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ppGpp-Mediated Stationary Phase Induction of the Genes Encoded by Horizontally Acquired Pathogenicity Islands and *cob*/*pdu* Locus in *Salmonella enterica* serovar Typhimurium

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***Salmonella enterica* is highly diverse in terms of genome structure, which is at least partly due to the horizontal transfer of genetic elements from various sources. In this study, we examined the expression profiles of such genes in *Salmonella* Pathogenicity Islands (SPIs) and the *cob*/*pdu* locus, horizontally acquired large DNA segments, during growth under standard growth conditions. Transcripts from exponentially growing and early stationary phase *Salmonellae* were compared using various methods including cDNA microarray analysis. Nearly all genes encoded by SPIs and the *cob*/*pdu* locus were induced at the onset of the stationary phase in a stringent molecule ppGpp-dependent but stationary phase σ, σ38-independent manner. Although, it has been suggested that ppGpp acts in concert with DksA, we found the stationary phase induction of those SPI genes was not DksA dependent. It is suggested that ppGpp stimulates the expression of these stress-inducible genes encoded by horizontally acquired DNA, by itself or in concert with DksA.**

***Keywords*:** *Salmonella*, ppGpp, microarray analysis, stationary phase, pathogenic genes, *dksA*

Bacteria under standard laboratory conditions proliferate exponentially, but eventually cease growth for various reasons, such as nutrient deprivation and/or overpopulation. Stationary phase gene expression is ascribed, in part, to RNA polymerase (RNAP) containing the stationary phase σ factor, RpoS, which is expressed specifically at entry into this phase (Hengge-Aronis, 1996; Zambrano and Kolter, 1996). RNA polymerase (RNAP) that is loaded with RpoS at entry into the stationary phase expresses a set of genes under its control. RpoS expression is regulated at many levels, including transcription initiation and elongation, translation, and protein stability (Hengge, 2008). The expression and function of RpoS requires the general stress signal molecule, ppGpp (Gentry *et al*., 1993; Kvint *et al*., 2000). ppGpp is synthesized by ribosome-bound protein RelA and cytosolic protein SpoT (Cashel *et al*., 1996). The accumulation of ppGpp at the end of the exponential phase has been considered to result in a reduction of stable RNA synthesis and the activation of those genes that are involved in the maintenance of growth arrested physiology and survival of environmental stresses (Chang *et al*., 2002; Potrykus and Cashel, 2008). Various models have been proposed to account for the mechanism by which ppGpp regulates gene expression (Cashel *et al*., 1996; Potrykus and Cashel, 2008). DksA, identified as *dnak* suppressor (Kang and Craig, 1990), has been shown to influence the regulatory activities of ppGpp by binding to RNAP along with ppGpp (Perederina *et al*., 2004; Potrykus *et al*., 2006). A recent model suggested that the binding of ppGpp and DksA to RNAP decreases stable RNA expression which consequently results in the liberation of RNAP for to those active promoters, which are rate-limited for RNAP binding (Barker *et al*., 2001; Paul *et al*., 2004).

Salmonella enterica serovar Typhimurium is a facultative intracellular pathogen that causes a systemic typhoid-like disease in mice. Approximately 4% of the *S. typhimurium* genome, which encodes over 200 virulence genes, is required for fatal infection in mice (Bowe *et al*., 1998). Virulence genes that are located on SPIs, which are composed of a series of genes and operons, play a crucial role in the pathogenesis of *Salmonella enterica* infections (Groisman and Ochman, 1996; Marcus *et al*., 2000). To date, five major SPIs have been identified. SPI-1 is a 40 kb chromosomal locus at centisome (cs) 63 in *Salmonella enterica* and is required for the invasion of host cells and the induction of macrophage apoptosis. SPI-2 is adjacent to the $tRNA^{val}$ gene at cs 30 and harbors genes that are required for systemic infection and replication within macrophages. The rest of the SPIs are smaller in size and remain to be characterized in detail. SPI-3 harbors 10 genes, including the *mgtCB* operon, and is required for survival in macrophages as well as growth in low Mg^{2+} environments. SPI-4 encodes 18 genes and is necessary for intramacrophage survival. The fifth pathogenicity island is located next to *serT* and contains six genes, four of which are involved in enteritis in calves. These SPIs, with a G+C composition that is lower (37%-47%) than the rest of the chromosome (~52%), are large cassettes of DNA that were probably acquired by horizontal transfer from unknown sources during the course

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of the organism's evolution (Groisman and Ochman, 1996).

