We have examined expression of the genes on *Salmonella* pathogenicity island 1 (SPI1) during growth under the physiologically well defined standard growth condition of Luria-Bertani medium with aeration. We found that the central regulator *hilA* and the genes under its control are expressed at the onset of stationary phase. Interestingly, the two-component regulatory genes *hilC*/ *hilD*, *sirA/barA*, and *ompR*, which are known to modulate expression from the *hilA* promoter (*hilAp*) under so-called “inducing conditions” (Luria-Bertani medium containing 0.3 M NaCl without aeration), acted under standard conditions at the stationary phase induction level. The induction of *hilAp* depended not on RpoS, the stationary phase sigma factor, but on the stringent signal molecule ppGpp. In the ppGpp null mutant background, *hilAp* showed absolutely no activity. The stationary phase induction of *hilAp* required *spoT* but not *relA*. Consistent with this requirement, *hilAp* was also induced by carbon source deprivation, which is known to transiently elevate ppGpp mediated by *spoT* function.

The observation that amino acid starvation elicited by the addition of serine hydroxamate did not induce *hilAp* in a RelA⁺ SpoT⁺ strain suggested that, in addition to ppGpp, some other alteration accompanying entry into the stationary phase might be necessary for induction. It is speculated that during the course of infection *Salmonella* encounters various stressful environments that are sensed and translated to the intracellular signal, ppGpp, which allows expression of *Salmonella* virulence genes, including SPI1 genes.

Infection with *Salmonella enterica* serovar Typhimurium can cause a systemic, typhoid-like disease in mice. Following ingestion, bacteria can colonize the intestinal tract, penetrate the intestinal epithelium, and access systemic sites such as the spleen and liver through lymphatic and blood circulation (1). Passage of the bacteria through the intestinal lining is initiated by bacterial invasion into enterocytes and M cells (1–4). The invasion is mediated by a bacterial type III secretion system (TTSS) encoded by genes on *Salmonella* pathogenicity island 1 (SPI1) (5). The TTSS translocates bacterial effector proteins, also encoded on a SPI, into the host cell cytosol to reorganize the cytoskeleton, resulting in membrane ruffling and eventual bacterial uptake (6). Expression of the SPI1 secretion system and its secreted effectors is coordinately regulated by HilA encoded on SPI1, a member of the Ompr/ToxR family of transcriptional regulators (7). The genes on SPI1 regulated by HilA include *invF* and *sirA* (8–10). *InvF*, a member of the AraC/XylS family of transcriptional regulators, in conjunction with SirA, a TTSS chaperone, takes part in the coordinated regulation of SPI1-encoded genes.

Regulation of *hilA* expression has been studied extensively because of its central role in invasion gene activation. Environmental signals such as oxygen concentration, osmolarity, and the growth state of bacteria have been shown to influence the expression of *hilA* and the secretion of invasion-associated proteins (11, 12). Thus, most studies have been carried out using bacteria grown under so-called inducing conditions, namely high osmolarity and low oxygen conditions (LB containing 0.3 M NaCl without aeration). Studies of bacteria grown under these conditions have, to date, revealed that *hilA* expression is regulated by a complex array of regulatory systems including *hilC*/ *sirC*/ *spra* (13–15), *hilD* (15), *sirA/barA* (16, 17), *fis* (18, 19), *envZ* (20, 21), *ompR* (21), *phoB* (7), *fadD* (7), *fluZ* (7), *hha* (22), *H-NS* (19), and *HU* (19). Two of these genes, *hilC* and *hilD*, encode AraC-like transcriptional activators that activate *hilA* transcription by binding upstream of the *hilA* promoter DNA (15, 23, 24). Members of the phosphorylated response regulator superfamily involved in *hilA* expression include *sirA/barA*, *envZ*/ *ompR*, *phoB*/ *phoB*, and *phoP*/*phoQ* (7, 12, 17, 21). However, none of these regulatory systems has been shown to directly relay environmental signals to *hilA* expression.

