

THE BACTERIAL SIGNAL MOLECULE, ppGpp, MEDIATES THE ENVIRONMENTAL REGULATION OF BOTH THE INVASION AND INTRACELLULAR VIRULENCE GENE PROGRAMS OF *Salmonella*

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Running Title: ppGpp and the Regulation of Gene Expression

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During infection of mammalian hosts, facultative intracellular pathogens have to adjust rapidly to different environmental conditions encountered during passage through the gastrointestinal tract and following uptake into epithelial cells and macrophages. Successful establishment within the host therefore requires the co-ordinated expression of a large number of virulence genes necessary for the adaptation between the extracellular and intracellular phases of infection. In this study we show that the bacterial signal molecule, ppGpp, plays a major role in mediating the environmental signals involved in the regulation of both the extracellular and intracellular virulence gene programs. Under oxygen-limiting conditions, we observed a strong ppGpp-dependence for invasion gene expression, the result of severe reductions in expression of the *Salmonella* pathogenicity island 1 (SPI1) transcriptional regulator genes *hilA*, *C*, *D* and *invF*. Over-expression of the non-SPI1-encoded regulator, *RtsA*, restored *hilA* expression in the absence of ppGpp. SPI2-encoded genes, required for intracellular proliferation in macrophages, were activated in the wildtype strain under aerobic, late-log phase growth conditions. The expression of SPI2 genes was also shown to be ppGpp-dependent under these conditions. The results from this study suggest a mechanism for the alternate regulation of the opposing extracellular and intracellular virulence gene programs, and indicate a remarkable specificity

for ppGpp in the regulation of genes involved in virulence compared to the rest of the genome. This is the first demonstration that this highly conserved regulatory system is involved in bacterial virulence gene expression on a global scale.

Introduction

Salmonella enterica serovar Typhimurium (*S. typhimurium*) is a major pathogen of animals and man, causing gastroenteritis characterized by inflammatory diarrhea in humans. *Salmonella enterica* serovars are responsible for up to 120 million cases of gastroenteritis each year in both industrial and developing nations (1). In the United States and Great Britain, *Salmonella* causes more human deaths than any other food-borne pathogen, and may be responsible for an even higher mortality rate than previously realised (2).

As with a number of facultative intracellular pathogens, the course of infection and persistence of *Salmonella* in the host requires virulence gene products often found clustered within pathogenicity islands on the chromosome or on virulence plasmids (3, 4). These virulence factors allow *Salmonella* to invade and colonise host cells, to surmount antimicrobial and physical barriers during infection, and to manipulate and modify host cell activities (5). Currently, five pathogenicity islands have been defined in *S. typhimurium* (6). *Salmonella* pathogenicity islands 1 and 2 (SPI1, SPI2) encode type III secretion systems required for invasion and replication

within host cells, respectively (5-8). SPI3 encodes a high affinity magnesium transport system required for macrophage survival and full virulence in mice (9). The role of SPI4 in *Salmonella* pathogenicity is not as well-characterised, but it encodes products involved in the host-specificity of *Salmonella typhimurium* infections (10). One SPI5-encoded effector protein, SopB, activates the host cell Akt serine-threonine kinase, involved in the regulation of cell proliferation and survival of epithelial cells (11). *S. typhimurium* also harbors a 90 kb virulence plasmid containing the *spv* operon which is required for full virulence in the murine typhoid model (12).

Guanosine tetraphosphate (ppGpp) is the mediator of a highly conserved regulatory system involved in control of global changes in gene expression patterns for adaptation to altered growth conditions. The accumulation of ppGpp in Gram-negative bacteria depends on the products of two genes, *relA* and *spoT*. The ribosome-associated RelA protein is responsible for the high-level ppGpp synthesis during amino acid starvation (stringent response; reviewed in 13). In contrast to RelA, the SpoT protein is a cytoplasmic enzyme with both ppGpp-synthetic and degradative functions (14, 15). The SpoT-derived, basal ppGpp levels accumulate in response to stress and nutrient limitations other than amino acid starvation. In Gram-positive bacteria, a single RelA/SpoT protein is responsible for both functions (16).

There is growing evidence that ppGpp plays an important role in the virulence of pathogenic bacteria, including *Mycobacterium tuberculosis* (17), *Listeria monocytogenes*, (18) *Legionella pneumophila* (19, 20), *Vibrio cholerae* (21) and *Pseudomonas aeruginosa* (22). A recent study found that an *S. typhimurium* $\Delta relA\Delta spoT$ strain was severely attenuated in susceptible BALB/c mice, effectively non-invasive for epithelial cells *in vitro*, and played a crucial role in the regulation of SPI1 and the *spv* virulence plasmid genes in *S. typhimurium* (23, 24). These results suggested that ppGpp may play a larger role in virulence gene expression in *Salmonella*.

In the present study we show that ppGpp is required for the expression of nearly all known *Salmonella* virulence genes in response to growth conditions relevant to host infection. The

mechanism for the alternate regulation of invasion genes and genes required for intracellular survival and proliferation of *Salmonella* within the host has eluded discovery for many years. The results of this study show that ppGpp is required for regulation of both the extracellular (invasion) and intracellular virulence gene programs. One of the environmental signals involved in activation of SPI1 (invasion) gene expression is low-oxygen levels (25-27). In contrast, the environmental signals involved in activation of SPI2 gene expression include acidic pH, phosphate and divalent cation limitations, conditions thought to resemble the phagosomal milieu (28-30). Here, we show that the low-oxygen activation of SPI1 requires ppGpp. Surprisingly, we found that SPI2 genes also show a growth phase-dependent activation in the wildtype strain under aerated growth conditions, and are repressed under low-oxygen conditions. However, SPI2 gene expression was severely reduced under all conditions in the absence of ppGpp. The results suggest that elevated oxygen tension may be an additional signal involved in a ppGpp-dependent activation of SPI2 gene expression. This novel regulatory role of ppGpp in virulence gene expression is shown to be largely independent of changes in the transcription of known global regulators. Since ppGpp is part of a highly conserved regulatory system for mediating the growth response to environmental conditions, this mechanism may represent a common strategy whereby facultative intracellular pathogens regulate the virulence gene programs required for invasion and later survival and persistence within host cells to match the capacity for growth.

Experimental Procedures

Bacterial strains and growth conditions. Strains and plasmids used in this study are listed in Table S4 in supporting information. The wildtype, virulent *Salmonella typhimurium* strain SL1344 (31) was provided by F. Norel (Institut Pasteur) and re-isolated from the spleens of infected Balb/c mice. The SL1344 $\Delta relA71::kan spoT^+$ and $\Delta relA71::kan \Delta spoT281::cat$ strains have previously been described (32). SL1344 harboring a complete deletion of SPI1 was constructed by P22 transduction of the $\Delta SPI1::kan$ allele from

strain SD11 (33). The Δ SPI2 deletion strain, KT3736, was constructed in the same manner, but using the Δ SPI2::*aph(kan)* strain MvP371 (34) as the donor for the P22 lysates, followed by elimination of the kanamycin-resistance cassette induced by plasmid pCP20 (35) yielding strain KT3824. The Δ SPI1::*kan* Δ SPI2 double mutant, KT3930, was constructed by transduction of the Δ SPI1::*kan* allele into KT3824. The SL1344 *phoP60::Tn10d* mutant (strain KT3980) was constructed by P22 transduction of strain SL1344 using strain TT13215 (J.R. Roth) as the donor for the lysates.

Bacterial cultures were cultivated in Lennox broth (36) at 37°C. Pre-cultures were inoculated from -70°C frozen stock cultures into L-broth (containing 0.085M NaCl) and grown to mid-log phase (OD₆₀₀ of approximately 0.5) for inoculation of experimental cultures. Oxygen-limited cultures were grown in filled, stoppered 15 ml Bijou bottles. Aerobic cultures were grown in 50 ml L-broth in 500 ml flasks with shaking. Media were supplemented with carbenicillin (100 µg/ml), kanamycin (50 µg/ml), chloramphenicol (20 µg/ml) or tetracycline (20 µg/ml) for selection of strains during construction. Antibiotics were omitted from experimental cultures.

Nucleic acid extraction and purification.

Bacterial culture samples (5 or 10 ml) were added to one-fifth of the sample volume of a solution of 5% (v/v) phenol in ethanol maintained on ice to stabilise total RNA. Total RNA was extracted and purified as described (37). Chromosomal DNA was removed by digestion with 50 to 100 units of RNase-free DNase (Boehringer), and re-extracted with phenol and chloroform to obtain purified total RNA. The absence of contaminating DNA was verified by PCR reactions involving primers targeting bacterial house-keeping genes.

Microarray construction. The *S. typhimurium* microarrays consisted of 4686 protein coding regions or ORFs (CDS) derived from the complete *S. typhimurium* LT2 genome sequence (38). The construction of the *S. typhimurium* microarrays has previously been described (39-41).

Template labelling and hybridization. RNA for microarray analysis was reverse transcribed into cDNA according to protocols described on the IFR microarray website (www.ifr.bbsrc.ac.uk/safety/microarrays/). Hybridisations were performed as

indirect comparison ('type II') experiments using genomic DNA as the common reference and internal hybridization efficiency control (42).

