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Regulation of Sigma S Degradation in *Salmonella enterica* Var Typhimurium: *in vivo* Interactions Between Sigma S, the Response Regulator MviA(RssB) and ClpX

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Abstract

The alternate sigma factor σ^S plays an important role in the survival of *Salmonella typhimurium* following sudden encounters with a variety of stress conditions. The level of σ^S is very low in rapidly growing cells but dramatically increases as those cells encounter environmental stress or enter into stationary phase. This increase is due in large measure to the stabilization of σ^S protein against degradation by the ClpXP protease. The MviA protein, also known as RssB or SprE in *Escherichia coli,* is a putative member of a two component signal transduction system that plays a central role in facilitating σ^S degradation by ClpXP. In contrast to most two-component systems, MviA does not appear to regulate gene expression but is believed to interact directly with σ^S and somehow facilitate degradation. We now provide evidence that MviA(RssB) directly interacts both with σ^S and ClpX *in vivo*, presumably enabling presentation of σ^S to the ClpP protease. Interactions were demonstrated using a bacterial two-hybrid system in which σ^S, MviA, and ClpX were fused to separate moieties of *Bordetella pertussis* CyaA (adenylate cyclase). Paired hybrid plasmids containing Cya'-MviA/RpoS-'Cya or Cya'-MviA/ClpX-'Cya successfully reconstituted adenylate cyclase activity in both *S. typhimurium* and *E. coli*. However, no direct interactions were detected between ClpX and RpoS. A second series of experiments has indicated that the interaction between MviA and σ^S requires the N-terminus but not the C-terminus of MviA. Cellular levels of MviA appear to be very low in the cell based on *lacZ* fusion, Western blot and Northern blot analyses suggesting a catalytic role for MviA in σ^S degradation. Mutagenesis of MviA residue D58, a canonical residue subject to phosphorylation in many two-component systems, decreased the ability of MviA to facilitate σ^S turnover *in vivo* confirming that phosphorylation of MviA increases MviA activity.

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Introduction

Acid is a stress commonly encountered by enteric microorganisms such as *Salmonella typhimurium.* Although it prefers to grow at neutral pH, *S. typhimurium* can adapt to endure a wide variety of pH conditions. Its ability to adapt to low pH environments is important to the pathogenesis of this organism as it encounters acid in the stomach and in the macrophage phagolysosome (Rathman *et al.,* 1996). *In vitro, S. typhimurium* adapts to survive low pH stress by inducing what is referred to as the acid tolerance response (ATR). This complex response involves the synthesis of over 50 acid shock proteins (ASPs) which are believed to prevent or repair macromolecular damage caused by an acidic internal pH (Foster, 1991; Foster, 1993). Efforts designed to identify ATR regulators exposed a subset of 10 ASPs controlled by the alternate sigma factor σ^S, encoded by the gene *rpoS* (Hengge-Aronis, 1993; Lee *et al.,* 1995; Loewen and Hengge-Aronis, 1994; Loewen *et al.,* 1998; Prince *et al.,* 1994). Originally discovered to accumulate in stationary phase cells, σ^S levels were also found to increase in log phase cells of *S. typhimurium* and *Escherichia coli* exposed to acid shock and other environmental stresses (Hengge-Aronis *et al.,* 1993; Lee, *et al.,* 1995). This stress-induced sigma factor is an important component of the ATR not only because it regulates 10 ASPs but because *rpoS* mutants are acid sensitive and avirulent (Fang *et al.,* 1992; Lee, *et al.,* 1995; Swords *et al.,* 1997; Wilmes-Riesenberg *et al.,* 1997). Therefore, an understanding of how acid and other stresses induce σ^S accumulation will lead to a better understanding of *S. typhimurium* pathogenesis.