Enteric bacteria elicit stringent control of ribosome production during the transition from exponential growth to the stationary phase (25, 26). The effector molecule of the stringent control modulation is the alarmone guanosine tetraphosphate ppGpp (27, 28). The ppGpp is synthesized by two synthetases, PSI and PSII, which are encoded by the *relA* and *spoT* genes, respectively. These two enzymes respond differently to environmental conditions. PSI is activated during amino acid starvation but is largely inactive during exponential growth; in contrast, PSII is mostly inactive during amino acid starvation but is active during exponential growth to determine basal conditions.
levels and to respond to certain environmental stress factors, including deprivation of carbon or energy (29–34). Accumulation of ppGpp during the exponential phase of growth results in the reduction of stable RNA synthesis and the activation of certain types of mRNA synthesis.

In this study, we examined expression of SPI1 genes, including hilA under physiologically well defined standard growth conditions (LB with vigorous aeration), and observed that these genes were induced at the onset of the stationary phase. This stationary phase induction was, however, not dependent on the stringent σ factor RpoS but on the stringent signal molecule ppGpp. Most interestingly, we found that the reduction of stable RNA synthesis and the activation of ppGpp during the exponential phase of growth results in induction of SPI1 genes, including deprivation of carbon or energy (29–34). Accumulation of ppGpp during the exponential phase of growth results in the reduction of stable RNA synthesis and the activation of certain types of mRNA synthesis.

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ACTAACGTGATT-3', which is complementary to positions 134–114 of the transcription start site of hilA, and 5'-ACTGGAAGATCTGGAATTGCTTTACAGCACAC-3', which is complementary to positions 124–95 of the transcription start site of hisG, were used. 32P-labeled primers (50,000 cpm) were co-precipitated with 30 μg of total RNA. Primer extension reactions were performed as described by Shin et al. (41).

**Invasion Assay**—The assays were performed essentially as described previously (42). Monolayers for bacterial invasion were prepared by seeding 5 × 10^6 HEP-2 cells into each well of 24-well plates. The HEP-2 cells were grown in Dulbecco's modified Eagle's modified (Invitrogen) plus 10% fetal bovine serum (Invitrogen) at 37 °C with 5% CO_2. Salmonellae prepared as described in the text were added to HEP-2 cells at a ratio of 10:1, and the mixture was incubated at 37 °C under 5% CO_2 for 30 min. Infected cells were washed three times with phosphate-buffered saline (pH 7.4), Dulbecco's modified Eagle's medium containing gentamicin (5 mg/ml; Sigma) was added, and the mixture was incubated for an additional 60 min. Intracellular bacteria were harvested by extraction with lysis buffer (0.05% Triton X-100 in phosphate-buffered saline, pH 7.4), and replica was plated for colony counting on brain heart infusion agar plates.

**RESULTS**

**Growth Phase-dependent Invasiveness of Bacteria Grown under Standard Conditions**—The ability of Salmonellae to invade cultured nonphagocytic cells has been correlated with the expression of SPI1-encoded genes (43). In an attempt to investigate the regulation of invasion genes under a physiologically well defined standard growth conditions, we determined the invasiveness of bacteria grown under standard conditions (LB with aeration). In this experiment, an overnight culture of bacteria grown in LB was diluted 40-fold in the same media or a high salt media (LB plus 0.3 M NaCl) and grown with or without aeration, respectively. The high salt media without aeration was considered the “inducing condition” for the expression of SPI1 genes (11, 12). Fig. 1A shows Salmonellae growth under the two conditions. Under the standard condition, bacteria grew rapidly and reached the stationary phase in ~4 h. Under the inducing condition, the culture entered into the stationary phase at a much lower A_600~ <1 A_600. Bacteria were sampled from the middle of the exponential phase (~2 h) and from the early (~4 h) and late (~12 h) stationary phases grown under the two conditions, and the invasiveness of each growth phase culture was determined using HEP-2 cells. In this experiment, the number of bacteria from various growth phases was adjusted to a multiplicity of infection of 10 (bacteria, 5 × 10^6; host cells, 5 × 10^5). Fig. 1B shows the actual number of intracellular bacteria that survived gentamicin treatment (10 μg/ml) and were recovered from the host cells following a 1-h incubation of bacteria and host cells. When grown under the standard condition (Fig. 1B, filled bars), the early stationary phase bacteria were found to be ~10–20-fold more invasive than the exponential phase and late stationary phase bacteria. By contrast, the invasiveness of bacteria grown under the inducing condition showed a different pattern; the early stationary phase culture was ~3-fold more invasive than the exponential phase culture but slightly less invasive than the late stationary phase culture (Fig. 1B, open bars). The maximum invasion was obtained with the early stationary culture grown under the standard condition. It was thought that the loss of invasiveness with the late stationary culture grown under the standard condition was due to the destruction of TTSS by continuous agitation of the culture. Thus, although the inducing condition might closely represent the intestinal milieu (11, 12), bacteria grown under the standard condition were used in the subsequent experiments to identify the factor(s) conferring the maximum invasiveness at the early stationary phase.

