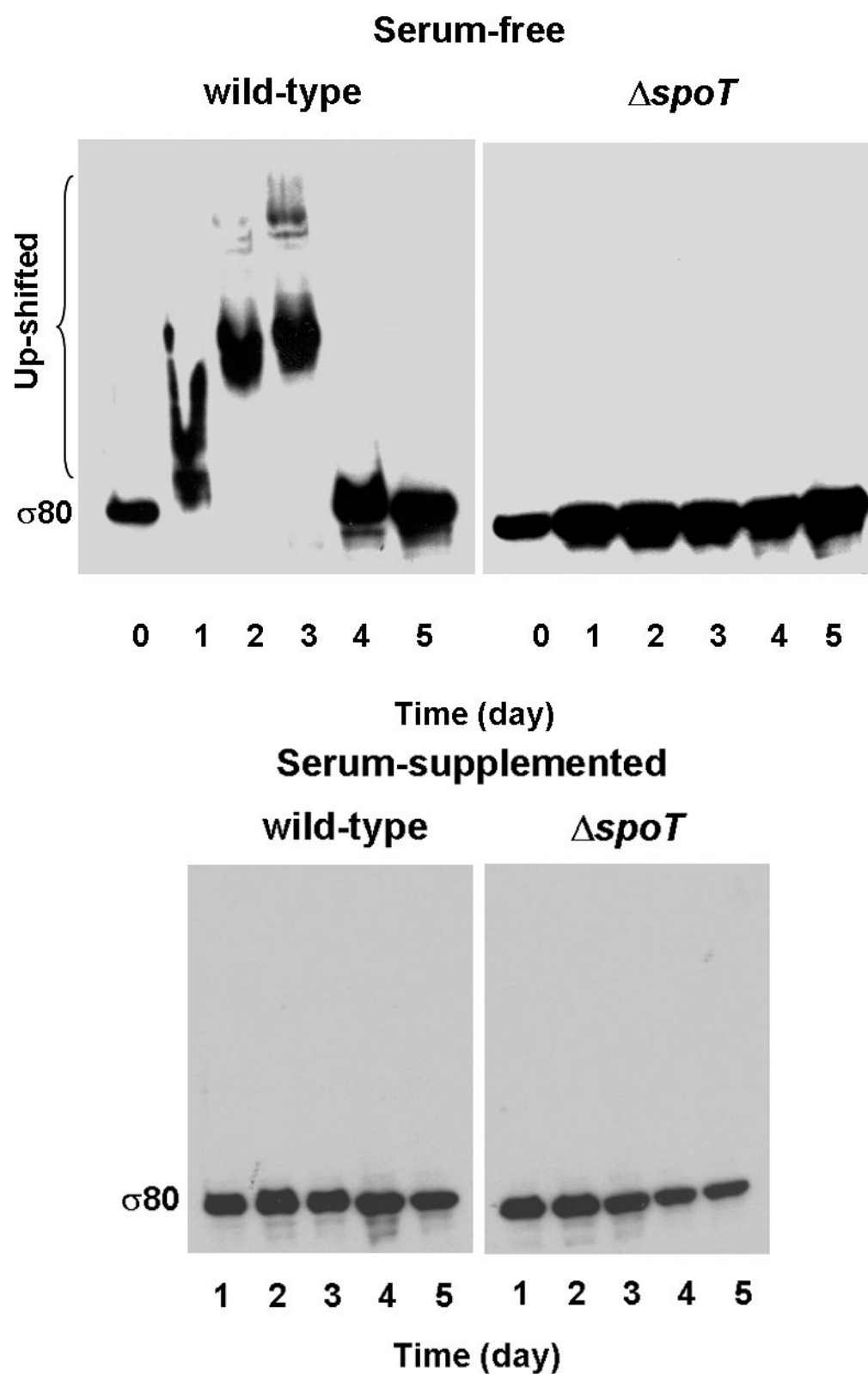
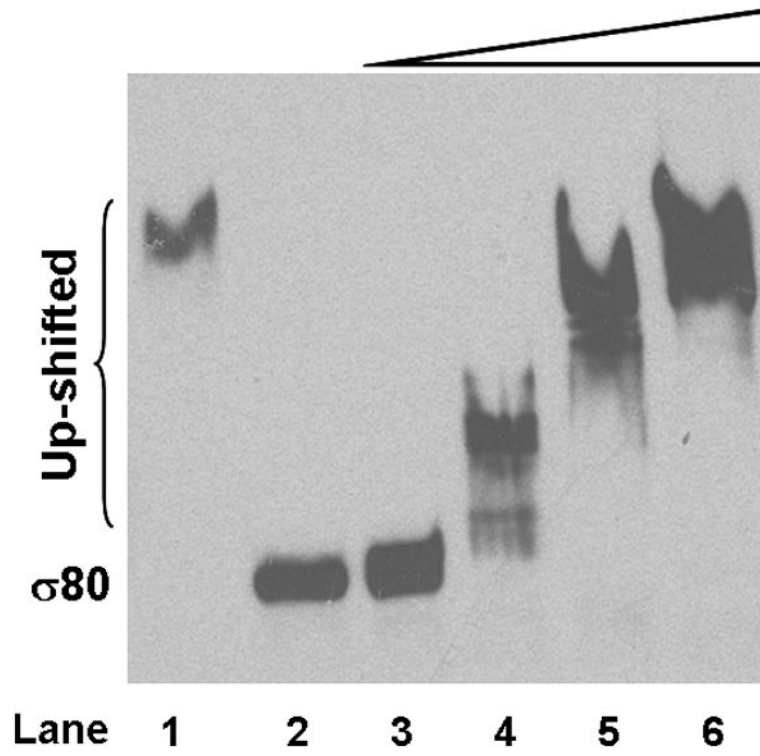