Another such locus in *Salmonella enterica* is *cob*/*pdu* at cs 41. This locus, which is located adjacent to a $tRNA^{asn}$ gene and has an unusually high G+C content (57%-58%), was presumably acquired by horizontal gene transfer as a single fragment (Roth *et al*., 1996). The *cob* operon is required for the biosynthesis of adenosylcobalamin (Ado-B_{12}), and the *pdu* operon encodes proteins for the B_{12}-dependent degradation of propanediol. These two operons are arranged in tandem to form a single regulon. The *cob* operon consists of three contiguous clusters of functionally related genes (Bobik *et al*., 1992). Genes in the CobI and CobII clusters are required for the biosynthesis of adenosyl-cobinamide and dimethylbenzimidazole, respectively. The CobIII gene products mediate the covalent joining of adenosyl-cobinamide and dimethylbenzimidazole to form adenosyl-cobalamin (Jeter and Roth, 1987).

In this study, we have examined the expression profile of the genes that were acquired by horizontal transfer during *Salmonella* growth under standard laboratory conditions in Luria-Bertani (LB) medium with vigorous aeration. Transcripts from exponentially growing and early stationary phase *Salmonellae* were compared. Our data showed that these genes are induced at entry into the stationary phase in a ppGpp-dependent but RpoS-independent manner.

Materials and Methods

Strains and plasmids

The *Salmonella* strains that were derived from Serovar Typhimurium 14028s used in this study are listed in Table 1. Bacterial strains were constructed by P22HT *int* transduction. The *dksA*::*cat* strain was prepared following the method of Datsenko and Wanner (2000). The *dksA* deletion-insertion allele was generated by amplification using a pair of 60 nt primers that included 40 nt homology extensions and 20 nt priming sequences with pKD3 as a template (5′ primer, TCGTCCCTGAGTATTCTCGCCATCGCTGGGGTGGAGCCGTTGTAGGCTGGAGCTGCTTC and 3′ primer, CAGCCAGCGTTTTGCAGTCGATGCACAGATCGGCTGTTGGCATATGAATATCCTCCTTAG, with the *dksA* sequences underlined). The 1.1 kb (for *dksA*::*cat*) PCR products were purified and transformed into bacteria containing a Red helper plasmid (pKD46) by electroporation.

cDNA Microarray and data analysis

Total RNA was isolated from wild type (WT), $RpoS^-$, $DksA^-$, and ppGpp-defective mutant *Salmonella* grown in the exponential phase and to the stationary phase. *S. typhimurium* LT2-specific DNA-DNA microarray chip that consisted of 4622 predicted ORFs was kindly provided by the Sidney Kimmel Cancer Center (San Diego, USA) (McClelland *et al*., 2001). The integrity of bacterial total RNA was assessed by capillary electrophoresis with an Agilent 2100 Bioanalyzer (Agilent, USA), and further purified with an RNeasy Mini kit (QIAGEN, USA). cDNA probes for microarray analysis were prepared by reverse transcription of total RNA (50 μg) in the presence of aminoallyl-dUTP and 6 μg of random primers (Invitrogen, USA) for 3 h. The cDNA probes were purified using a Microcon YM-30 column (Millipore, USA), followed by coupling to Cy3 (for reference) or Cy5 dye (for test sample) (Amersham Pharmacia, Sweden). The Cy3 or Cy5-labeled cDNA probes were purified using a QIAquick PCR Purification kit (QIAGEN). Dried Cy3 or Cy5-labeled cDNA probes were resuspended in hybridization buffer containing 30% formamide, 5× SSC, 0.1% SDS, and 0.1 mg/ml salmon sperm DNA, mixed, and hybridized to a microarray slide. After overnight incubation at 42°C, the slide was washed twice with washing solution 1 (2× SSC, 0.1% SDS) for 5 min at 42°C, and once with washing solution 2 (0.1× SSC, 0.1% SDS) for 10 min at room temperature, and finally four times with 0.1× SSC for 1 min at room temperature.