Next, we determined the presence of secreted effector proteins encoded within SPI1 (38) in the cultures at different times grown under the standard condition (see Fig. 1C). Total supernatants of cultures at different phases were collected, precipitated with trichloroacetic acid, and analyzed on 7.5% SDS-PAGE gel (Fig. 1C). The volume of the supernatant was proportionally adjusted to the number of bacteria at each growth phase. The secreted effector proteins, namely SipA (89 kDa), SipB (67 kDa), SigD (62 kDa), and SipC (42 kDa), were detected only in the supernatant of cultures entering the stationary phase (4 h) and thereafter (6 h). Thus, Salmonellae at the entry of the stationary phase were most invasive because SPI1-encoded genes, including those constituting the TTSS apparatus and effector proteins, were expressed exclusively at this growth phase under the standard condition (see below).

**Growth Phase-dependent Expression from the Promoters in SPI1**—We analyzed the activity of the promoters driving expression of the genes involved in Salmonella invasion of host cells encoded on SPI1, namely the hisG, invF, and sicA promoters (abbreviated hilAp, invFp, and sicAp, respectively), during growth under the standard condition. To determine activity of these promoters, Salmonella typhimurium strains carrying lacZ genes transcriptionally fused to individual promoters on the chromosome (SMR2063; Fig. 2A) or a plasmid in the 14028s
strain background (Fig. 3) were used. Bacteria were taken at regular time intervals, and \( \beta \)-galactosidase activity representing the activity of each promoter during the course of growth was determined. Fig. 2 shows the \( hilA \) activity determined under the standard and inducing growth conditions. Under the inducing condition, \( hilA \) activity was about the same throughout the exponential and stationary phases. By contrast, \( hilA \) activity under the standard growth condition was induced 30-fold when the culture entered the stationary phase. \( hilA \) activity under the standard condition was 10-fold less during the exponential phase but 3.5-fold more at the entry of the stationary phase as compared with the activities under the inducing condition. Thus, this result accounts for the different invasiveness of the cultures grown under the two conditions; the central regulator \( hilA \) is selectively expressed at the onset of the stationary phase under the standard condition but is maintained at more or less the same level irrespective of the growth phase under the inducing condition. To further verify the \( hilA \) induction under the standard growth condition, the \( hilA \) specific transcript was monitored during the course of growth by the primer extension assay (Fig. 2B). The \( hilA \) specific RNA was detected at 3 h as culture entered the stationary phase, peaked at 5 h, and disappeared at 7 h.