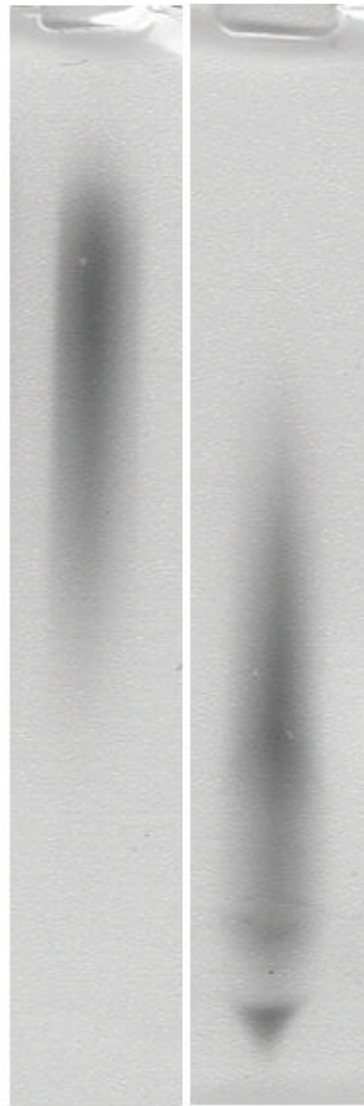