Table 1. Strains and plasmids

Strains	Description	Reference or source
S. typhimurium		
14028s	Wild-type	
SCH2006	*rpoS*::*Amp*, Amp^r	Song *et al*. (2004)
SHJ2037	*relA*::*kan*, *spoT*::*cat*, Kan^r, Cam^r	Song *et al*. (2004)
SMR2147	*cob-24*::*mudJ* (*cobI*), Kan^r	This work
SMR2148	*rpoS*::*Amp*, *cob-24*::*mudJ* (*cobI*), Amp^r, Kan^r	This work
SMR2149	Δ*relA*Δ*spoT*, *cob-24*::*mudJ* (*cobI*), Kan^r	This work
SMR2150	*cob-62*::*mudJ* (*cobII*), Kan^r	This work
SMR2151	*rpoS*::*Amp*, *cob-62*::*mudJ* (*cobII*), Amp^r, Kan^r	This work
SMR2152	Δ*relA*Δ*spoT*, *cob-62*::*mudJ* (*cobII*), Kan^r	This work
SMR2153	*cob-66*::*mudJ* (*cobIII*), Kan^r	This work
SMR2154	*rpoS*::*Amp*, *cob-66*::*mudJ* (*cobIII*), Amp^r, Kan^r	This work
SMR2155	Δ*relA*Δ*spoT*, *cob-66*::*mudJ* (*cobIII*), Kan^r	This work
SMR2170	*dksA*::*cat*	This work
SMR2171	*dksA*::*cat*, *cob-24*::*mudJ* (*cobI*), Kan^r, Cam^r	This work
SMR2172	*dksA*::*cat*, *cob-62*::*mudJ* (*cobII*), Kan^r, Cam^r	This work
SMR2173	*dksA*::*cat*, *cob-66*::*mudJ* (*cobIII*), Kan^r, Cam^r	This work
TT10852	*metE*205 *ara*9 *cob-24*::*mudJ* (*cobI*), Kan^r	Bobik *et al*. (1992)
TT10857	*metE*205 *ara*9 *cob-62*::*mudJ* (*cobII*), Kan^r	Bobik *et al*. (1992)
TT10858	*metE*205 *ara*9 *cob-66*::*mudJ* (*cobIII*), Kan^r	Bobik *et al*. (1992)

The slide was dried by centrifugation at 650 rpm for 5 min. The hybridization image on the slide was scanned with Scanarray lite (Packard Bioscience, USA), and analyzed using GenePix Pro 3.0 software (Axon Instrument, USA) to obtain gene expression ratios (reference vs test sample). Expression ratios were normalized using GenePix Pro 3.0 software (Axon Instrument). An image was obtained from hierarchical clustering (Eisen *et al.*, 1998), which involves computing 'distances' between data elements.

Primer extension analysis

Total RNA was isolated from statically grown *Salmonella* using TRIzol reagent (Life Technologies, USA). To analyze *hilA*, ssa*G*, *pipC*, and *sopB* transcription, the following primers were used: the primer (5′-TAATAATATTGTTATAACTAACTGTGATTA-3′) was complementary to +134 to +114 of *hilA*; the primer (5′-AAATCATTACCA TTCATTTTGTCATTAATG-3′) was complementary to +124 to +95 of *ssaG*; the primer (5′-TTCATTAAAATAAACCTGTATCCCATCATC-3′) was complementary to +170 to +141 of *pipC*; and the primer (5′-TGTATAAGGTTTTTTGTAGGCTTTTAAAAG-3′) was complementary to +137 to +108 of *sopB*. ^{32}P-labeled primers (50,000 cpm) were co-precipitated with 30 μg of total RNA. Primer extension reactions were performed as described in Shin *et al*. (2001).

β-Galactosidase assays

β-Galactosidase assays were performed as described by Song *et al*. (2004), and β-galactosidase specific activity was expressed as Miller units (A_{420}/min/A_{600}/ml x1,000). To measure β-galactosidase levels in bacteria at different stages of growth, fresh overnight cultures were diluted (1:50) with LB (Difco Laboratories, USA) and grown at 37°C until the stationary phase. Each strain was assayed in triplicate, and average enzyme activities were plotted as a function of time.