Data acquisition and analysis. Fluorescence intensities of scanned microarrays were quantified using GenePix Pro software, version 3.0 (Axon Instruments, Inc.). Data was filtered and spots showing a reference signal lower than background plus 2 standard deviations were discarded. Unequal dye incorporation was compensated by median centering. Significance of the data at $P=0.05$ was determined using a parametric-based statistical test adjusting the individual P -value with the Benjamini and Hochberg false discovery rate multiple test correction (43). All expression data for genes discussed in the text have passed this filter and are therefore statistically significant. These tests are features of the GeneSpring™ 6.2 (Silicon Genetics) microarray analysis software package which was used for both data visualisation and analysis. All microarray data used in this study has been made available at NCBI (GEO accession number GSE4631).

Cell culture and invasion assays. LoVo (ATCC CCL-229) intestinal epithelial and J774A.1 (ATCC TIB-67) murine macrophage-like cell lines were grown and maintained in DMEM/Ham's F12 salts medium or Iscove's modified DMEM (Biochrom, Germany), respectively, supplemented with 10% fetal calf serum (FCS) at 37°C and 5% CO₂. Bacteria were grown with aeration to an OD₆₀₀ of between 2 and 3 (late log phase), collected by centrifugation, resuspended in cell culture medium and diluted to the appropriate concentrations for infection. Duplicate wells of monolayers of cells grown in 12-well culture plates (2×10^5 cells/well) were infected to yield an MOI of ≤ 1 per host cell except for the Δ relA Δ spoT and Δ spi1 Δ spi2 strains (MOI of 10). After a centrifugation step (5 min., 250 x g), infected cells were incubated for 30 min. (macrophage) or 60 min. (epithelial cells) prior to a change of medium containing 50 µg/ml of gentamycin. The medium was again replaced after 30 or 60 min., respectively, with medium containing 10 µg/ml of gentamycin for the remainder of the experiment. At the times indicated in the figures, wells were washed twice with PBS, and lysed by addition of 0.1% Triton X-100 in distilled water. Serial dilutions of lysates

were plated for determination of intracellular bacteria. The data shown are representative of at least three, independent assays for all strains.

Results

A ppGpp-dependent switch between expression of virulence genes involved in invasion and intracellular growth.

In a previous study, a $\Delta relA\Delta spoT$ strain of *S. typhimurium* showed severe reductions in the expression of genes involved in invasion of host cells and an avirulent phenotype in susceptible mice (23). However, since non-invasive strains of *Salmonella* are also capable of causing infection (44, 45), it was likely that the expression of additional virulence factors were likely to be affected in this mutant. We therefore used microarray gene expression profiling to assess the full effects of ppGpp on the expression of *S. typhimurium* virulence genes as well as global gene expression patterns. Since it was not known which of the environmental signalling pathways involved in virulence gene expression were affected by the absence of ppGpp, we compared the expression profiles obtained from wildtype and $\Delta relA\Delta spoT$ strains grown under four different growth conditions: low-oxygen cultures grown in L-broth containing 0.3M NaCl, previously reported to activate SPI1 invasion genes (25, 26), and the separated growth conditions of either L-broth with low oxygen or aerated L-broth, with or without 0.3M NaCl.

The role of ppGpp on pathogenicity island gene expression was first placed into context by comparing transcript levels of the wildtype strain for pathogenicity island genes encoded within SPI1 through SPI5 (Fig. 1). Genes encoded within SPI1 and SPI5 were expressed under all growth conditions examined. Additionally, SPI4 was found to be activated under aerated L-broth growth conditions (Fig. 1A). SPI3 signal intensities were low under all growth conditions. Surprisingly, SPI2-encoded genes showed significant levels of transcription under aerated growth conditions, irrespective of the salt concentration in the medium (Fig. 1A,C). These observations were unexpected, since SPI2 genes are not thought to be activated under these growth conditions (28, 29). Under low-oxygen conditions, the wildtype strain showed continued expression of SPI1-encoded invasion genes as expected (Fig.

1B,D), but a near complete repression of SPI2-encoded genes.

The transcriptional profiles of the the ppGpp-deficient $\Delta relA\Delta spoT$ strain was compared with the wildtype strain to determine the role of ppGpp in virulence gene expression under the same, four environmental conditions. The $\Delta relA\Delta spoT$ mutant showed reduced transcript levels of SPI1-encoded (invasion) genes under aerated growth conditions (Fig. 1B and F), but essentially a complete loss of invasion gene expression under oxygen-limited growth conditions (Fig. 1, panels D and H). In the high salt, low-oxygen (SPI1-activating) growth conditions, genes encoding the major transcriptional activators of SPI1 genes, *hilACD* and *invF*, showed 2- to 6-fold reduced expression in the $\Delta relA\Delta spoT$ strain, explaining the loss of invasion gene expression (23). These results indicated that the severe reductions in SPI1 gene expression in the $\Delta relA\Delta spoT$ strain were independent of the salt concentration in the medium; rather, the response to low-oxygen levels appeared to have been lost. This was of particular interest since no regulatory factor has been identified to explain the activation of *hilA* expression under low-oxygen conditions (26, 46). Large reductions in the expression of the SPI5 effector protein genes *sopE2*, *pipC* and *sopB* were also observed (Fig. 2D). The secretion of these proteins is dependent on the SPI1 type III secretion apparatus (46).

While the low-oxygen growth conditions mainly affected the expression of SPI1-encoded invasion genes in the $\Delta relA\Delta spoT$ strain (Fig 2BD), under oxygenated growth conditions loss of ppGpp had the greatest effect on virulence genes involved in intracellular growth and/or survival (Fig 2AC). We observed up to 10-fold reduced expression of SPI2-encoded genes, and reduced expression of other virulence-related genes (*pipB*, *pipB2*, *sopE2*, *sifA*, *sopD* and the PhoPQ-regulated *pagCK*) involved in phagosome biogenesis or intracellular survival (47, 48; Table S2 in supporting information). Whereas the wildtype strain showed activation of SPI2-encoded genes in the presence of oxygen, the $\Delta relA\Delta spoT$ strain showed no expression of SPI2 genes under any of the growth conditions tested. These observations indicated that ppGpp was required for the response

to environmental signals involved in the regulation of both SPI1 and SPI2 pathogenicity islands. Under low-oxygen growth conditions, SPI1 gene expression was strongly ppGpp-dependent, whereas under aerated conditions SPI1 genes showed only reduced expression, but there was no activation of SPI2 gene expression as seen in the wildtype. Therefore, ppGpp was required for both the low oxygen-dependent activation of SPI1 genes, and the oxygen-dependent activation of SPI2 genes. However, since the growth rates for the wildtype strain were different under these two conditions, the involvement of other previously reported factors or components of the media in the ppGpp-dependent effect on SPI2 activation cannot be excluded. Nevertheless, the results indicated that the expression of both SPI1- and SPI2-encoded genes were dependent on ppGpp. One curious exception to the general reduction in virulence gene expression was the SPI3-encoded *slsA* gene, which has been annotated only as a conserved, hypothetical inner membrane protein. Under aerated growth conditions, *slsA* showed up to two- to five-fold higher levels of expression in the $\Delta relA\Delta spoT$ strain (Figs. 1B,F and 2A,C, Table S2).

In contrast to virulence-related genes, no major reductions in the expression of genes encoding components of the transcriptional and translational apparatus were observed in the $\Delta relA\Delta spoT$ strain (Fig S5 and Table S5 in supporting information). There were also no significant differences in the growth rates under all four conditions examined (data not shown). These results indicated that the severe ppGpp-dependent reductions in SPI1 and SPI5 gene expression were not the result of a general repression of gene expression.

The effects on regulatory gene expression in the $\Delta relA\Delta spoT$ strain is limited. One goal of this study was to determine whether the ppGpp-dependent loss of virulence gene expression reflected altered levels of known or previously unidentified regulators of virulence gene expression. As noted above, regulatory genes which showed reduced expression in the $\Delta relA\Delta spoT$ strain under low-oxygen conditions included the major SPI1 and SPI2 pathogenicity island regulators *hilACD*, *invF*, and *ssrA/B* (Fig. 3B,D). In addition to pathogenicity island-encoded

regulatory genes, many other global regulators play significant roles in maintaining the virulence of *Salmonella* (reviewed in 26, 46, 49, 50). A comparison of the expression patterns of known regulatory genes revealed only four global regulatory genes with significant ppGpp-dependent reductions in expression under the low-oxygen conditions (Fig. 3B,D and Table S3 in supporting information). Of these four global regulators (Dps, Lrp and RtsA/B), only RtsA/B have been shown to be involved in *hilA* gene expression (51, 52), and both genes showed 3- to 5-fold reductions in expression in the absence of ppGpp under low-oxygen conditions (Fig. 3B,D).

To determine whether the loss of expression of *dps*, *lrp* or *rtsAB* contributed to reduced invasion gene expression in the $\Delta relA\Delta spoT$ strain, we examined the effects of both mutations and over-expression of Dps, Lrp and RtsA/B on the expression of a *hilA-lacZ* fusion in the wildtype and $\Delta relA\Delta spoT$ strains. As shown in Fig. 4, transcriptional fusions with *rtsA* and *rtsB* verified a strong ppGpp-dependence for expression, consistent with the microarray data. Over-expression of RtsA or RtsA/B only partially compensated for the loss of *hilA* gene expression in the $\Delta relA\Delta spoT$ strain (Fig. 5), presumably due to the reduced expression of *hilA* gene co-activators, HilC and HilD (52). These results identified RtsA and/or RtsA/B as the first, non-SPI1-encoded regulators whose expression could be directly linked to loss or severe reductions in *hilA* and SPI1 gene expression in the $\Delta relA\Delta spoT$ strain.