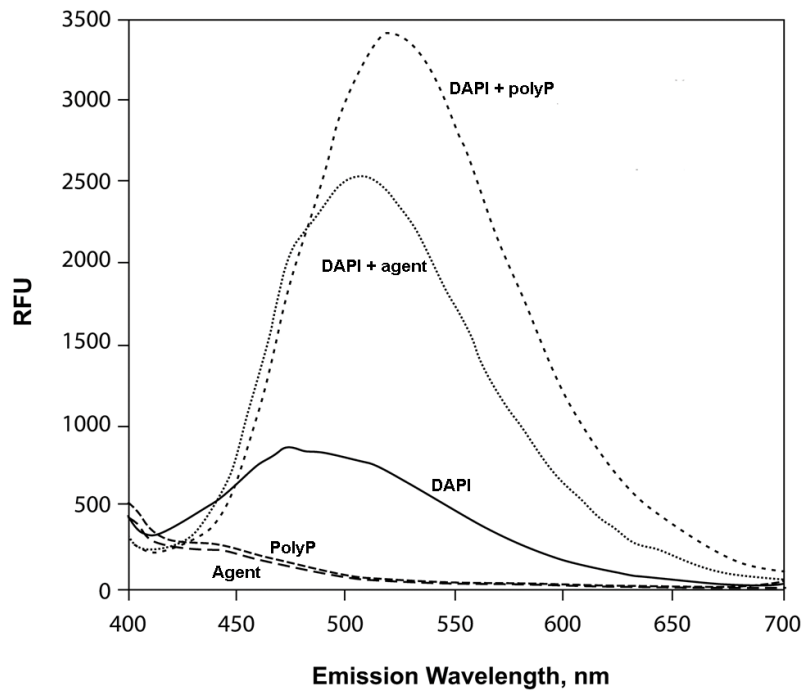
Figure 1. The electrophoretic mobility of $\sigma 80$ is greatly reduced during a SpoT-mediated serum starvation response