Results

Experimental design

To determine the expression profiles of genes that were acquired by horizontal transfer, particularly those that were encoded by SPIs and the *cob*/*pdu* locus, we compared the transcripts that were obtained from exponential and early stationary phase *Salmonellae* grown under rich medium conditions (see Fig. 3 for growth curve). Global gene analysis was performed using microarrays that contained the 4622 predicted open reading frames (ORFs) of *S. typhimurium* (McClelland *et al*., 2001). Exponential and stationary phase *Salmonellae* were removed for analysis at about A_{600}≤1 and 3.5, respectively. Of the 4622 ORFs, 3969 were detected above the internal control level with wild type *Salmonellae*. SAM analysis of data revealed that, upon entry into the stationary phase, 1250 ORFs (31.5%) were decreased by 1.5-fold or more, 282 ORFs (7.1%) were increased by 1.5-fold or more, and 2437 ORFs (61.4%) did not change, with a *q*-value<0.5 (Fig. 1) (Larsson *et al*., 2005). The *q* value provides a measure of each feature's significance from the optimization of p-value with False Discovery Rate (FDR) (Storey and Tibshirani, 2003)

Expression profiles of individual genes in the SPIs and at the *cob*/*pdu* locus were analyzed. Interestingly, all these genes were induced at entry into the stationary phase with varying degrees of expression (Table 2). Subsequently, we determined the extent of RpoS- and ppGpp-dependent stationary phase

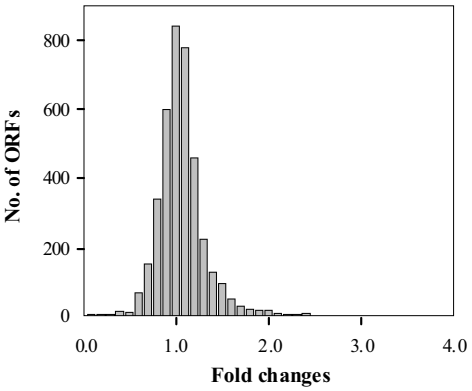

Fig. 1. Expression profile of total ORFs during *Salmonella* entry into the stationary phase under standard conditions. The ORF number was plotted as a function of fold changes in expression at entry into the stationary phase relative to that during exponential phase. A number equal to 1 represents no change, numbers below 1 indicate reduced expression, and numbers above 1 signify activation of gene expression at entry into the stationary phase. In total, 35 genes displaying activation values >4 were omitted in this plotting. These include *fruF* (4.1), *cbiK* (4.1), *phsC* (4.2), *grxB* (4.3), *gabT* (4.4), *ybdQ* (4.5), *ssrB* (4.6), *pipC* (5), *dmsA* (5), *melA* (5.2), *iadA* (5.2), *yceP* (5.2), *yeaG* (5.3), *sseA* (6), *rpsV* (6.2), *pagC* (6.4), *yjiG* (6.6), *yjbJ* (6.8), *sopD* (7.1), *sodB* (7.4), *manY* (8.3), *manZ* (8.3), *yjiH* (9.0), *yfiD* (11.2), *ynaF* (12.9), *yfiA* (15.8), *yahO* (23.7), *yliH* (25.5), *rmf* (35.1), *ompW* (122.9), *ymdF* (459.3), and *ychH* (490.8). The numbers in parentheses represent fold change.

gene induction by examining the expression profiles in RpoS$^-$ and ppGpp-defective mutants (Δ*relA*Δ*spoT*). Virtually all the horizontally acquired genes were induced more or less normally in the RpoS$^-$ mutant but displayed significantly reduced expression in the ppGpp-defective mutant. Therefore, this class of genes should be considered exponential phase σ, RpoD-dependent. Previous studies reported that RpoD-dependent stationary phase genes are superinduced in an RpoS$^-$ mutant background and underinduced in the presence of excess RpoS because there is competition between these two σ factors for a limited amount of RNAP cores during the stationary phase (Farewell *et al*., 1998). However, we observed variations in the induction of horizontally acquired genes in the RpoS$^-$ mutant compared to the wild-type. Most of these genes were induced at a higher level in the RpoS$^-$ mutant background with a few exceptions, such as *sptP*, *sscB*, *sseB*, and *mgtC*. Some genes, such as *sseC*, *siiE*, *siiF*, *cbiD*, *pduD*, and *pduF*, were induced much more than two-fold in the RpoS$^-$ mutant than in the wild type *Salmonellae.* These differences may be ascribed to the complexity of gene modulation, which includes cascades of multiple regulatory steps (Marcus *et al*., 2000). Notably, those genes with unusual G+C contents were induced under stress conditions in *Salmonellae* that were entering the stationary phase in a ppGpp-dependent manner.