Subsequently, \( invF \) and \( sicA \) as well as \( hilA \) promoters on the pRS415 plasmid (44) were determined during growth under the standard condition (Fig. 3). The episomal \( hilA \) activity (Fig. 3A) showed a similar pattern of induction at the onset of the stationary phase, but the magnitude of induction was significantly reduced to ~4-fold. The reduction was ascribed to the huge increase in the basal level activity at the exponential phase, as if a repressor acting at the exponential phase was titrated out by the episomal \( hilA \) DNA. The \( invF \) and \( sicA \) activities were determined using the strain carrying the individual promoters fused to \( lacZYA \) on the pRS415. Both \( invF \) (Fig. 3C) and \( sicA \) (Fig. 3B) were increased >50-fold as the culture entered the stationary phase. The extension in the activation of downstream activators \( invF \) and \( sicA \), as compared with that of the upstream activator \( hilA \), might represent a magnification of the physiological response in cascade regulation. Taken together, these results clearly establish that the early stationary phase bacteria grown under the standard condition are the most invasive because of the selective expression of the central activator, \( hilA \), and the consequent expression of the downstream activators \( invF \) and \( sicA \) under its control.
Regulation of Stationary Phase Induction of hilA Expression—We then set out to establish the molecular mechanism underlying the stationary phase induction of hilA under the standard growth condition. Stationary phase induction of gene expression in enteric bacteria is due at least in part to the stationary phase sigma factor σ^SS, the rpoS gene product (45). The heat shock sigma factor (σ^H), the rpoE product, has also been shown recently to be strongly induced upon the entry of Salmonellae into the stationary phase (46, 47). We determined the chromosomal hilAp activity in the RpoS^- mutant background and found that the stationary phase activation pattern was even greater than in the wild type (WT) (Fig. 4). In the RpoE^- mutant background, no difference was observed. Thus, the stationary phase induction of hilAp is apparently independent of rpoS or rpoE.

Subsequently, we examined the regulation of hilAp by those two-component regulatory systems that are known to activate hilA under the inducing condition, namely hilC/hilD, sirA/barA, and envZ/ompR (7, 48) (Fig. 5). Under the standard growth condition, hilAp activity in the HilC^- mutant in the exponential phase was equivalent to that in the WT but was not induced at the entry of the stationary phase. On the other hand, hilAp activity in the HilD^- mutant in the exponential phase was ~10-fold lower than that in the WT, and the activity remained reduced throughout the course of growth. The hilAp activity in either the SirA^- or the BarA^- mutant in the exponential phase was not much different from that in the WT but was only partially induced at the entry of the stationary phase. The hilAp activity was virtually undetected in the OmpR^- mutant throughout the growth period. However, in the EnvZ^- mutant strain hilAp was induced at the entry of the stationary phase, although ~2.5-fold lower than in the WT. The differences in hilAp induction in OmpR^- and EnvZ^- suggest that OmpR could be phosphorylated by a protein(s) other than EnvZ (49) if phosphorylated OmpR induces hilAp activity. Nevertheless, a defect in the two-component regulatory systems resulted in a failure to induce hilAp at the entry of the stationary phase under the standard condition. It is speculated that these activators might respond to a certain signal at the entry of the stationary phase.

ppGpp-dependent Induction of hilA and SPI1 Genes—In an attempt to identify the global regulatory system responsible for the stationary phase induction of hilAp, we examined hilAp activity in a strain lacking ppGpp, the effector molecule of the stringent response (28). ppGpp is produced and maintained by FIS and PSII, the respective relA and spoT gene products. We examined the hilAp activity in the ΔrelA and ΔrelA::spoT strains, which lack PSII or both PSII and FISII, respectively (Fig. 6). Growth of the mutants did not differ much from the WT strain under the standard growth condition in LB. We observed that, in the ΔrelA mutant strain, hilAp activity was indistinguishable from that in the WT strain. However, hilAp activity was completely silent throughout the growth phase in the ΔrelA::spoT mutant strain lacking ppGpp. These observations suggest that hilAp induction at the entry of the stationary phase is mediated by ppGpp, which is synthesized primarily by SpoT activity. To further verify the route of ppGpp synthesis leading to hilAp induction, hilAp activity was determined during carbon source starvation, which is known to elevate ppGpp in a SpoT-dependent manner (34, 50–52). Carbon starvation was elicited by the addition of 2.5% α-MG, a competitive inhibitor of glucose uptake, into LB containing 0.1% glucose (Fig. 7). The addition of α-MG only slightly reduced the growth rate; generation time shifted from ~30 min to ~40 min for all three strains, i.e., WT, ΔrelA, and ΔrelA::spoT. Fig. 7A shows a representative growth curve for all three strains. The basal hilAp activity levels prior to α-MG addition in the strains fell within a 2-fold range, with WT > ΔrelA > ΔrelA::spoT in order (Fig. 7B). Upon the addition of α-MG, hilAp activity increased drastically in the WT and ΔrelA strains but not in the ΔrelA::spoT strain. We also examined the hilAp activity during amino acid starvation that elevates ppGpp levels in a RelA-dependent manner (37) (Fig. 8). The condition of amino acid starvation was elicited by the addition of 2 mM SerHX to a culture grown in nutrient broth supplemented with 0.75 mM serine. Both WT and ΔrelA::spoT strains grew with more or less the same generation time (~30 min) prior to the addition of SerHX (Fig. 8A). The addition of SerHX in the middle of exponential phase of growth immediately reduced the growth rate for the WT strain (Fig. 8A, top panel). By contrast, the cell mass (A_600) of ΔrelA::spoT (Fig. 8A, bottom panel) increased at the same rate for some period (~1 h) as the rate prior to the addition of SerHX.
ppGpp-dependent Induction of SPI1 Genes