Cells of *H. pylori* wild type strain G27 and its isogenic $\Delta spoT$ mutant were grown in BLBB medium, which was either serum-free (**A**) or supplemented with 10% FBS (**B**). Cells were sampled at the indicated times and lysed. The $\sigma 80$ protein in cell lysates was separated by 4–12% SDS-PAGE, followed by protein immunoblotting with the $\sigma 80$ -specific polyclonal antibodies. The positions of $\sigma 80$ with normal mobility or with reduced electrophoretic mobilities (up-shifted) are indicated. The up-shifted $\sigma 80$ appeared to be smeared bands, which is likely due to polyP being heterogeneous in size (see text).



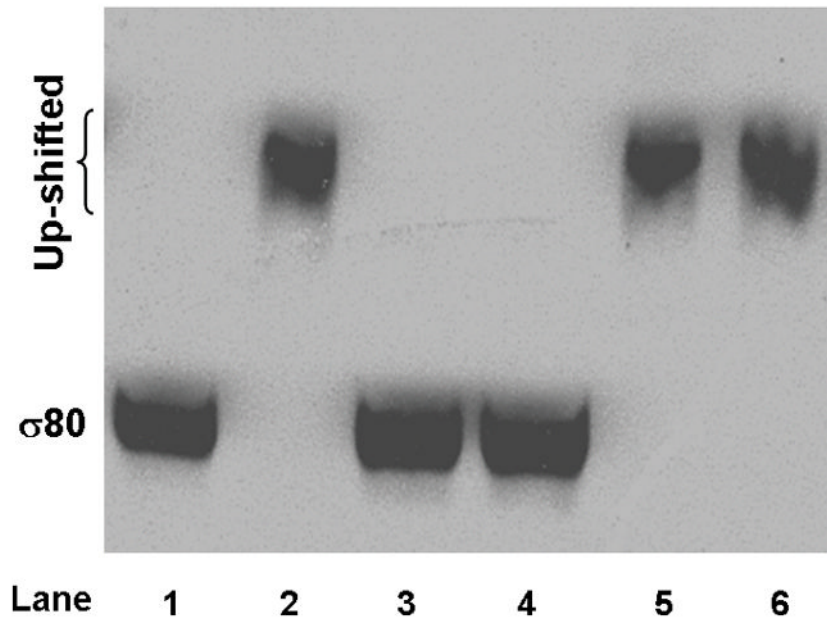


Lane 1 2



Treatment of the agent prior to the addition

None CIAP PPX DNase RNase



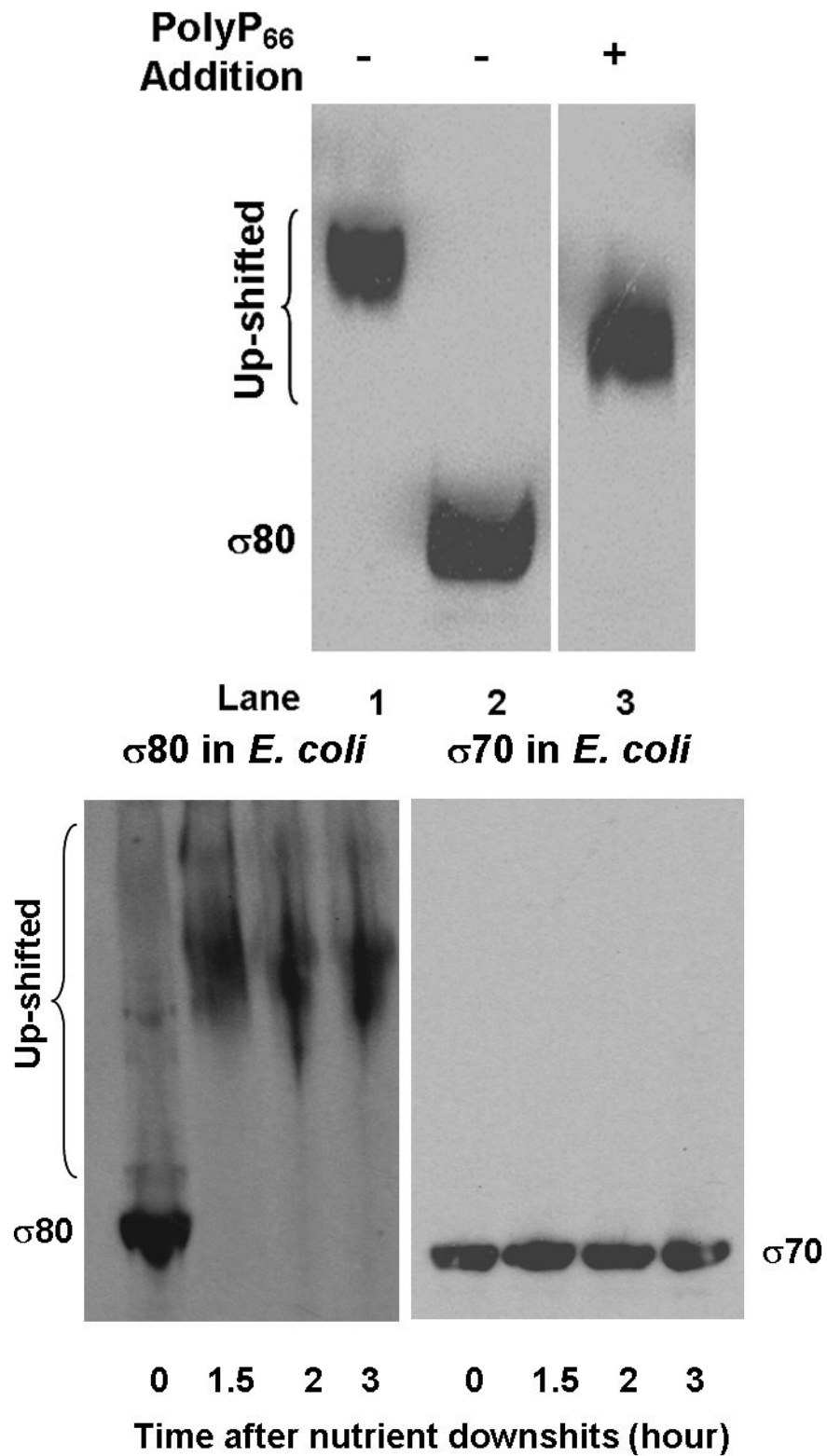


Figure 2. The agent that caused σ_{80} up-shift is polyP
 (A) Addition of a shifted lysate reduced the mobility of σ_{80} from an un-shifted lysate. A shifted lysate (as 1X amount) (lane 1); an un-shifted lysate (2X amount) either was alone

(lane 2) or mixed with increasing amounts of the shifted lysate of approximate 0.5X (lane 3), 1X (lane 4), 2X (lane 5), and 4X quantities (lane 6). **(B)** Both the agent (lane 1) and polyP₆₆ (lane 2) were analyzed by 6% urea-PAGE. They stained pink by Toluidine blue O. **(C)** The fluorescence spectrum of DAPI