Genes encoded by SPIs

As determined by microarray analyses, nearly all the SPI-encoded transcripts were induced during the stationary phase

Table 2. Expression profiles of individual genes in SPIs and the *cob*/*pdu* locus

Gene name	Fold change in WT	Fold change in RpoS$^-$	Fold change in *ΔrelAΔspoT*	G+C Content (%)[a]
Virulence Genes in SPI				
SPI1				
hilA	1.48	1.60	0.4	43
iacP	1.71	2.32	0.4	38
iagB	1.53	1.48	0.4	41
invF	1.49	1.50	0.2	46
sicA	1.70	2.46	0.3	43
sicP	1.89	2.06	0.3	39
spaS	1.46	1.77	0.4	43
sptP	1.66	1.58	0.4	44
SPI2				
ssaC	2.27	4.53	0.4	42
ssaD	2.31	3.58	0.4	44
ssaE	2.84	4.71	0.3	43
ssaG	3.35	3.44	0.3	38
ssaJ	2.33	4.47	0.4	42
ssaK	2.29	4.39	0.4	43
ssaL	2.55	5.79	0.4	46
ssaN	2.02	5.01	0.5	51
ssaQ	1.54	1.78	0.6	47
ssaV	1.58	2.75	0.6	47
sscA	2.23	4.75	0.4	51
sscB	1.84	1.70	0.4	48
sseA	5.98	5.38	0.2	41
sseC	3.21	8.26	0.3	46
sseD	3.60	6.66	0.3	49
sseE	3.36	6.37	0.3	45
sseF	1.81	3.85	0.6	49
sseG	1.93	2.49	0.5	48
ssrA	2.74	6.27	0.3	42
ssrB	4.65	8.76	0.2	40
SPI3				
mgtC	1.49	1.39	0.6	50
SPI4				
siiE	1.90	5.43	0.29	48
siiF	2.03	4.22	0.33	35
yjcB	1.40	1.28	0.62	54
SPI5				
pipB	1.51	2.58	0.5	39
pipC	4.97	5.86	0.1	44
pipD	2.10	4.05	0.4	49
sopB	1.62	1.73	0.56	47
cob*/*pdu locus				
cbiA	1.47	1.71	0.6	56
cbiB	1.94	2.00	0.5	59
cbiC	1.50	2.18	0.5	55
cbiD	1.85	3.83	0.5	56
cbiE	1.74	2.74	0.6	58
cbiF	2.60	3.72	0.5	57
cbiG	2.78	3.87	0.4	56
cbiH	2.57	4.19	0.4	57
cbiJ	2.65	2.98	0.5	59
cbiK	4.14	6.37	0.4	56
cbiL	3.86	6.03	0.4	58
cbiM	3.13	6.34	0.4	54
cbiN	3.10	5.55	0.4	50
cbiO	2.93	4.88	0.4	52
cbiP	2.33	4.30	0.5	56
cbiQ	2.16	4.24	0.5	54
cbiT	2.26	2.93	0.5	56
cobS	1.68	2.66	0.7	56
cobU	1.72	2.75	0.6	55
pduC	2.03	3.68	0.5	57
pduD	1.62	4.18	0.6	57
pduE	1.52	2.18	0.8	56
pduF	1.51	3.58	0.6	51
pduM	2.50	2.97	0.4	61
pudB	4.00	5.31	0.3	60