The mechanisms regulating the expression and synthesis of σ^S are complex, encompassing transcriptional (only in complex media), translational and post-translational controls (Barth *et al.,* 1995; Bohringer *et al.,* 1995; Hengge-Aronis, 1996; Klauck *et al.,* 1997; Lange *et al.,* 1993; Lange and Hengge-Aronis, 1994; McCann *et al.,* 1993; Muffler *et al.,* 1996; Muffler *et al.,* 1996). There is also some evidence that σ^S activity is regulated (Zhou and Gottesman, 1998). However, the accumulation of σ^S is most profoundly affected by a post-translational control mechanism involving proteolytic turnover (Zgurskaya *et al.,* 1997). During exponential growth, σ^S is rapidly degraded with a half life of less than 3 minutes. When cells enter stationary phase or are challenged with acid or other specific stresses, the half-life of σ^S increases to greater than 30 minutes (Bearson

et al., 1996; Lange and Hengge-Aronis, 1994). This increased stability enables σ^S to accumulate which, in turn, increases the expression of σ^S-dependent genes.

Degradation of σ^S in *S. typhimurium* and *E. coli* requires the ClpXP protease (Schweder *et al.,* 1996; Webb *et al.,* 1999) and is regulated by the product of the mouse virulence gene *mviA* in *S. typhimurium* (Bearson, *et al.,* 1996), also known as *rssB* or *sprE* in *E. coli* (Muffler *et al.,* 1996; Pratt and Silhavy, 1996). Mutants defective in *clpPX* or *mviA* (*rssB*) accumulate large amounts of this sigma factor in log phase cells. Amino acid sequence analysis of the MviA(RssB) amino terminus suggests that it is the response regulator component of a previously unrecognized two-component signal transduction system (Benjamin *et al.,* 1996). The MviA protein is unusual in that the C-terminus bears no obvious homology to known response regulator output domains. Because of this unique structure, it has been proposed that MviA(RssB) represents a new family of two component signal transduction systems that control the proteolytic degradation of key regulatory factors in the cell (Bearson, *et al.,* 1996).

The homology between the N-terminus of MviA(RssB) and the OmpR family of response regulators includes the canonical DDL motif in which the second aspartate (D58 in this case) is the phosphoacceptor residue in a phospho-relay system (Parkinson and Kofoid, 1992). Recently it was shown that the *E. coli* RssB protein can be phosphorylated *in vitro* and that phosphorylation requires residue D58 (Bouche *et al.,* 1998). However, the role of RssB phosphorylation on σ^S turnover *in vivo* was not defined. A study with *S. typhimurium* also found that *mviA* mutants harboring changes at residue D58 exhibited increased expression of *katE*, a σ^S -dependent gene, but again a direct effect on *in vivo* σ^S levels was not demonstrated (Cunning and Elliott, 1999). We now confirm that in *S. typhimurium* residue D58 is required for the optimum activity of MviA in the σ^S degradation pathway when MviA is present at low intracellular levels but not when the protein is overexpressed. In addition, we demonstrate direct *in vivo* interactions between MviA(RssB), σ^S and ClpX that support a model in which MviA chaperones σ^S to the ClpXP protease.