shifted by the addition of the purified agent, a result similar to the addition of polyP (polyP₆₆). Excitation wavelength: 372 nm; and the emission maximum of DAPI alone was ~ 475 nm. RFU: relative fluorescence unit. **(D)** The σ 80 up-shifting activity of the agent was sensitive to the treatments of CIAP and PPX (lanes 3–4) and resistant to DNase and RNase treatments (lanes 5–6). An un-shifted lysate (lane 1); the un-shifted lysate was mixed with the purified agent, which was untreated (lane 2); or pre-treated with an indicated enzyme (lanes 3–6). **(E)** A polyP addition up-shifted σ 80 from an un-shifted lysate. A shifted lysate as a control (lane 1); and an un-shifted lysate was either alone (lane 2) or mixed with polyP₆₆ (lane 3). **(F)** The *H. pylori* σ 80 protein expressed in nutrient downshifted *E. coli* cells was up-shifted while the mobility of the host σ 70 protein was unchanged under the same conditions. At time 0, the cultures were nutrient downshifted from LB to a minimal medium containing a low concentration of Pi and no amino acids for three hours. The samples in **(D to F)** were analyzed as described in the legend of Figure 1, and the *E. coli* σ 70 protein in **(F)** was detected by σ 70 specific polyclonal antibodies. The up-shifted σ 80 appeared to be smeared bands, which is likely due to polyP being heterogeneous in size (see text).