[a] G+C contents were calculated based on the DNA sequence of each ORF acquired from the genome sequence of *S. typhimurium* LT2 (Accession number: NC 003197)

in an RpoS-independent manner. In total, eight genes in SPI-1, twenty in SPI-2, one in SPI-3, three in SPI-4, and four in SPI-5 were induced in the stationary phase in the wild-type and the RpoS$^-$ mutant, but not in the ppGpp-defective mutant. We further validated this observation by identifying the SPI-encoded transcripts by primer extension analysis. The genes that were identified included *hilA* in SPI-1, *ssaG* in SPI-2, and *pipC* and *sopB* in SPI-5. Wild-type and ppGpp-defective mutant *Salmonellae* were grown in LB under standard conditions, and total RNA samples were extracted at the indicated time-points for analysis (Fig. 2). Transcription from *hilA*p and *ssaG*p was initiated from the same position as previously described (Song *et al*., 2004; Lim *et al*., 2006) (panel B). The transcription from *pipC* and *sopB* initiated from the nucleotide (As) at 64 bp and 58 bp upstream of the translation start sites. However, the DNA sequences at and around the -10 region of these promoters were not particularly discernable. Nevertheless, the transcripts from *hilA*p, *pipC*p, and *sopB*p1 were induced from 4 h and the transcript from *ssaG*p was induced from 6 h, when the culture entered stationary phase in wild-type bacteria (left panels) but not for the ppGpp-defective mutant (middle panels). The levels of these transcripts decreased from those points onward.

Since it has been proposed that ppGpp activates gene expression in concert with DksA (Paul *et al*., 2004). We also examined these transcripts in a DksA$^-$ mutant background. The stationary phase activation of the transcripts from SPIs was virtually unaffected by the mutation in the *dksA* gene (Fig. 2). It seems that the mechanism underlying the stationary phase gene activation of SPI genes by ppGpp might be different from that following amino acid starvation in relation to DksA.

Stationary phase induction of *cob*/*pdu* locus genes

The genes for cobalamin (vitamin B12) biosynthesis (*cob*) are coregulated with the genes for the degradation of propanediol (*pdu*) (Roth *et al*., 1996). In our analysis of the microarray data, most of the genes in the *cob* and *pdu* operons were found to be induced at entry into the stationary phase in an RpoS-independent manner (Table 2). In all cases, the degree of stationary phase induction in the RpoS$^-$ mutant was greater than in the wild-type and significantly reduced in the ppGpp-defective mutant. To further verify the microarray results, we

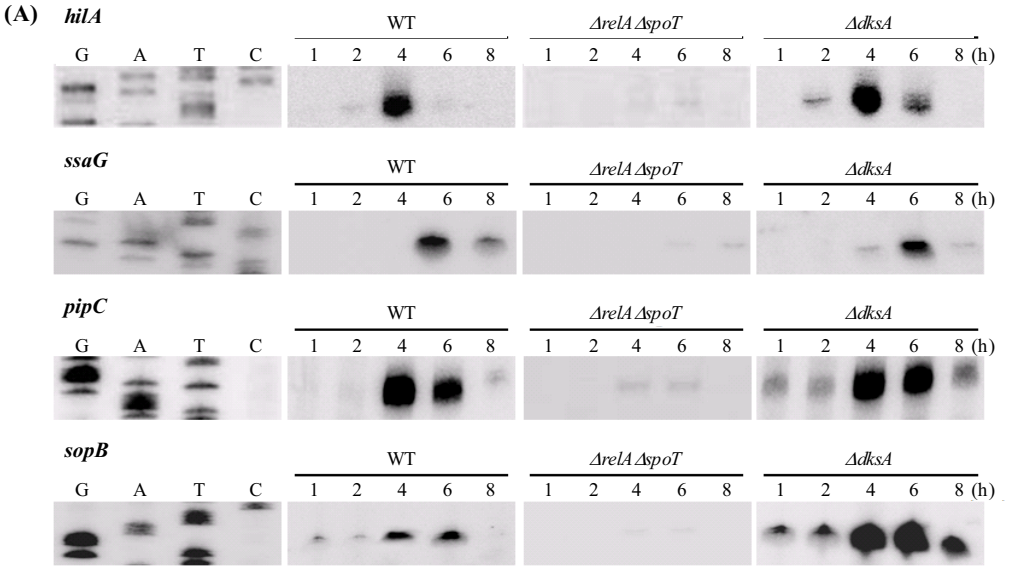

Fig. 2. Transcripts of *hilA* in SPI-1, s*saG* in SPI-2, *sopB* and *pipC* in SPI-5, as determined by primer extension analysis in WT (SCH2005), Δ*relA*Δ*spoT* mutant (SHJ2037), and DksA$^-$ (SMR2170) mutant backgrounds during growth under standard conditions (A). Samples were taken at the indicated time. Panels on left show DNA sequencing ladders. (B) shows DNA sequences around the promoters that were studied, in which the -10 region is underlined and the +1 region is in bold.