In contrast, neither the over-expression of Dps or Lrp, nor the introduction of mutations in *dps* or *lrp* showed any effect on the loss of *hilA* expression in the $\Delta relA\Delta spoT$ strain, indicating that the reduced expression of Dps and Lrp was not involved in loss of SPI1 gene expression. Indeed, over-expression of these regulatory genes resulted in reduced *hilA* expression in the wildtype strain, suggesting both can act as repressors of *hilA* expression (supplementary Figs. S1 and S2). The SirA/BarA two-component system has also been found to play a role in the activation of *hilA* expression (53). Although the microarray data indicated no significant effects on the expression of the *sirA(uvrY)/barA* genes in the $\Delta relA\Delta spoT$ strain under any of the growth conditions, we also

examined the effects of SirA over-expression. Despite an up to two-fold activation of *hilA* expression in the wildtype strain, the over-expression of SirA did not compensate for reduced *hilA* expression in the $\Delta relA\Delta spoT$ strain, suggesting that SirA levels were not limiting (supplementary Fig. S3).

It remained possible that the over-expression of a number of global regulatory genes in the $\Delta relA\Delta spoT$ strain might also have been responsible for loss of SPI1 gene expression (Fig. 3). The two-component PhoP/Q system functions as a negative regulator of *hilA* and invasion gene expression (reviewed in 46), and *phoP* gene expression showed significant elevations under the SPI1-activating growth conditions in the $\Delta relA\Delta spoT$ strain (Fig. 3D). However, a previous study had shown that the elevated *phoP* expression in the $\Delta relA\Delta spoT$ strain did not contribute to loss of *hilA* expression (23), observations which we verified (data not shown). Genes encoding the global regulators *fis* and *fur* also showed elevated expression levels in the $\Delta relA\Delta spoT$ strain under low-oxygen growth conditions (Fig. 3B,D). While Fis has been found to activate *hilA* expression (26), both Fis and Fur are also known to have repressor activity. The introduction of mutations in either of these genes reduced *hilA* expression in both the wildtype and $\Delta relA\Delta spoT$ strains, indicating that the loss of *hilA* expression in the $\Delta relA\Delta spoT$ strain was not due to repression by these regulators (supplementary Fig. S4). The loss of *hilA* and invasion gene expression in the $\Delta relA\Delta spoT$ strain therefore appeared to result from the concomitant loss of expression of both SPI1-encoded (*hilC*, *hilD* and *invF*; 23, 24; and this study) and the non-SPI1-encoded regulators, RtsA/B (Figs. 3 and 4).

The expression of SPI2-encoded genes, involved in establishing intracellular growth, is also dependent upon a number of regulators. Encoded within SPI2, SsrA/B are the key regulators for the majority of SPI2-encoded genes (7, 54, 55), and *ssrA/B* expression is in turn dependent upon the OmpR/EnvZ two-component regulators (30, 56) and SlyA, a global transcriptional regulator affecting a number of functions including flagellar biosynthesis (57). To determine whether over-expression of SsrA/B would compensate for the reduced SPI2

expression observed in the microarray study for the $\Delta relA\Delta spoT$ strain (Fig. 1B,F; Fig. 2A,C), we examined the effects of SsrA/B over-expression on a chromosomally-encoded SPI2 *ssaG* promoter-GFP transcriptional fusion in the wildtype and $\Delta relA\Delta spoT$ strains (Fig. 6). The expression from the *ssaG* promoter was low in both strains compared to a control, ribosomal protein gene promoter (Fig. 6A). However, there was an apparent growth phase-dependent activation late in the growth phase in the wildtype as previously reported (58), but which was at least two-fold reduced in the $\Delta relA\Delta spoT$ strain (Fig. 6B). The reduced expression of *ssaG* in the $\Delta relA\Delta spoT$ strain was fully compensated by the over-expression of plasmid-encoded SsrA/B (Fig. 6C). The high background level of expression of *ssaG* in both the wildtype and $\Delta relA\Delta spoT$ strains was likely due to incomplete repression of the arabinose-inducible promoter. Nevertheless, these results supported the suggestion that the reduced levels of SPI2-encoded gene expression observed in the microarray analysis for the $\Delta relA\Delta spoT$ strain was likely due to the parallel loss or reduction in *ssrAB* expression.

Since *ssrA/B* expression is dependent on OmpR, we also determined the expression levels of *ompR* as well as a non-virulence related OmpR-dependent gene, *ompC*, in both the wildtype and $\Delta relA\Delta spoT$ strains. Under normal, aerated growth conditions where the levels of expression of SPI2-encoded genes were highest in the wildtype strain (Fig. 1A), the levels of *ompR* expression in the $\Delta relA\Delta spoT$ strain were similar to that of the wildtype (supplementary Fig. S5). Likewise, the level of expression of *ompC* was essentially identical in both strains, indicating that OmpR was both expressed and active under these growth conditions. These results indicated that the reductions observed for *ssrA/B* and SPI2 gene expression in the $\Delta relA\Delta spoT$ strain were not the result of loss of either the expression or activities of OmpR. Interestingly, under the high salt, low oxygen growth conditions, where SPI2 gene expression was repressed in the wildtype strain (Fig. 1G), both *ompR* and *ompC* expression was activated in the wildtype strain (Fig. S5B,D). In contrast, the expression levels in the $\Delta relA\Delta spoT$ strain remained the same regardless of the growth conditions.

ppGpp is required for intracellular growth. A number of *Salmonella* virulence genes in addition to those encoded by SPI2 are required for intracellular proliferation within macrophage and other cell types. To determine whether the expression of genes involved in this aspect of pathogenesis were also affected in the $\Delta relA\Delta spoT$ strain, we compared the intracellular growth phenotypes of the wildtype and $\Delta relA\Delta spoT$ strains in both intestinal epithelial and murine macrophage cell lines. Consistent with the reduced levels of SPI1-encoded invasion genes, at least ten-fold higher levels of infecting $\Delta relA\Delta spoT$ bacteria were required to achieve similar initial uptake levels as the wildtype strain in both cell types. As shown in Fig. 7, panels A and B, the intracellular growth phenotype of the $\Delta relA\Delta spoT$ strain varied with cell type. In epithelial cells, the mutant showed only a growth defect, with no increase in intracellular colony forming units for up to 24 hours (Fig. 7A), whereas the wildtype strain showed a 20-fold increase in the same time period.

We also compared the intracellular growth kinetics with a number of known mutants affecting proliferation and survival within macrophage. As shown in Fig. 7, panels C and D, strains harboring mutations or complete deletions of *phoP* or SPI2, respectively, showed phenotypes distinct from that of the $\Delta relA\Delta spoT$ strain. Whereas the former strains showed an intracellular growth defect, *i.e.* no increase in intracellular bacteria with time, the $\Delta relA\Delta spoT$ strain showed a rapid loss in viable, colony-forming units, indicating that both intracellular growth as well as survival mechanisms were affected (Fig. 7B). These results indicated that the loss of ppGpp affected mechanisms involved in proliferation and intracellular survival in addition to those dependent upon either the PhoP regulon or the SPI2 type III secretion system.

Discussion

Virulence gene regulation in *Salmonella* is complex and overlapping regulatory networks have evolved to enable *Salmonella* to adapt and survive in both the intracellular and intestinal environments (28, 30, 46, 48-50). Previous studies have shown that subsets of virulence genes are

expressed in response to various extra- and intracellular signals. The current study shows that ppGpp is required for the expression of these, and most of the other known virulence-related genes in *Salmonella*. In addition, the reduced expression of virulence genes occurs in the absence of major effects on the expression of other, known regulatory genes. Indeed, for the two growth conditions of aerated L-broth and high salt, low oxygen, 56% and 37% of the total virulence genes, respectively, showed ppGpp-dependent reductions of greater than two-fold. This was in marked contrast to the remainder of the genome which showed two-fold reduced expression for only 8% and 10% of the total genes under the same growth conditions (Supplementary Table S1). The results of this study therefore define ppGpp as the first regulatory effector found to play a comprehensive role in the expression of virulence genes in *Salmonella*.

The results show that ppGpp is required to mediate the environmental signals involved in the activation of either the invasive or intracellular virulence gene programs in *Salmonella* (Figs. 1 and 2), and identify the low-oxygen response for SPI1 activation as a key ppGpp-dependent pathway. The data also identify a possible mediator for the ppGpp-dependent regulatory pathway involved in SPI1 expression under low-oxygen conditions. To date, no regulatory factor has been identified to explain the activation of *hilA* expression under low-oxygen conditions (25, 26, 46). Under low-oxygen conditions, the large reductions in SPI1 gene expression correlated with up to a 10-fold loss in expression of the SPI1-encoded regulators *hilA* and *hilC,D* (Fig. 3BD). Prior studies showed that RtsA/B was capable of increasing the expression of *hilA*, *hilC*, *hilD* and *invF* as well as other virulence genes dependent upon these regulators (51, 52), results also verified in this study (Figs. 4 and 5). RtsA/B are the first non-SPI1 encoded regulators shown to be directly involved in *hilA* and SPI1 gene expression in a ppGpp-dependent manner. The observation of a strong ppGpp-dependence for the expression of the *rtsA/B* genes under low-oxygen growth conditions may therefore provide the missing, additional components explaining the loss of SPI1 (invasion) gene expression in the $\Delta relA\Delta spoT$ strain.