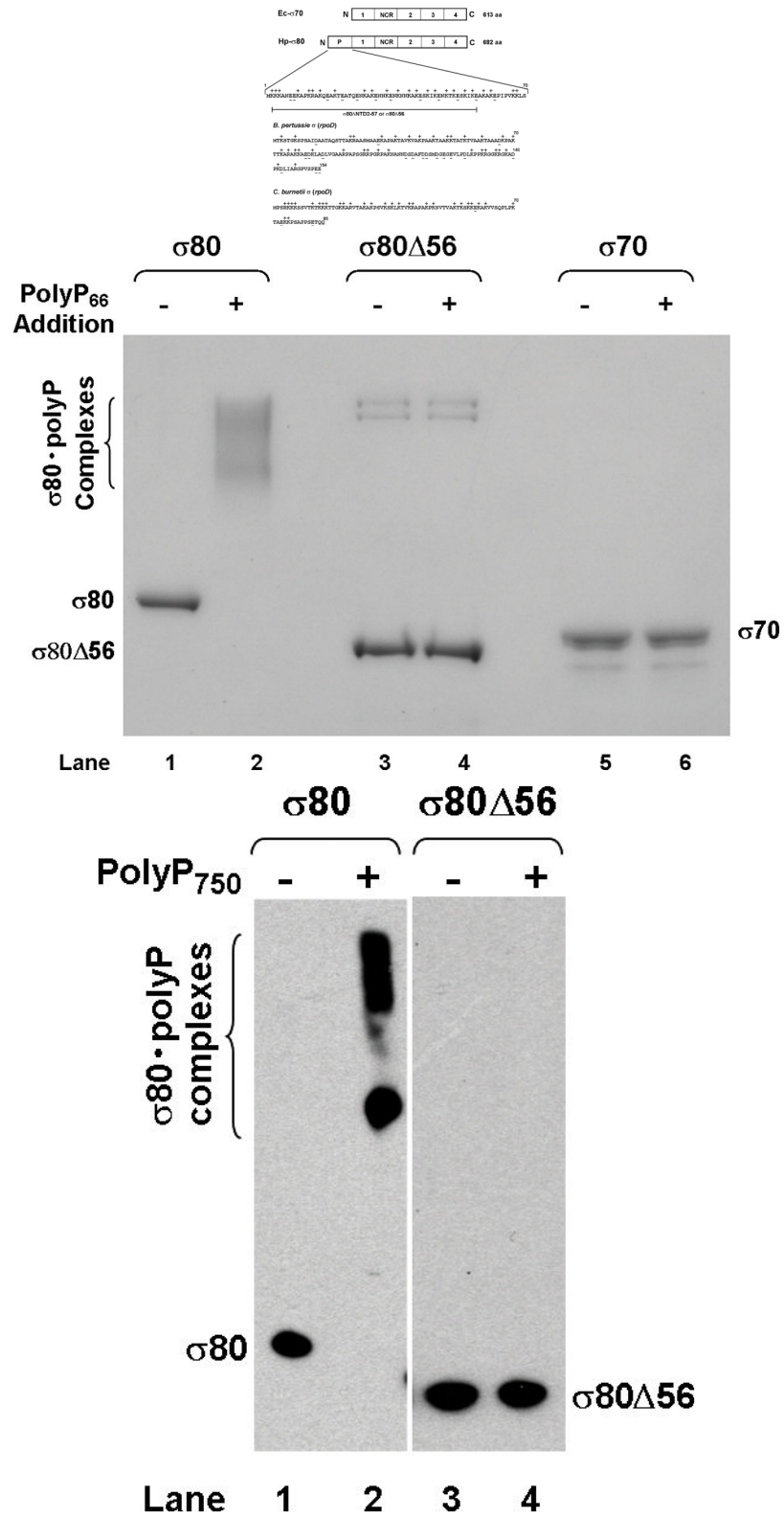


Figure 3. PolyP binds to the Lys-rich region at the NTD of σ 80

(A) There is an extra region of about 70 amino acid residues (aa) at the NTD of the *H. pylori* [NC_011333] σ 80 compared with the *E. coli* [U00096] σ 70. This extra region is concentrated with positively charged (+) aa including 23 Lys and one Arg, compared with 12 negatively charged (–) Glu. This extra region is named “region P” because it is a positively-charged-Lys-rich region and the target for polyP (see text). The conserved regions and non-conserved region (NCR) of members of the σ 70 family proteins (Gruber & Gross, 2003) are shown. The *rpoD* mutation that deleted the aa 2–57 (σ 80 Δ NTD2–57 or σ 80 Δ 56) is indicated. (B) A putative “region P” of the primary σ factor from *Bordetella pertussis* [NC_002929] or *Coxiella burnetii* (NC_002971). For simplicity, only the positively-charged-Lys-rich extra region (aa before conserved “region 1”) at the NTD of the σ factor indicated is shown. Positively charged (+) and negatively charged (–) aa are indicated. (C) Region P of σ 80 is important for binding polyP. Purified recombinant σ 80, σ 80 Δ 56 and σ 70 proteins in the absence (–) or presence (+) of polyP₆₆ were analyzed by 4–12% SDS-PAGE. The gels were stained with QuickBlue Stain. The positions of σ 80, σ 80 Δ 56, σ 70 and the σ 80-polyP complexes are indicated. The other minor bands reflected impurities in protein preps. In particular, the higher molecular weight bands in lanes 3 & 4, were not related to σ 80 Δ 56 as they were not immunostained by the σ 80-specific polyclonal antibodies (data not shown). (D) PolyP is coimmunoprecipitated with the wild type σ 80 protein but not with the NTD deletion under native non-denaturing condition. Purified recombinant σ 80 and σ 80 Δ 56 proteins in the absence (–) or presence (+) of polyP₇₅₀ were immunoprecipitated by anti-polyHistidine antibody, and the samples were analyzed as described in the legend of Figure 1. The positions of σ 80, σ 80 Δ 56 and the σ 80-polyP complexes are indicated.