performed an assay with *Salmonellae* that contained a Mud–*lac* fusion at CobI, II, and III (Fig. 3) (Bobik *et al*., 1992). *Salmonellae* with Mud-*lac* fusions were grown in LB under standard conditions, and β-galactosidase activity was determined during entry into the stationary phase. In all cases, β-galactosidase activities culminated during entry into the stationary phase. This stationary phase induction remained unaffected in the RpoS$^-$ mutant, but was abolished in the ppGpp-null mutant. Clearly, *cob* operon expression is induced during this growth phase in an RpoS-independent manner. To determine whether or not DksA was also involved in the stationary phase induction of these promoters, the fusions were moved to DksA$^-$ mutant *Salmonellae*, and β-galactosidase activity was determined. It remained at the basal level in the DksA$^-$ mutant background. In contrast to the SPI genes, the stationary phase induction of *cob* operon expression was dependent upon both ppGpp and DksA.

Discussion

The results that are presented in this study show that virtually all SPI-encoded genes are induced at entry to the stationary phase in a ppGpp-dependent but RpoS-independent manner (Table 2 and Fig. 2). In a previous investigation (Song *et al*., 2004), we demonstrated that *hilA*p in SPI-1 is induced in a ppGpp-dependent manner at the stationary phase. HilA, which is a master transcription regulator, controls two major promoters, *invF*p and *sicA*p, in SPI-1 (Song *et al*., 2004). Thus, all SPI-1 encoded genes that are expressed from these two major promoters should also be induced selectively at entry into the stationary phase under standard growth conditions. Expression of the genes that are encoded by the *sse* and *ssa* operons in SPI-2 is known to be modulated by a putative two-component regulatory system, SsrAB, which is also encoded by SPI-2, under growth-adverse conditions, such as low osmolarity and low calcium concentration (Hensel, 2000). The expression of *ssrAB* is, in turn, regulated by the OmpR-EnvZ two-component system and SlyA. SlyA levels were reported to increase upon entry into the stationary phase *in vitro* independently of RpoS and during infection of macrophages (Buchmeier *et al*., 1997). We observed similar stationary phase induction of *slyA* but not of *ompR* (data not shown). *mgtBC* in SPI-3 is required for intramacrophage survival and growth in low Mg^{2+} media, and it has also been reported to increase at the end of active growth in an RpoS-independent manner (Blanc-Potard and Groisman, 1997; Tao *et al*., 1998). This is similar to what we have observed with *mgtC* (Table 2). Limited information is available on the transcriptional organization of genes that are encoded in SPI-4 and SPI-5. Among the SPI-5 genes, which include *pipA*~*D* and *sopB* (Wood *et al*., 1998), primer extension analysis revealed that *sopB* and *pipC* were induced at entry into the stationary phase under standard growth