and then ceased (data not shown). It has been shown that under this growth condition the addition of SerHX drastically increased the ppGpp level ~10-fold in a RelA-dependent manner (37). Under this condition, we first determined an amino acid histidine biosynthesis operon promoter (hisGp), which is a classical promoter known to respond in parallel with the change in ppGpp level (37, 62, 63). The promoter activity was determined by measuring the transcripts. The addition of SerHX increased hisGp activity in WT (~30-fold) within 5 min but not in the \( \Delta relA \Delta spoT \) mutant strain (Fig. 8B, top). Under the same condition, hilAp activity remained unchanged by the addition of SerHX in either WT or the \( \Delta relA \Delta spoT \) strain (Fig. 8B, bottom). These results confirm that the induction of hilAp and, consequently, those genes under its control at the entry of the stationary phase results from the elevation of ppGpp levels in a SpoT-dependent manner.

Lastly, we evaluated the WT and \( \Delta relA \Delta spoT \) strains for their abilities to invade HEp-2 cells to access the in vivo in Salmonella virulence (Table II). The early stationary phase bacteria were used in this assay. The analysis revealed that the invasion by the \( \Delta relA \Delta spoT \) strain was \(<1\%\) of the level of invasion by the WT bacteria. Thus, the lack of hilA expression in the \( \Delta relA \Delta spoT \) strain and, consequently, the lack of expression of those genes under its control, including SPI1-encoded TTSS and effector proteins, caused an apparent reduction in invasiveness.

**DISCUSSION**

**Stationary Phase Induction of hilA under the Standard Growth Condition**—In this study we reported that hilA and, therefore, those genes under its control are expressed under the standard growth condition at the onset of the stationary phase based on the following observations: 1) invasiveness culminating at the early stationary phase culture (Fig. 1); 2) some representative secreted proteins encoded in SPI1 being detected in the supernatant from early stationary phase cultures but not in supernatant from exponential phase cultures (Fig. 1); and 3) hilAp and those promoters under its control, i.e. sicAp and \( \text{insFP} \), being induced at the onset of the stationary phase (Fig. 2 and 3). Similarly, early stationary phase bacteria grown under the standard condition are reportedly most cytotoxic to cultured mammalian cells (53). The primer extension analysis revealed that hilAp was transitionally expressed during transition from the exponential phase to the stationary phase, demonstrating a pattern of growth phase-dependent expression. The induction of hilA was, however, independent of the stationary phase sigma factor, \( \sigma^{26} \), or the heat shock sigma factor, \( \sigma^{34} \), which have both been implicated in Salmonella pathogenesis in animals and are induced at the entry of the stationary phase (45–47) (Fig. 4). Therefore, hilAp seems to be induced in response to an unidentified environmental signal built up as culture enters the stationary phase.

We have observed that hilA induction on a multicopy plasmid was lower than that on the chromosome (~4-fold versus ~30-fold), largely due to a huge increase in hilAp activity at the exponential phase (~1000-fold). Therefore, the stationary phase induction of hilAp activity could, at least in part, be ascribed to the removal of a repressor acting during the exponential phase. In this case, the hypothetical repressor might be titrated out by episomal hilAp DNA, resulting in the elevation of hilA activity during the exponential phase. Because a plethora of regulatory systems has been proposed to regulate hilA (7), the titratable factors should include an activator(s) as well as a repressor(s).