It was unexpected that significant levels of transcription of SPI2 genes were observed in the wildtype strain under aerated growth conditions (Fig. 1A), since the environmental signals involved in regulation of this pathogenicity island are not assumed to be present in these growth media. Acidic pH, limiting divalent metal ions, and phosphate limitation have all been reported as signals involved in SPI2 gene activation (28, 29, 59). The signals involved in the observed activation of SPI2 gene expression in the wildtype strain in aerated, L-broth cultures is not clear; however, the activation appears to require oxygen and is clearly ppGpp-dependent (Figs. 1 and 2). SPI2-encoded genes are activated intracellularly in macrophages, an apparently oxygen-rich environment (40, 54). One of the ppGpp-dependent components in the signalling pathway leading to SPI2 activation was found to be SsrB, the response regulator for SPI2 genes. The loss of activation of SPI2 expression in the $\Delta relA\Delta spoT$ strain under aerated growth conditions correlated with a two-fold reduction in *ssrB* gene expression (Fig. 3), and SsrA/B over-expression was able to compensate for reduced levels of *ssaG* (Fig. 6C). Since the expression levels and activities of the *ompR* gene product did not appear to be affected under these conditions (Figs. 3 and S5), it remains unclear whether the reductions in *ssrB* expression represent a direct or indirect result of loss of ppGpp. The $\Delta relA\Delta spoT$ strain also showed extensive defects in macrophage survival and proliferation in host cells, as indicated by the distinct intracellular phenotype of the $\Delta relA\Delta spoT$ strain compared to other known regulatory and virulence mutants (Figs. 8 and S6). These observations support a major role for ppGpp in regulation of the intracellular gene expression program in addition to its role in invasion gene expression.

A recent study showed that SPI2 genes are activated extracellularly prior to invasion of the intestinal epithelium, indicating that at least some of the signals involved in activation of intracellular virulence genes are also present in the intestinal lumen (60). Based on the observed loss of SPI1 activation under oxygen-limiting conditions, and the lack of activation of SPI2 genes under aerated growth conditions in the $\Delta relA\Delta spoT$ strain, we speculate that oxygen

tension may serve as an additional signal mediating the switch between the SPI1 and SPI2 gene expression programs. Activation of SPI2-encoded genes outside of the intracellular environment may result from proximity to the intestinal wall, where diffusion of oxygen from underlying tissues may be sensed by *Salmonella*. Invasive *Salmonella* in close contact with the intestinal epithelia would therefore activate genes required for intracellular growth and survival prior to or at the same time as invasion. This might also explain why *in vitro* growth conditions have generally not been able to show simultaneous activation of both of these pathogenicity islands. As noted previously (26, 46), it is likely that the role of oxygen in the regulation of SPI1, and possibly SPI2, is indirect, a result of changes in bacterial metabolism and global regulatory responses. The observation of a growth phase (cell density) dependence for both SPI1 and SPI2 gene expression would be consistent with this idea.

Apart from reduced *ssrA/B* and *rtsA/B* gene expression, we observed no consistent ppGpp-dependent differences in the expression of other, known regulatory genes that would explain the observed loss of global virulence gene expression. Indeed, for a number of regulatory systems, we noted large reductions in virulence-related gene expression, but little or no effects on house-keeping genes controlled by the same regulators. Supportive of this observation is a recent study which examined the immunogenicity of the $\Delta relA\Delta spoT$ strain in mice (61). Despite the avirulent phenotype of the mutant in mice, immunization with the strain was able to elicit both systemic and mucosal antibody responses against a subsequent challenge with the wildtype strain. This observation also indicates that the majority of all other epitopes and bacterial components were expressed and available for antigen presentation as part of an immune response. The observation that deletion of either or both of the major pathogenicity islands, a gene involved in establishment and maintenance of the phagosome (*sifA*) or a regulator of genes involved in bacterial defense mechanisms (*phoP*), were unable to reproduce the reduced survival and/or intracellular growth of the $\Delta relA\Delta spoT$ strain (Fig. 7 and S6), supports a global role for ppGpp in the regulation of both extra- and intracellular virulence gene expression.

The results of this study are consistent with a model wherein the horizontal acquisition of virulence determinants by facultative, intracellular pathogens is brought under control of the same regulatory system involved in growth rate control and regulation of global gene expression in response to changing growth conditions. As noted in a previous study (23), the simplest explanation for the strong ppGpp-dependence for invasion gene expression might be related to the higher AT-content of these acquired genes and islands relative to the remainder of the genome. AT-rich promoters have been found to show a ppGpp-dependence for expression (62). Consistent with this idea, a whole genome comparison of gene expression levels versus GC-content indicates a skew toward reduced expression of genes with high AT-content in the $\Delta relA\Delta spoT$ strain. While the AT-content of coding regions may not necessarily reflect the base composition of promoter regions, for the few virulence gene promoters which have been mapped, the correlation appears to hold true for the promoters as well (unpublished observations). This model would also explain the apparently inconsistent expression patterns of genes dependent upon the various regulators. Since the major function of ppGpp is the re-allocation or partitioning of limiting RNA polymerase among promoters in the cell (63), the specificity for a given gene's expression will also depend on the presence of accessory proteins and regulators involved in RNA polymerase recruitment for activation of promoters in the various regulons. However, in a $\Delta relA\Delta spoT$ strain, promoters which require either ppGpp-bound RNA polymerase for transcription or unusually high local concentrations of RNA polymerase would still be poorly expressed despite the presence of accessory proteins, due to

insufficient free RNA polymerase available for mRNA synthesis.

Attention has recently been focussed on an additional RNA polymerase-associated protein involved in the regulation of gene expression by ppGpp, DksA (64). DksA has been described to enhance both the inhibitory (rRNA promoters) and activating (*e.g.* amino acid gene promoters) regulatory effects of ppGpp (65). The absence of significant changes in *dksA* expression under any of the growth conditions examined here (Table S1) is consistent with observations in *E. coli*, and further suggests the effects observed here for virulence gene expression is due to the loss of ppGpp, although DksA likely plays a co-regulatory role in the wildtype strain.

The lack of significant effects on expression of "house-keeping" genes coupled with large reductions in virulence-related genes dependent upon the same regulators may therefore simply reflect the high AT-content of the promoters of the latter, horizontally-acquired gene sets. We suggest that promoters for house-keeping genes may have been optimized during evolution for a certain degree of ppGpp-independence to assure basal levels of expression under all growth conditions, whereas recently acquired virulence gene promoters remain strongly dependent on ppGpp.

Acknowledgements

We thank B. Ahmer, G.F.-L. Ames, J.M. Calvo, J. Foster, T. Henry, M. Hensel, K. Hughes, D.G. Kehres, M.E. Maguire, F. Norel and J.M. Slauch for strains and plasmids used in this study, and I. Hautefort and L. Scharek for assistance with the flow cytometry determinations. This study was funded by a BBSRC core strategic grant to JH. KT was supported by a grant from the Fondation pour la Recherche Médicale and the Deutsche Forschungsgemeinschaft (DFG).

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Figure Legends

Fig. 1. Expression levels of genes encoded within SPI1 to 5 in the wildtype and $\Delta relA\Delta spoT$ strains. Data shown is the log of the signal intensity ratio of Cy5-labelled cDNA to Cy3-labelled genomic DNA for the wildtype (panels A,C,E and G) and $\Delta relA\Delta spoT$ strains (panels B,D,F and H). Transcript levels were determined under four growth conditions: (A,B) L-broth with aeration; (C,D) L-broth with low-oxygen; (E,F) L-broth containing 0.3M NaCl with aeration; and (G,H) L-broth 0.3M NaCl and low-oxygen conditions. Data is summarized in Table S2 in supporting information.

Fig. 2. Comparison of the relative expression levels of SPI1 to 5 in the wildtype and $\Delta relA\Delta spoT$ strains. Data shown is the log of the relative signal ratios of the $\Delta relA\Delta spoT$ strain relative to the wildtype. Increased or decreased expression is indicated by red or blue bars, respectively. Growth conditions were (A) L-broth with aeration; (B) L-broth with low-oxygen; (C) L-broth containing 0.3M NaCl with aeration; and (D) L-broth 0.3M NaCl and low-oxygen conditions. Data is summarized in Table S2 in supporting information.

Fig. 3. Relative expression levels of global and virulence-related regulatory genes in the wildtype and $\Delta relA\Delta spoT$ strains. Increased or decreased expression is indicated by red or blue bars, respectively. Growth conditions in panels A - D are ordered as in Fig. 2. Data is summarized in Table S3 supporting information.

Fig. 4. Loss of *rtsAB* gene expression in the $\Delta relA\Delta spoT$ strain. β -galactosidase activities were determined for the wildtype (open bars) or $\Delta relA\Delta spoT$ (filled bars) strains harboring *lacZ* fusions to either the *rtsA* or *rtsB* genes (indicated below). Shown are the results for either (A) aerated L-broth cultures or (B) L-broth 0.3M NaCl low oxygen conditions. Data shown are representative of at least three, independent experiments.