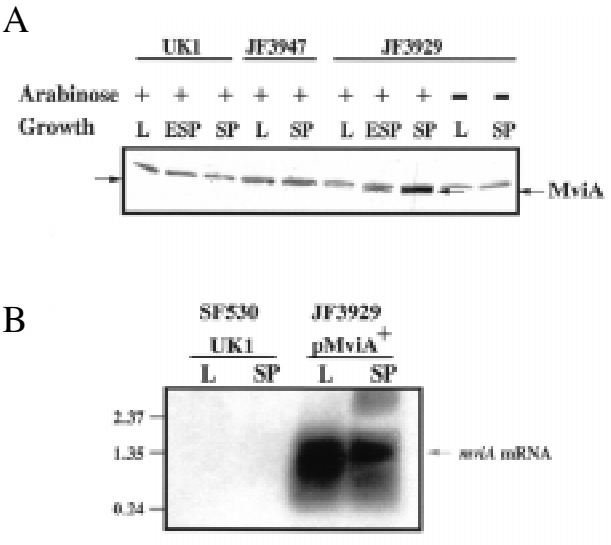

Figure 1. Relative MviA levels produced in wild-type cells and from an arabinose-inducible promoter (P_{BAD}) as detected by Western and Northern blot. Cells were grown in EG+0.05%CAA media to OD_{600} of 0.4 for log phase cells (L), OD_{600} of 3.5 for early stationary phase growth (ESP), and 24 hours for stationary phase growth (SP). A. Western blot. Protein samples (5 μg) were separated on a 10% Tris-HCl SDS PAGE gel and immunoblotted with MviA polyclonal antibody. MviA protein is the lower band indicated by the arrow. The upper, cross- reacting band is not related to MviA. B. Northern blot. Cells were grown in LB supplemented with 0.2% arabinose to induce pMviA$^+$. Cells were harvested at mid log (OD_{600} 0.4) and early stationary phase (OD_{600} 3.5). RNA was isolated and 5 μg total RNA loaded onto a denaturing agarose gel (Experimental Procedures). Blots were hybridized with a radiolabeled DNA probe specific for *mviA*. SF530 (UK1), JF3947 (pBAD18-Cm) and JF3929 (pMF298 *mviA*$^+$).

Results

MviA Production in *S. typhimurium*

In an attempt to assess the *in vivo* levels of MviA(RssB) present during different phases of growth, His-tagged MviA was purified and used to raise polycolonal antisera for Western blot analysis. Figure 1A illustrates that MviA is normally produced at levels undetectable by Western blot but was readily detected if overproduced from an arabinose-inducible promoter (UK1 vs JF3929). Furthermore, MviA(RssB) is undetectable in either log or stationary phase cells suggesting that its level in the cell remains low during all phases of growth. Construction of an *mviA-lacZ* fusion strain (JF4297, See Experimental Procedures) confirmed that expression of this gene is extremely low. ß-Galactosidase levels produced in log phase and stationary phase were 1 and 10 Miller units respectively (data not shown). This was confirmed using Northern blot analysis probing for MviA message. Regardless of the phase of growth, MviA mRNA was generally not detected in cell extracts but easily observed when expressed from a plasmid (Figure 1B). The results suggest that MviA is normally present at very low intracellular levels.

Mutations at the Putative Phosphoacceptor Site Diminish MviA *in vivo* Activity

Since MviA is the predicted response regulator of a putative two-component signal transduction system, residue D58 is thought to play a pivotal role in regulating MviA activity. Two previous studies using *E.coli* and *S. typhimurium* support this idea (Bouche, *et al.,* 1998; Cunning and Elliott, 1999). We have confirmed and extended those studies using site-directed amino acid substitutions introduced into MviA at D58. The substitutions included asparagine(D58N) and glutamate (D58E), neither of which will undergo phosphorylation. Glutamate was used to address the possibility that removing aspartate would destroy an essential salt bridge within the protein. Glutamate should at least partially substitute for aspartate in forming such a bridge. Mutant forms of *mviA* were placed under the control of the arabinose-inducible promoter (P_{BAD}) and the derivative plasmids were introduced into an *mviA::Km* insertion mutant. The effects of wild type and mutant forms of MviA(RssB) on the log phase levels of σ^S were analyzed via Western blot. At low arabinose concentrations, enough MviA was produced to enable σ^S degradation but not enough for MviA to be detected by Western blot (Figure 2, JF3929 vs JF3947). When the site-directed mutants were similarly induced neither could efficiently degrade σ^S (Figure 2, JF3950 and JF3930). However, at higher induction levels where MviA was present in sufficient quantities to be observed on a Western blot (Figure 2, panel

B), both the D58E and D58N mutant forms of MviA targeted σ^S for degradation, although not with the same efficiency as MviA$^+$. Since the two mutant forms of MviA were produced to the same level as MviA$^+$ when induced with 0.2% arabinose, the difference in efficiency was due to a less active protein, not protein instability. This confirms the previous report with *S. typhimurium* which demonstrated the effects of D58 substitutions in MviA(RssB) on the ability of the cell to express a σ^S-dependent gene, *katE* (Cunning and Elliott, 1999). The current work extends the previous report by showing a direct effect on σ^S-levels.