Interestingly, it was noted that the two-component regulatory systems known to activate hilA under the inducing condition acted at the level of its induction at the entry of the stationary phase under the standard growth condition (Fig. 5). Among the regulatory components, hilC/hilD has been shown to exert its regulatory effect by directly binding to a site upstream of hilA (15, 23, 54). We observed under the standard growth condition that hilA activity remained at the basal level in the HilC– or HilD– mutant background, although the defect was more severe in the HilD– mutant. In fact, hilA activity was reduced ~10-fold in the HilD– mutant background even during the exponential phase. This result suggests that the mechanism of hilAp activation by hilC/hilD might be different depending on whether the cells are in the exponential growth phase or at the entry into the stationary phase. We obtained similar results with the strains lacking the two-component regulatory systems reported to up-regulate hilA activity under the inducing condition, i.e. barA/sirA and envZ/lomPR (16, 20, 48, 55). Under the standard growth condition, hilA in the BarA– or SirA– mutant was defective at the level of its induction in the HilC– or HilD– mutant background, although the peak activity was ~2.5-fold less than that in the WT. It is well established that EnvZ phosphorylates OmpR upon sensing an increase in osmolarity in the environment (49). Under the standard condition, if the phosphorylated OmpR was responsible for hilA induction, the phosphorylation must occur through a route(s) other than EnvZ. In fact, an EnvZ-independent mechanism of OmpR phosphorylation has been postulated (56, 57). OmpR might take part in hilA induction at the onset of the stationary phase by sensing an unknown environmental signal through a currently unknown sensor. In this case, OmpR is unlikely to be responding to a change in media osmolarity, because we did not detect any noticeable change in the media osmolarity as the culture entered stationary phase (data not shown). Further study is required to elucidate the underlying mechanism of hilA induction and its regulation by two-component regulatory systems under the standard growth condition.

**Implication of ppGpp in hilA Induction**—After establishing hilA induction at the entry of the stationary phase under the standard growth condition, we searched for the global regulatory signal responsible for the induction and found ppGpp. hilAp activity remained at the basal level in the \( \Delta relA \Delta spoT \)
strain, although it was normally induced in the ΔrelA strain (Fig. 6). SPI1-encoded *sicA* and *invF* promoters also remained at their basal levels in the ΔrelAΔspoT mutant strain (data not shown). Consistently, the ΔrelAΔspoT strain showed reduced invasiveness by >100-fold as determined by an *in vitro* assay using HEP-2 cells (Table II). RelA is known to sense an imbalance or lack of amino acid supply and to synthesize ppGpp, resulting in the reduction of stable RNA synthesis, the phenomenon known as “stringent response” (29, 26, 28). Alternatively, the basal level of ppGpp during balanced growth is regulated by *spoT*, which carries both ppGpp synthetase (PSII) and hydrolase (34, 58). Therefore, the basal level of ppGpp depends on the balance of two activities. Some conditions, including carbon and energy starvation, have been shown to result in the accumulation of ppGpp in a SpoT-dependent manner (34, 59, 60). Under the standard laboratory growth condi-

### Table II

| Strain                  | WT          | ΔrelAΔspoT |
|-------------------------|-------------|------------|
| Invasiveness (%)        | 100         | <1         |
ppGpp-dependent Induction of SPI1 Genes

...the transition from the exponential phase to the stationary phase presumably represents a stressed condition. It was reported recently that changes in the proteome pattern of *Escherichia coli* entering the stationary phase were significantly different between WT and the ΔrelAΔspdt mutant, which had a proteome pattern that appeared to be locked in the exponential growth mode (61). We speculate that the transitional stress at the entry of stationary phase must be sensed and translated to ppGpp synthesis primarily by the SpoT function.