Fig. 5. RtsAB over-expression compensates for loss of *hilA* expression in the $\Delta relA\Delta spoT$ strain. Wildtype or $\Delta relA\Delta spoT$ strains harboring chromosomal *hilA-lacZ* fusions and the indicated plasmids were grown in either (A) aerated L-broth or (B) L-broth 0.3M NaCl low oxygen conditions and shifted to the same medium with (filled bars) or without (open bars) arabinose for induction of either *rtsA* or *rtsAB* expression under transcriptional control of the BAD promoter. The vector, pBAD30, served as control. Data shown are representative of at least three, independent experiments.

Fig. 6. Reduced SPI2 gene (*ssaG*) expression in the $\Delta relA\Delta spoT$ strain and compensation by SsrA/B over-expression. (A) Wildtype or $\Delta relA21 spoT-211::Tn10$ strains harboring transcriptional *gfp* fusions with either no promoter (JH3008 and KT3456 respectively), the *ssaG* promoter (JH3009 and KT3458 respectively) or the *rpsM* promoter (strains JH3016 and KT3462 respectively) were grown in aerated L-broth and the fraction of cells expressing GFP was determined by flow cytometry. (B) Wildtype (JH3009) or $\Delta relA21 spoT-211::Tn10$ (KT3458) strains harboring the *ssaG* promoter *gfp* fusion grown in aerated L-broth and culture samples examined for GFP expression levels at the indicated optical densities. In panels A and B, open bars indicate the SL1344 strain background, filled bars the $\Delta relA21 spoT-211::Tn10$ strain. (C) *ssaG*-GFP expression in wildtype or $\Delta relA\Delta spoT$ strains (indicated below) harboring plasmid p1437-1 encoding the *ssrAB* genes under transcriptional control of the BAD promoter. Wild type strains are JH3008 (promoterless), JH3009 (*PssaG-gfp*⁺) and JH3016 (*PrpsM-gfp*⁺); $\Delta relA21 spoT-211::Tn10$ strains are KT3456 (promoterless), KT3458 (*PssaG-gfp*⁺) and KT3462 (*PrpsM-gfp*⁺). Open bars indicate the non-induced cultures, filled bars represent cultures induced by addition of 0.1% arabinose. Results shown are representative of at least three, independent experiments.

Fig. 7. Comparison of the intracellular growth characteristics of the $\Delta relA\Delta spoT$ strain with known mutations affecting growth in macrophage. Comparison of the intracellular growth kinetics of the wildtype (open symbols) and the $\Delta relA\Delta spoT$ strain (filled symbols) in (A) intestinal epithelial or (B) murine macrophage cell lines. Intracellular growth kinetics of *phoP* (KT3980, panel C) and Δ SPI2 (KT3824, panel D) mutants were determined in murine macrophage. In all panels, the wildtype strain is

indicated by open symbols, the various mutants by filled symbols. Results shown are representative of at least three, independent experiments.

Supplementary Figure Legends

Fig. S1. Reduced *dps* expression does not play a role in loss of *hilA* expression in the $\Delta relA\Delta spoT$ strain. Shown are the β -galactosidase activities for a *hilA-lacZ* fusion in the wildtype (CL87, open bars) and $\Delta relA\Delta spoT$ strain (KT2786, filled bars) harboring either the plasmid pDps containing the *dps* gene under transcriptional control of the *tac* promoter (panels A, B) or a *dps::kan* allele (panels C, D). In panels A and B, β -galactosidase activities were determined in either the absence (open bars) or presence (filled bars) of IPTG. In panels C and D, expression levels of the *hilA-lacZ* fusion were determined in either the wildtype and *dps::kan* (CL87 and KT3040, resp.) or *relA21::Tn10* $\Delta spoT$ and *relA21::Tn10* $\Delta spoT$ *dps::kan* (KT2786 and KT3166, resp.) strain backgrounds. Strains were grown either in L-broth with aeration (panels A and C) or L-broth 0.3M NaCl under limiting oxygen conditions (panels B and D). Strain backgrounds are indicated below the figure. Results shown are representative of at least three, independent experiments.

Fig. S2. Reduced *lrp* expression does not play a role in loss of *hilA* expression in the $\Delta relA\Delta spoT$ strain. Shown are the β -galactosidase activities for a *hilA-lacZ* fusion in the wildtype (CL87, open bars) and $\Delta relA\Delta spoT$ strain (KT2786, filled bars) harboring either the plasmids pKS(-) (control, open bars) or Lrp-expressing plasmid pCV192 (filled bars). Expression of Lrp from plasmid pCV192 is constitutive. In panels C and D, expression levels of the *hilA-lacZ* fusion were determined in either the wildtype CL87 or KT2786 strain backgrounds (open bars) or in wildtype and *relA21::Tn10* $\Delta spoT$ derivatives harboring in addition the *lrp::Tn5(kan)* allele (strains KT3558 and KT3562, respectively) (filled bars). Strains were grown either in L-broth with aeration (panels A and C) or L-broth 0.3M NaCl under limiting oxygen conditions (panels B and D). Strain backgrounds are indicated below the figure. Results shown are representative of at least three, independent experiments.

Fig. S3. Over-expression of SirA does not compensate for loss of *hilA* expression in the $\Delta relA\Delta spoT$ strain. Shown are the β -galactosidase activities for a *hilA-lacZ* fusion in the wildtype (CL87) or $\Delta relA\Delta spoT$ (KT2786) strains harboring either plasmid pBA324 containing the *sirA* gene under transcriptional control of the arabinose-inducible pBAD promoter or control plasmid pBAD30. β -galactosidase activities were determined in either the absence (open bars) or presence (filled bars) of 0.1% arabinose. Strains were grown either in L-broth with aeration (panel A) or L-broth 0.3M NaCl under limiting oxygen conditions (panel B). Strain backgrounds are indicated below the figure. Results shown are representative of at least three, independent experiments.

Fig. S4. Over-expression of Fis or Fur is not responsible for loss of *hilA* expression in the $\Delta relA\Delta spoT$ strain. Shown are the β -galactosidase activities for a *hilA-lacZ* fusion in the wildtype and $\Delta relA\Delta spoT$ strains (strains CL87 and KT2786, resp., open bars) harboring either a mutation in *fis* (strains KT3628 and KT3632, resp., panels A and B, filled bars) or a deletion of the *fur* gene (strains KT3584 and KT3588, resp., panels C and D, filled bars). Strains were grown either in L-broth with aeration (panels A and C) or L-broth 0.3M NaCl under limiting oxygen conditions (panels B and D). Strain backgrounds harboring the *fis* or *fur* mutations are indicated below the figure. Results shown are representative of at least three, independent experiments.

Fig. S5. OmpR and OmpC expression in the $\Delta relA\Delta spoT$ strain. Shown are the β -galactosidase activities determined for the wildtype (open bars) and $\Delta relA\Delta spoT$ strains (filled bars) harboring *lacZ* fusions to either *ompR* (strains KT3868 and KT3872, resp.; panels A and B) or *ompC* (strains KT4018 and KT4022, resp.; panels C and D). Strains were grown in aerated L-broth to an OD₆₀₀ of approximately 2 and shifted to either aerated L-broth (A and C) or L-broth 0.3M NaCl low oxygen (B and D) and incubated for an additional 2 or 4 hours, respectively. Results shown are representative of at least three, independent experiments.

Fig. S6. Comparison of the intracellular growth characteristics of the $\Delta relA\Delta spoT$ strain with known mutations affecting growth in macrophage. The intracellular growth kinetics in the J774A.1 murine macrophage cell line of the wildtype (open symbols) and the $\Delta relA\Delta spoT$ strain (KT2160, panel A) are shown compared to the growth kinetics of $\Delta sifA::kan$ (KT3960, panel B), $hilA::kan \Delta SPI2$ (KT3914, panel C), and $\Delta SPI1::kan \Delta SPI2$ (KT3930, panel D) mutants. In all panels, the wildtype strain is indicated by open symbols, the various mutants by filled symbols. Results shown are representative of at least three, independent experiments.