Although a recent report indicates that low levels of arabinose do not yield uniformly low levels of induction of P_{BAD}-driven genes in all cells within a population (Siegele and Hu, 1997), this phenomenon does not affect the design or conclusions of this experiment. A concentration of arabinose of 0.01% (equal to 6 X 10^{-4} M), as used in our experiments, has been shown to induce pBAD in 90% of cells (Siegele and Hu, 1997). Thus, in a situation where 90% of the cells were induced for MviA, there remained a distinct difference in the ability of mutant forms of MviA to stimulate σ^S degradation (Figure 2). Consequently, our conclusion that the mutant proteins were considerably less active than wild type remains valid.

MviA Does not Affect the Production of ClpX

Although MviA/RssB does not affect ClpXP production in *E. coli*, the situation in *S. typhimurium* has not been addressed (Muffler, *et al.,* 1996; Zhou and Gottesman, 1998). Consequently, Western blot analysis was used to determine if *mviA* mutants produce less ClpX than do *mviA*$^+$ cells. Since *clpX* is the downstream member of the *clpPX* operon, it is reasonable to monitor *clpXP* expression by probing for ClpX. The results presented in Figure 3 reveal that *mviA* status has no effect on the production of ClpX in *S. typhimurium*. Since acid shock as well as entry into stationary phase triggers the accumulation of σ^S we tested the effects of these conditions on ClpX production. Figure 3 reveals that neither acid shock nor stationary phase affected ClpX levels. The results are consistent with models in which MviA either directly affects ClpXP activity or is involved in the presentation of σ^S to ClpXP. However, a recent report argues against the former since *E. coli* RssB(MviA) does not affect the degradation of other ClpXP substrates (Zhou and Gottesman, 1998).

MviA(RssB) Interacts with σ^S and ClpX *in vivo*

An *in vitro* interaction between *E. coli* MviA(RssB) and σ^S has been demonstrated but *in vivo* interactions were not explored (Becker *et al.,* 1999). We have used a novel bacterial two-hybrid system to determine whether *S. typhimurium* MviA(RssB) interacts with σ^S or ClpX *in vivo*. The system utilizes N-terminal (T25) and C-terminal (T18) halves of *B. pertussis* adenylate cyclase (CyaA) produced on separate plasmids (Karimova *et al.,* 1998). If proteins that interact *in vivo* are fused to the CyaA subunits, the two CyaA subunits will be brought into proximity, forming an active adenylate cyclase. In initial experiments, σ^S and MviA were fused to the T18 C-terminal (RpoS-'CyaA) and the T25 N-terminal (CyaA'-MviA) portions of CyaA, respectively (Experimental Procedures). The plasmids were introduced individually and together into a *cya clpP* mutant of *S. typhimurium*. Two assays were used to determine adenylate cyclase activity in *Salmonella*, acid production on MacConkey mannitol media (red colonies) and growth in mannitol as a sole carbon source. A *cya* mutant appears white on MacConkey mannitol agar and fails to grow in mannitol broth. Individual plasmids did not complement the *cyaA* mutation, nor did the parent plasmids, pT18 and pT25, when placed individually or together in the *cya* mutant. However, the strain containing both pMF379 (RpoS-'CyaA[T18]) and pMF383 (CyaA[T25]'-MviA) formed red colonies on MacConkey mannitol plates and allowed growth in minimal mannitol media indicating that MviA(RssB) and σ^S interact *in vivo* (Table 1).