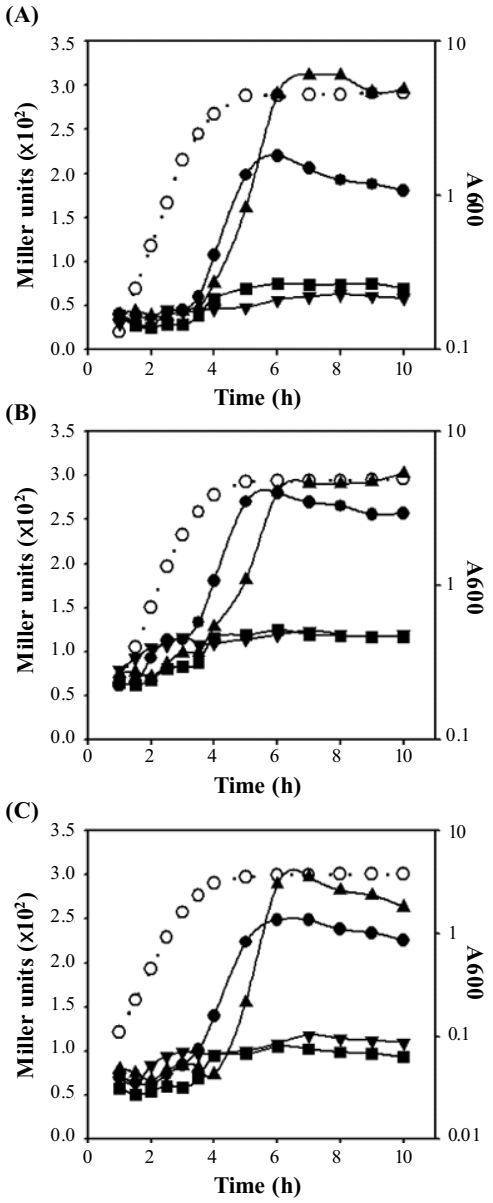

Fig. 3. Expression from *cobI*p (*cob-24*::*mudJ*, A), *cobII*p (*cob-62*::*mudJ*, B) and *cobIII*p (*cob-66*::*mudJ*, C) in WT (closed circles), RpoS$^-$ (closed triangles), Δ*relA*Δ*spoT* (closed squares), and DksA$^-$ (closed reverse triangles) mutant backgrounds during *Salmonella* growth under standard growth conditions. Open circles indicate growth (A_{600}; representative data), and closed symbols signify promoter activities as determined by the β-galactosidase assay (Miller units).

conditions independently of RpoS. Taken together, the results suggested that SPI genes are induced during growth-adverse conditions, which would result in an elevation of the internal stress sensor, ppGpp. The rationale behind stationary phase induction of genes that are encoded by the *cob*/*pdu* locus remains to be established, although it may constitute a reaction to the special environment that is created during entry into the stationary phase: presence of propanediol under anaerobic conditions. Presumably, the entry into the stationary phase would create a somewhat anaerobic condition. In fact, we detected the induction of several anaerobic genes by microarray analysis (data not shown). It has been suggested that anaerobic growth on propanediol requires enzymes that are encoded by the *pdu* operon, which can convert propanediol to propionyl-CoA (Bobik *et al*., 1992).

Here we report that the genes that are not ancestral to the *Salmonella* chromosome and that were introduced by horizontal transfer at or following the divergence of *Salmonellae* from *Escherichia* species are induced at entry into the stationary phase in an RpoS-independent manner. It is unlikely, however, that mere differences in the G+C content determine the nature of gene expression regulation, since genes with both high (*cob*/*pdu* operons) and low G+C content (SPIs) were induced at stationary phase entry. Rather, these genes that are of foreign origin may be stress-inducible and thus expressed only under specific conditions. It seems that a stressful environment is created at entry into the stationary phase under otherwise normal growth conditions. Thus, these stress-inducible genes of foreign origin are induced non-specifically at entry into the stationary phase. We propose that the environmental stress at entry into the stationary phase is conveyed and translated into ppGpp, which serves as an internal signal to trigger the expression of stress-inducible genes that are encoded by horizontally acquired DNA.

A striking observation that was made in this study was that the stationary phase induction of *cob*/*pdu* operon required both ppGpp and DksA but that the induction of the SPI genes only required ppGpp. It has been recently reported that DksA$^-$ and ppGpp-defective mutants shared similar effects on many of the observed relaxed phenotypes with some exceptions, such as cell adhesion (Magnusson *et al*., 2007). The DksA$^-$ mutant increased while the ppGpp-defective mutant abolished the adhesion, as analyzed by yeast cell agglutination. It is suggested that ppGpp would work independently of DksA, depending on the promoters. We have shown previously that, in addition to ppGpp, some other alteration that accompanied entry into the stationary phase may be necessary for *hilA*p induction (Song *et al*., 2004). Further study is necessary to elucidate the mechanism that underlies ppGpp-dependent stationary phase gene induction.

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