Table S4. Bacterial Strains and Plasmids^a

| Strain | Genotype/Phenotype | Source or Reference ^b |
|---------|--|----------------------------------|
| SL1344 | <i>hisG46 rpsL</i> | (1) |
| CL87 | SL1344 <i>iagB87::lacZY</i> | (2) |
| DH215K | <i>S. typhimurium</i> ATCC 14028 Δ <i>sifA::kan</i> (Km) | T. Henry |
| GA445 | <i>S. typhimurium</i> LT2 <i>dhuA1 lrp-1::Tn5</i> (Km) | G.F.-L. Ames |
| JF4178 | <i>ompC::MudJ</i> (Km) | J.W. Foster |
| JF4484 | <i>putPA1303::kan::ompR-lacZ</i> | J.W. Foster |
| JH3008 | SL1344 (promoterless <i>gfp</i> ⁺) (Cm) | (3) |
| JH3009 | SL1344 Φ (<i>PssaG-gfp</i> ⁺) (Cm) | (3) |
| JH3016 | SL1344 Φ (<i>PrpsM-gfp</i> ⁺) (Cm) | (3) |
| JS324 | <i>S. typhimurium</i> ATCC 14028 (<i>rtsA::lac</i>)5 (Km) | (4) |
| JS325 | <i>S. typhimurium</i> ATCC 14028 (<i>rtsB::lac</i>)6 (Km) | (4) |
| MM2635 | SL1344 Δ <i>fur41::kan</i> (Km) | D.G. Kehres |
| MM2819 | SL1344 <i>dps::kan</i> (Km) | D.G. Kehres |
| MvP371 | <i>S. typhimurium</i> ATCC 14028 Δ SPI2:: <i>aph</i> (Km) | (5) |
| SD11 | SL1344 Δ SPI1:: <i>kan</i> (Km) | (6) |
| TH1763 | <i>S. typhimurium</i> LT2 <i>fis-2::kan</i> (Km) | K. Hughes |
| TT7542 | <i>S. typhimurium</i> LT2 <i>relA21::Tn10</i> (Tc) | J.R. Roth |
| TT13215 | <i>S. typhimurium</i> LT2 <i>phoP60::Tn10d</i> (Tc) | J.R. Roth |
| TT22386 | <i>S. typhimurium</i> LT2 <i>spoT-211::Tn10</i> (Tc) | J.R. Roth |
| VV302 | SL1344 Δ <i>hila523</i> | (7) |
| KT2146 | SL1344 Δ <i>relA71::kan</i> | (8) |
| KT2160 | SL1344 Δ <i>relA71::kan</i> Δ <i>spoT281::cat</i> | (8) |
| KT2786 | SL1344 <i>iagB87::lacZY</i> Δ <i>relA</i> Δ <i>spoT</i> | (9) |
| KT2792 | SL1344 <i>iagB87::lacZY</i> Δ <i>relA</i> <i>spoT-211::Tn10</i> | (9) |
| KT3040 | SL1344 <i>iagB87::lacZY</i> <i>dps::kan</i> | This study |
| KT3158 | SL1344 <i>iagB87::lacZY</i> <i>relA21::Tn10</i> Δ <i>spoT</i> | This study |
| KT3166 | SL1344 <i>iagB87::lacZY</i> <i>dps::kan</i> <i>relA21::Tn10</i> Δ <i>spoT</i> | This study |
| KT3456 | SL1344 (promoterless <i>gfp</i> ⁺ :: <i>cat</i>) Δ <i>relA</i> <i>spoT-211::Tn10</i> | This study |
| KT3458 | SL1344 Φ (<i>PssaG-gfp</i> ⁺ :: <i>cat</i>) Δ <i>relA</i> <i>spoT-211::Tn10</i> | This study |
| KT3462 | SL1344 Φ (<i>PrpsM-gfp</i> ⁺ :: <i>cat</i>) Δ <i>relA</i> <i>spoT-211::Tn10</i> | This study |
| KT3558 | SL1344 <i>iagB87::lacZY</i> <i>lrp-1::Tn5</i> | This study |
| KT3562 | SL1344 <i>iagB87::lacZY</i> <i>lrp-1::Tn5</i> <i>relA21::Tn10</i> Δ <i>spoT</i> | This study |
| KT3584 | SL1344 <i>iagB87::lacZY</i> Δ <i>fur41::kan</i> | This study |
| KT3588 | SL1344 <i>iagB87::lacZY</i> Δ <i>fur41::kan</i> <i>relA21::Tn10</i> Δ <i>spoT</i> | This study |
| KT3610 | SL1344 <i>rtsA::lac</i> | This study |
| KT3614 | SL1344 <i>rtsA::lac</i> <i>relA21::Tn10</i> Δ <i>spoT</i> | This study |
| KT3616 | SL1344 <i>rtsB::lac</i> | This study |
| KT3620 | SL1344 <i>rtsB::lac</i> <i>relA21::Tn10</i> Δ <i>spoT</i> | This study |
| KT3628 | SL1344 <i>iagB87::lacZY</i> <i>fis-2::kan</i> | This study |
| KT3632 | SL1344 <i>iagB87::lacZY</i> <i>fis-2::kan</i> <i>relA21::Tn10</i> Δ <i>spoT</i> | This study |
| KT3736 | SL1344 Δ SPI2:: <i>aph</i> (Km) | This study |
| KT3824 | SL1344 Δ SPI2 (cured of kanamycin cassette) | This study |
| KT3868 | SL1344 <i>putPA1303::kan::ompR-lacZ</i> | This study |

| | | |
|--------|--|------------|
| KT3872 | SL1344 <i>putPA1303::kan::ompR-lacZ relA21::Tn10 ΔspoT</i> | This study |
| KT3914 | SL1344 <i>ΔhilA523 ΔSPI2::aph (Km)</i> | This study |
| KT3930 | SL1344 <i>ΔSPI1::kan ΔSPI2</i> | This study |
| KT3960 | SL1344 <i>ΔsifA::kan</i> | This study |
| KT3980 | SL1344 <i>phoP60::Tn10d (Tc)</i> | This study |
| KT4018 | SL1344 <i>ompC::MudJ(Km)</i> | This study |
| KT4022 | SL1344 <i>ompC::MudJ(Km) relA21::Tn10 ΔspoT</i> | This study |

Plasmids

| | | |
|---------|--|-------------|
| p1437-1 | <i>bla pBAD-ssrAB⁺</i> | M. Hensel |
| pBA324 | <i>bla pBAD-sirA⁺</i> | B. Ahmer |
| pCP20 | <i>bla cat pSC101ori(ts) cI857(ts) λpL-FLP⁺</i> | (10) |
| pCV192 | <i>bla pCon-Irp⁺</i> (constitutive expression) | (11) |
| pDps | <i>bla lacI ptac-dps⁺</i> | D.G. Kehres |
| pRtsA | <i>bla pBAD-rtsA⁺</i> | (4) |
| pRtsAB | <i>bla pBAD-rtsAB⁺</i> | (4) |
| pBAD30 | <i>bla pBAD</i> | (12) |

^a Unless noted otherwise, *ΔrelA* refers to the *ΔrelA71::kan* allele, and *ΔspoT* the *ΔspoT281::cat* allele. Abbreviations: Cm, chloramphenicol-resistance; Km, kanamycin-resistance; Tc, tetracycline-resistance.

^b References:

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Fig. 1

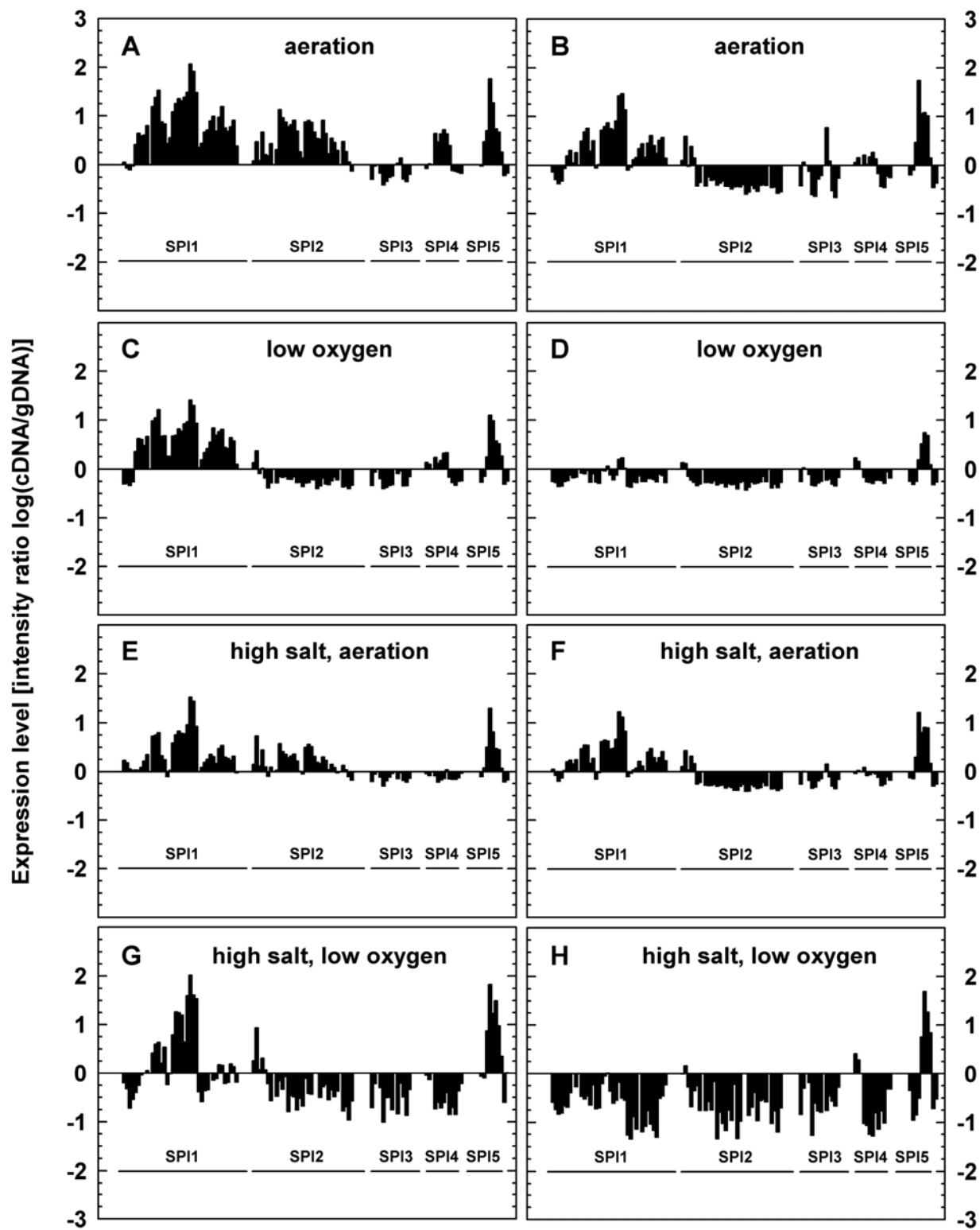


Fig. 2

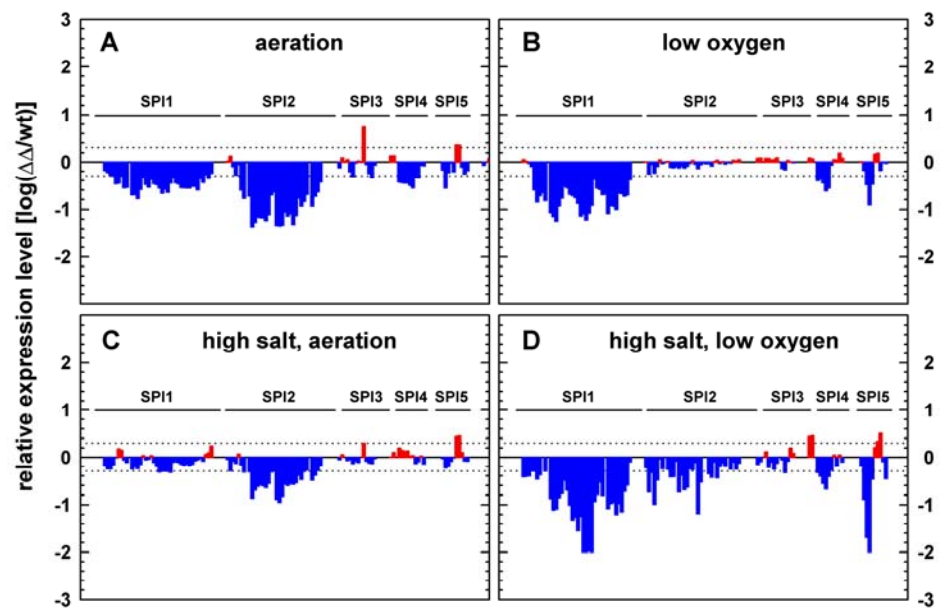


Fig. 3

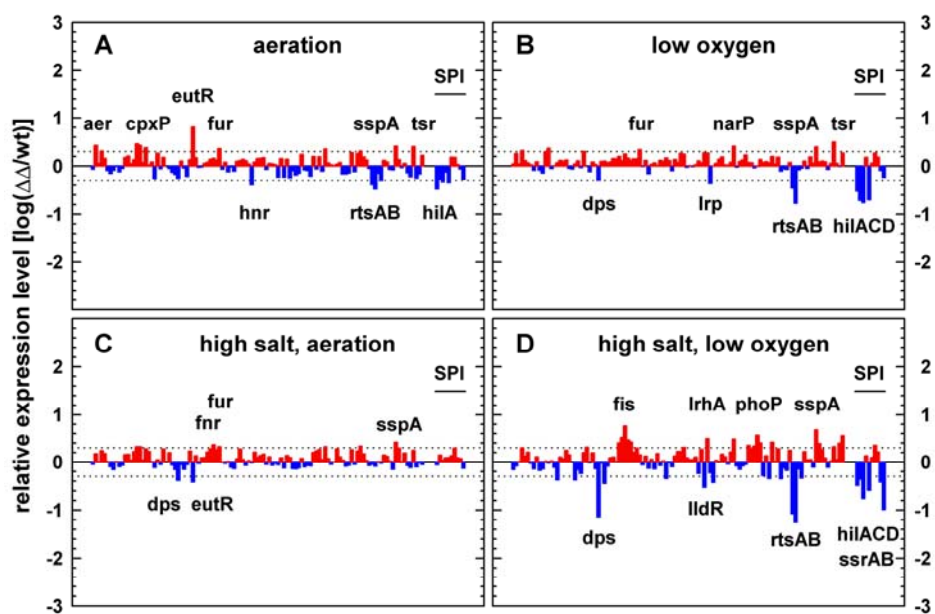


Fig. 4

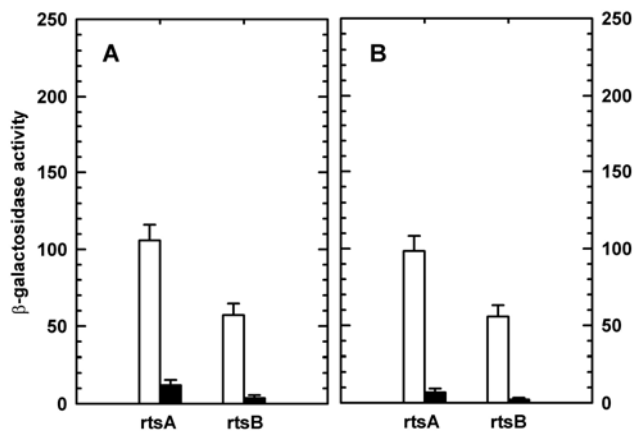


Fig. 5

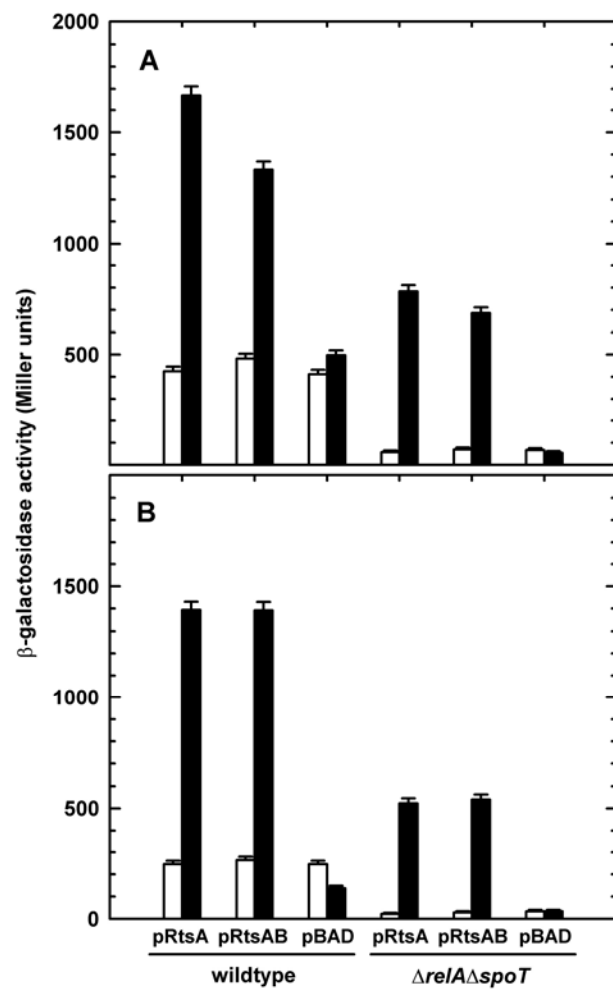


Fig. 6

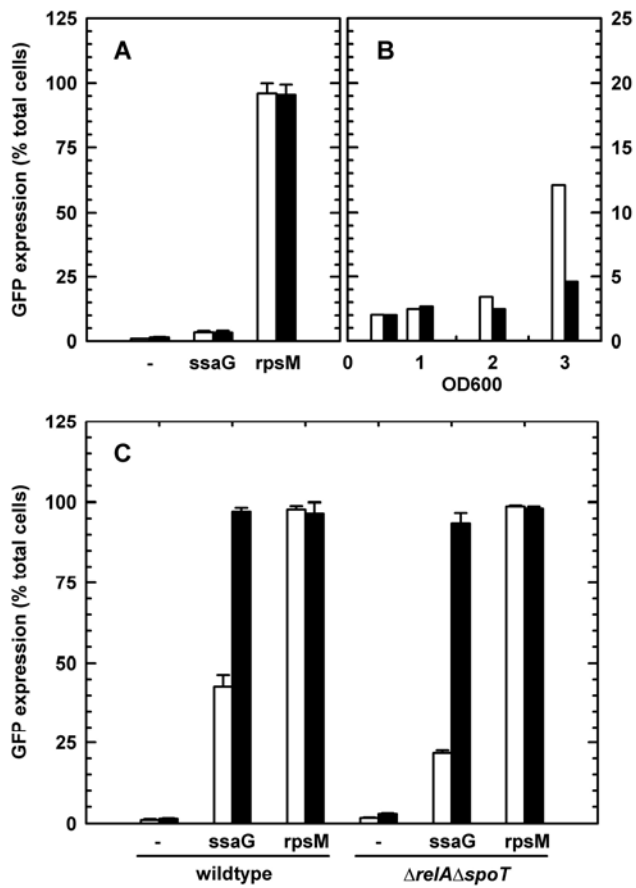