To study this interaction more closely, various combinations of two-hybrid plasmids were introduced into an *E. coli cya* mutant strain (EK395) and any interactions resulting in cAMP production were detected by measuring ß-galactosidase activity (Figure 4). The *cya* mutant (EK395) alone exhibited very low levels of ß-galactosidase activity, as expected. When the pT18-Zip/pT25-Zip positive control plasmids, whose hybrid proteins interact very strongly (Karimova, *et al.,* 1998), were introduced into this strain (EF646), activity increased to 800 and 1500 Miller Units in

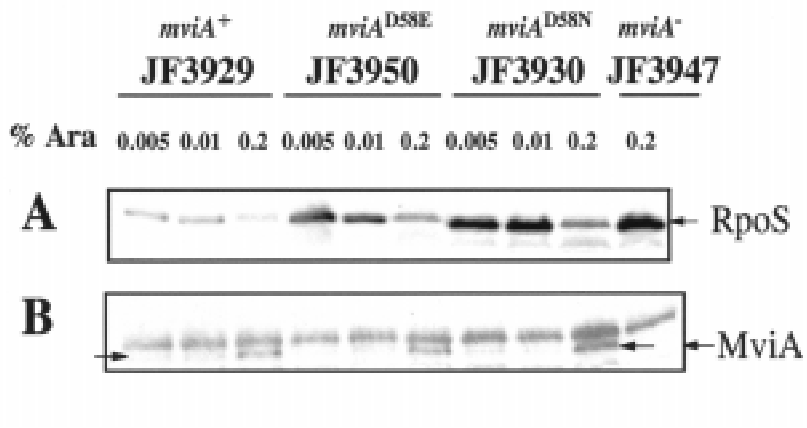

Figure 2. Effect of site-directed mutant proteins of MviA on RpoS levels as measured by Western blot analysis. Cells were grown in EG with 0.05% CAA media to an OD_{600} of 0.35- 0.4. Different concentrations of arabinose were used to induce P_{BAD}-driven *mviA* to different levels. Protein samples (5 μg) were separated on a 10% Tris-HCl SDS PAGE gel. Panel A, RpoS Western blot. Panel B, MviA Western blot. MviA is the lower band as indicated with an arrow.

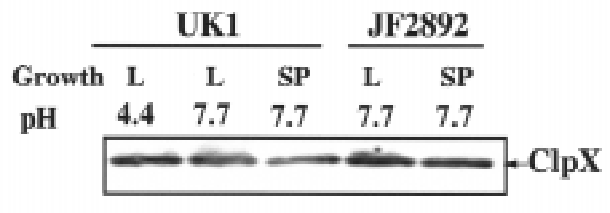

Figure 3. The effect of acid shock, growth phase and *mviA* on ClpX levels. Cells were grown in EG media to an OD_{600} 0.4 for log phase cells (L) and overnight (24 h) for stationary phase cultures (SP). Acid shocked cells were challenged at pH 4.4 for 20 minutes. Protein samples (3 μg) were separated on a 10% Tris-HCl SDS PAGE gel and immunoblotted with ClpX antibody. UK1 (wild-type *S. typhimurium*); JF2892 (*mviA4185*::Km)

log and stationary phases, respectively, demonstrating that the *cya* mutation could be complemented using the *B. pertussis* CyaA two-hybrid tethering technique (Figure 4, EF646). The data presented also demonstrate that when the RpoS-'CyaA(T18) and CyaA(T25)'-MviA-encoding plasmids were introduced into this strain, ß-galactosidase activity increased to 500 Miller Units in stationary phase cultures (Figure 4, EF644). This was a 10-fold increase over the *cya* mutant EK395. The results indicate that MviA (RssB) and σ^S interact *in vivo* in both *E. coli* and *S.typhimurium*.