Serum-free

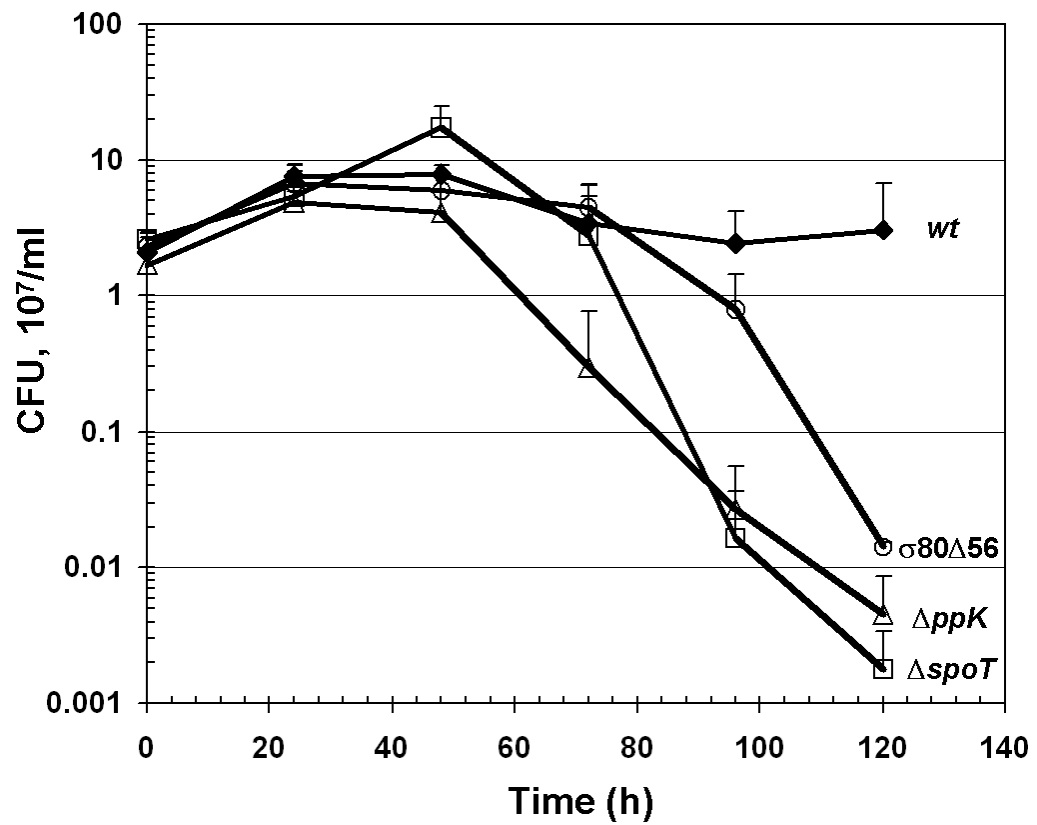
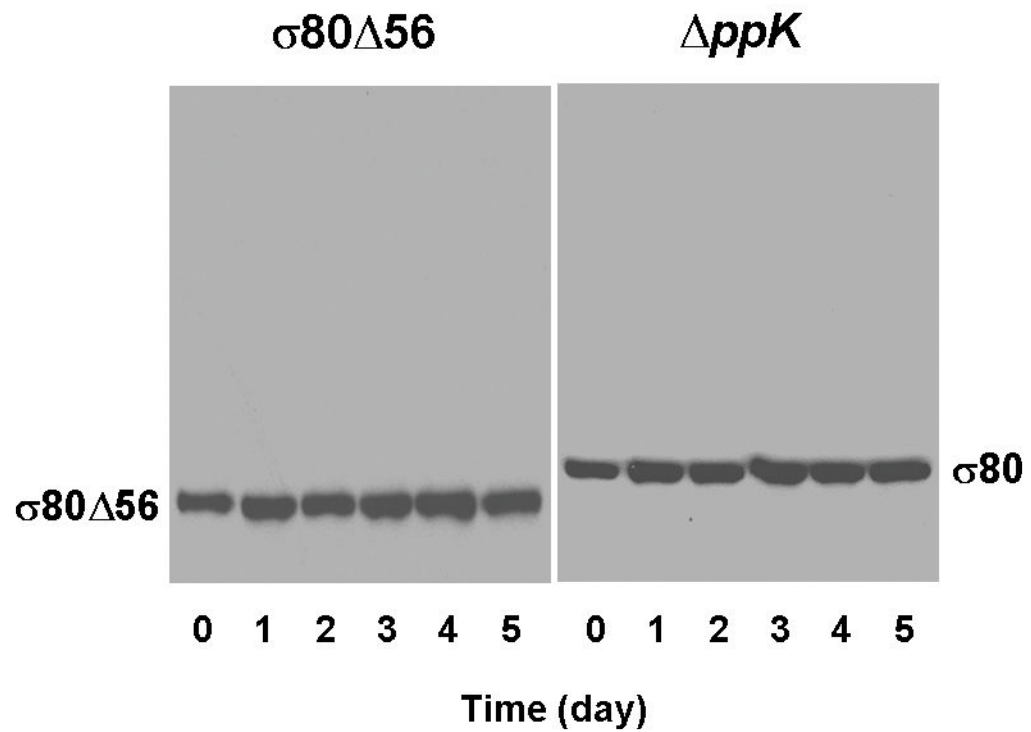


Figure 4. Mutational analysis of the determinants during serum starvation response
(A) Both the $\sigma 80\Delta 56$ and ΔppK mutants were defective in forming the $\sigma 80$ -polyP complexes during serum starvation. $\sigma 80$ was un-shifted in both the $\sigma 80\Delta 56$ and ΔppK mutant cells grown serum-free. Cells were analyzed as described in the legend of Figure 1. (B). Both the $\sigma 80\Delta 56$ and ΔppK mutants exhibited reduced survival during serum starvation, approaching the premature death phenotype of the $\Delta spoT$ mutant at day 5. The numbers of viable bacteria are expressed as colony-forming unit (CFU). The error bars indicate the deviations from the means, and there are no error bars indicated when the deviations were smaller than the symbol. The data are representative of three independent experiments.