Fig. 7

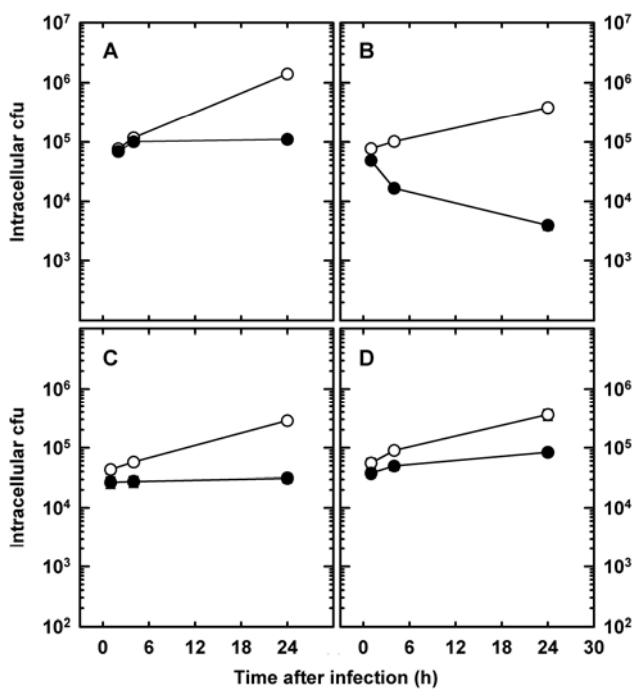


Fig. S1

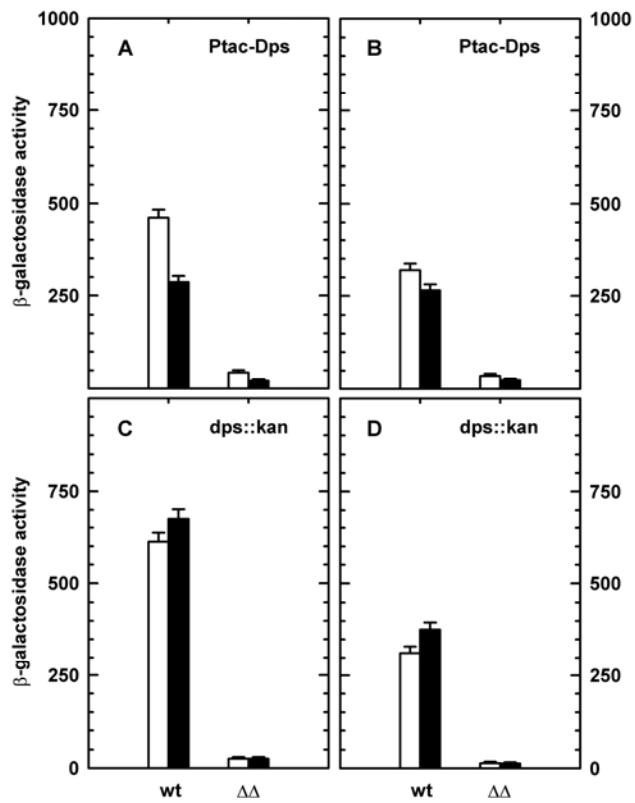


Fig. S2

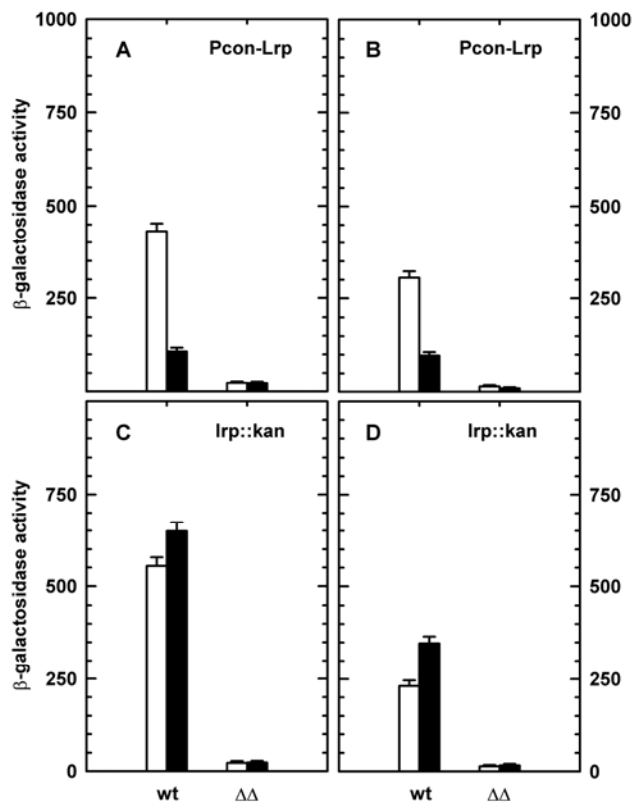


Fig. S3

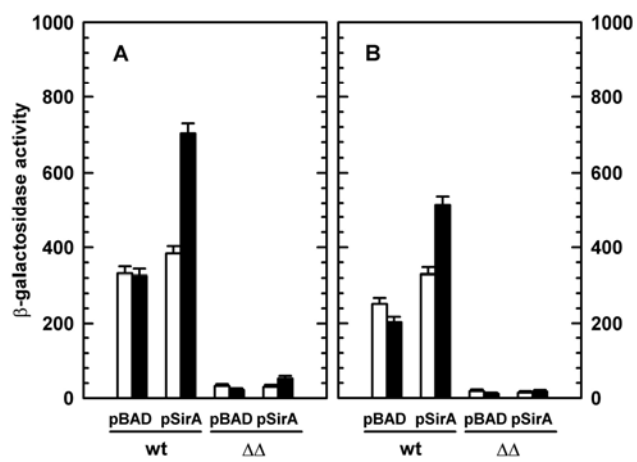


Fig. S4

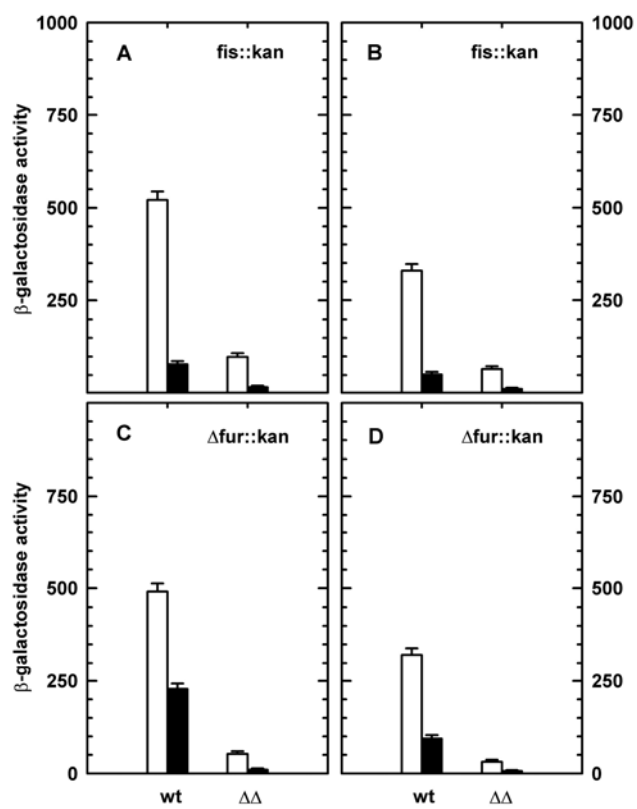


Fig. S5

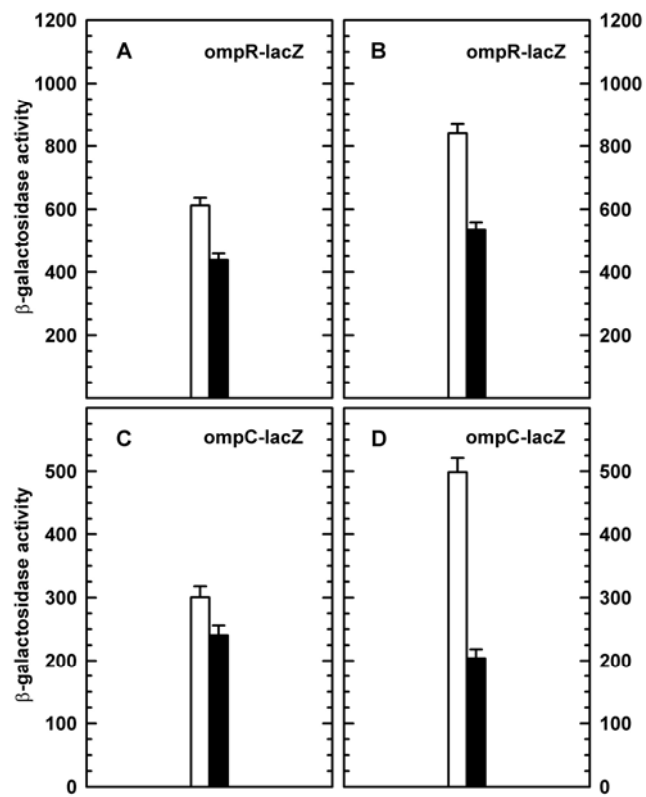


Fig. S6