We also tested whether MviA or RpoS might interact *in vivo* with ClpX. The products of the CyaA(T25)'-ClpX and MviA-'CyaA(T18) plasmids clearly interacted as exhibited by a 20-fold increase in ß-galactosidase activity (Figure 4, EF682). However, a CyaA(T25)'-ClpX, RpoS-'(T18)CyaA combination did not interact (Figure 4, EF689). These results suggest that MviA(RssA) serves as a molecular "lynch-pin" bringing the ClpX and RpoS proteins together.

MviA(RssB) is not Degraded by ClpXP

Because MviA(RssA) facilitates σ^S degradation and has been shown to interact with σ^S and ClpX, we questioned whether MviA(RssB) itself might be degraded by the ClpXP protease. When we tested this possibility in a *clpP* mutant, MviA did not accumulate (Figure 5A). We also induced MviA production in *clpP*$^+$ and *clpP* mutants containing pBAD-*mviA*$^+$ (pMF298). The results in Figure 5B revealed that although MviA was visible in *clpP*$^+$ cells, this protein did not accumulate to any greater extent in the *clpP* mutant. As discussed above, reports that low levels of arabinose may not yield uniformly low levels of induction in all cells within a population does not affect this conclusion since the concentrations used in Figure 5, 0.05 to 0.1%, induce 100% of pBAD-containing cells (Siegele and Hu, 1997).

The N-Terminus of MviA(RssB) Affects σ^S Activity

Zhou *et al.* reported that *E. coli* RssB interferes with σ^S activity even in *clpP* mutants that cannot degrade σ^S (Zhou and Gottesman, 1998). Inhibition of σ^S activity is likely due to direct binding of MviA(RssB) to σ^S based on evidence that these proteins successfully interact *in vivo* and *in vitro*. We have confirmed that MviA(RssB) inhibits σ^S activity in *S. typhimurium* using a strain which contains a *katE-lacZ* operon fusion, a *clpP1::*Tn*10* insertion and a plasmid-borne copy of the *mviA* gene under the control of an arabinose inducible promoter (P_{BAD}). In the absence of arabinose, this strain expressed high levels of ß-galactosidase activity during the stationary phase of growth, a result consistent with high σ^S activity. However, in the presence of 0.1 % arabinose, *katE-lacZ* expression decreased 5-fold even though Western blot analysis showed that σ^S cannot be degraded due to the *clpP* mutation (Figure 6, lanes 2 and 5). This suggests that MviA interaction with σ^S will prevent σ^S from initiating transcription from the *katE* promoter.

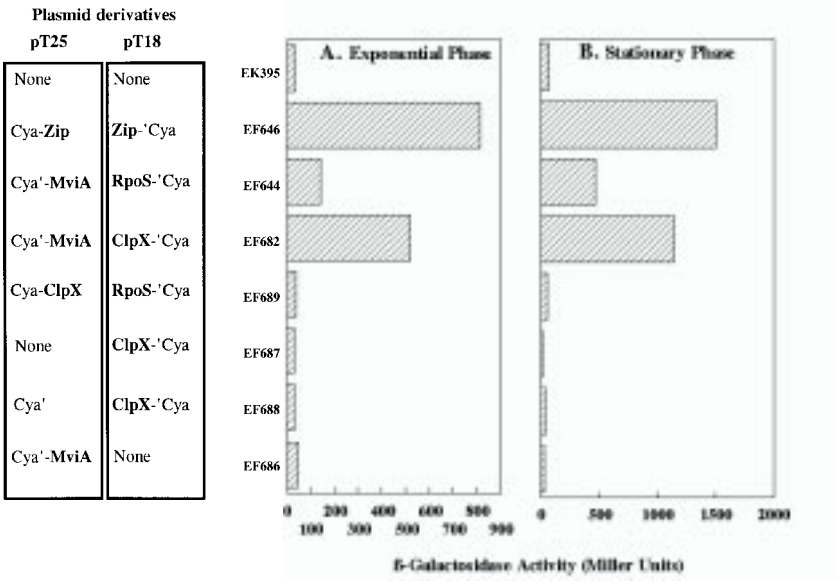

Figure 4. *In vivo* interactions between MviA(RssB), σ^S and ClpX. Derivatives of EK395 (*cya*) were grown at 30°C in LB media supplemented with appropriate antibiotics and 0.5 mM IPTG. ß-Galactosidase activity was assayed in log phase (OD_{600} 0.4) and stationary phase (24 h overnight) cultures. Strains and plasmid combinations are indicated in the Figure. When T25 and T18 hybrid proteins interact, the result is reconstitution of CyaA activity and induction of chromosomal *lacZ* expression.