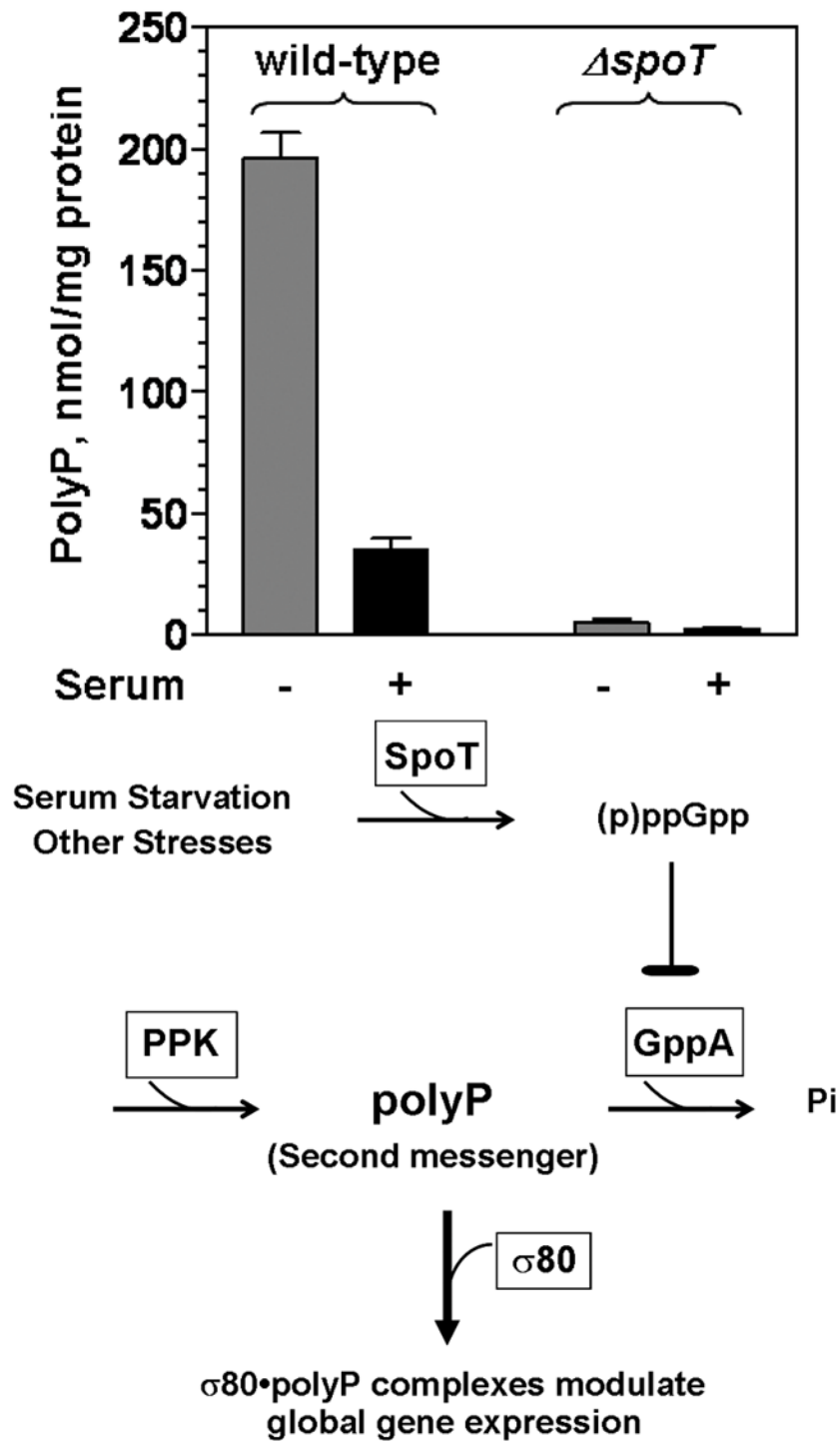


Figure 5. The determinants during starvation/stress response in *H. pylori*

(A) Accumulations of polyP are observed in wild type cells grown serum-free and the accumulation is regulated by (p)ppGpp in *H. pylori*. PolyP extracted from wild type and the *spoT* mutant cells grown either in the absence (-) or presence of serum (+) for 48 hours were quantified. The error bars indicate the deviations from the means. The data are representative of three independent experiments. (B) This simplified scheme proposes the

regulation of polyP and the role of polyP during SpoT mediated starvation/stress response *in H. pylori*. Serum starvation and other stresses promote SpoT to synthesize (p)ppGpp and/or to decrease the degradation of these alarmones, which can lead to accumulation of (p)ppGpp. *H. pylori* lacks the gene for the exopolyphosphatase PPX but contains a homologous guanosine pentaphosphate phosphohydrolase gene for GppA that is also an exopolyphosphatase (Keasling *et al.*, 1993). Inhibition of GppA by (p)ppGpp can in turn lead to accumulation of polyP, assuming that (p)ppGpp does not inhibit polyphosphate kinase (PPK). Increased levels of polyP serve as a second messenger by forming strong association with $\sigma 80$ to modify transcription machinery and global gene expression.