Most interestingly, it was observed that *hilA* was induced during the exponential phase of growth by carbon source starvation, which is known to elevate ppGpp level in a SpoT-dependent manner (34, 50–52), but it was not induced by amino acid starvation, which is known to elevate ppGpp level in a RelA-dependent manner (37) (Figs. 7 and 8). Whereas, the amino acid histidine biosynthesis operon promotor (hisGp), a classical promoter positively regulated by ppGpp, responded in parallel with the RelA-dependent increase in ppGpp level in this study (Fig. 8). It has been reported that the amino acid starvation elicited by the addition of SerHIX causes an immediate increase in ppGpp level in RelA-dependent manner, i.e., from 28.7 pmol A<sub>660</sub> to 1.042 pmol A<sub>660</sub> (37). The carbon source deprivation could also increase ppGpp concentration up to ~500 pmol A<sub>660</sub> in WT bacteria (51). However, it must be noted that *hilA* was induced normally in the ΔrelA strain following the carbon source starvation in which ppGpp pool was measured as being increased by only a few fold, a little more than 100 pmol A<sub>660</sub> (34). Thus, regulation of gene expression following carbon source starvation and amino acid starvation seem to be mechanistically different. Likewise, gene induction at the entry of the stationary phase and the induction observed during the classical stringent response following amino acid deprivation must also be different. In addition to ppGpp, some other alteration accompanying entry into the stationary phase may be necessary for gene induction, including *hilA* activation. The physiological consequence of the two routes of ppGpp elevation, the RelA- and SpoT-dependent mechanisms, remains unknown.

During the course of animal infection, *Salmonella* bacteria encounter diverse environments in the intestinal lumen and inside various host cells. Thus, it is imperative that *Salmonella* must be able to sense and respond to changing environments in order to survive (64). We speculate that environmental stress is sensed and translated to the intracellular signal ppGpp, which enables expression of various *Salmonella* virulence genes, including those encoded on SPI1 that are required for the invasion of host cells and induction of macrophage apoptosis (1, 65).

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REFERENCES

1. Carter, P. B., and Collins, F. M. (1974) *J. Exp. Med.* 139, 1189–1203
2. Hohmann, A. W., Schmidt, G., and Rowley, D. (1978) *J. Bacteriol.* 136, 1208–1209
3. Jones, B. D., Ghori, N., and Falkow, S. (1994) *Mol. Microbiol.* 13, 33–43
4. Takeuchi, A. (1967) *J. Bacteriol.* 94, 175–182
5. Darwin, K. H., and Miller, V. L. (2001) *Microbiol. 36, 635–646*
6. Hengge-Aronis, R. (1996) *J. Biol. Chem.* 271, 20233–20238
7. Lucas, R. L., and Lee, C. A. (2000) *Microbiol. 146, 4381–4385*
8. Simons, R. W., Houman, F., and Kleckner, N. (1987) *Genes 53, 85–96*
9. Harshman, R. B., and Yamazaki, H. (1971) *Biochemistry* 10, 3980–3982
10. Lagesky, P. A., and Chang, F. N. (1960) *J. Bacteriol.* 81, 449–458
11. Ryals, J., Little, R., and Bremer, H. (1982) *J. Bacteriol.* 151, 1425–1432
12. Murray, K. D., and Bremer, H. (1996) *J. Mol. Biol.* 259, 41–57
13. Davis, R. W., Botstein, D., and Roth, J. R. (1980) *Advanced Bacterial Genetics: A Manual for Genetic Engineering*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
14. Datenskin, R. A., and Wanner, B. L. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 6649–6654
15. Eichelberg, K., Gentry, D. R., Hernandez, V. J., and Vinella, D. (1996) in *Escherichia Coli and Salmonella: Cellular and Molecular Biology* (Neidhardt, F. C., Curtiss, R. III, Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaeffer, M., and Umbarger, H. E., eds), Vol. 1, pp. 1458–1496, ASM Press, Washington D. C.
16. Cashel, M., and Gallant, J. (1968) *J. Biol. Chem.* 244, 3133–3141
17. Lazzarini, R. A., Cashel, M., and Gallant, J. (1971) *J. Biol. Chem.* 246, 4381–4385
18. Altier, C. (2003) *Can. J. Microbiol.* 49, 2587–2587
19. Lawhon, S. D., Frye, J. G., Suyemoto, M., Porwollik, S., McClelland, M., and Altier, C. (2000) *J. Bacteriol.* 182, 552–556