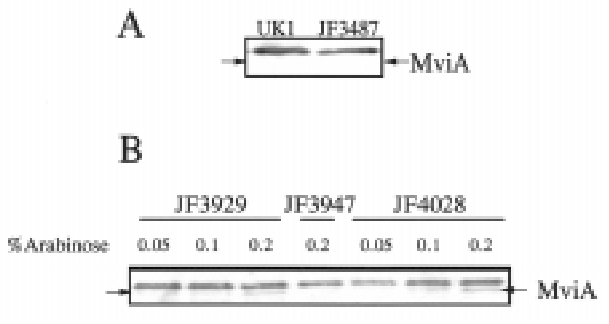

Figure 5. The effect of *clpP* on MviA levels. Cells were grown in EG with CAA to an OD_{600} of 0.4. (Panel A) UK1 (wild type); JF3487 (*clpP::Tn10dTc*). (Panel B) Arabinose was added at different concentrations to strains containing pMF298 *mviA*$^+$ to induce MviA to various levels in the cell. JF3929 (*mviA*/pMF298 *mviA*$^+$); JF3947 (*mviA*); JF4028 (*mviA clpP*/pMF298 *mviA*$^+$). Protein samples (5 μg) were separated on a 10% Tris-HCl SDS gel and immunoblotted with MviA antibody.

Since MviA is a putative response regulator that possesses a typical signal input N-terminal domain but whose C-terminus bears no homology to known output domains (Benjamin, *et al.,* 1996), one possible model to explain the interaction between MviA and σ^S is that the C-terminal domain may be involved with binding σ^S. To test this hypothesis we constructed plasmids that would express only the N-terminal domain (the first 111 amino acids containing the conserved DDL motif) or the C-terminal domain consisting of the remaining 226 amino acids of the protein and examined *katE-lacZ* expression in the *clpP katE-lac* strain (Figure 6, lanes 7 and 8). Contrary to expectations, the results obtained from this experiment indicate that the ability to inhibit σ^S activity resides in the N-terminal half of the MviA protein which, when overexpressed, decreased *katE-lacZ* expression 4 fold. Confirmation that these truncated MviA(RssB) proteins were expressed was obtained by Western blot analysis of extracts probed with anti-MviA antiserum (data not shown). A similar inhibition of σ^S activity was observed with the site-directed MviA (D58E) and MviA (D58N) mutant proteins, although to a lesser degree than wild-type MviA, indicating that phosphorylation at residue D58 is not required for the effect but does stimulate interaction (data not shown).

The demonstration that MviA interacts *in vivo* with ClpX and σ^S along with evidence indicating that the σ^S-inhibitory activity of MviA(RssB) resides within the N-terminus suggests a model for MviA(RssB) action in which the MviA N-terminus interacts with σ^S while the C-terminal domain interacts with ClpX. If true, then the interaction between the N-terminal domain of MviA and σ^S would not be sufficient to target σ^S for degradation. Plasmids encoding the N-terminal and C-terminal domains of MviA were transformed by electroporation into a strain of *S. typhimurium* containing an *mviA::*Km insertion and a functional ClpXP protease (JF3924). RpoS protein levels in cultures grown in the presence of 0.2% arabinose were determined using Western blot analysis (Figure 7). The results confirm that while full-length MviA(RssB) was able to efficiently target σ^S for degradation (JF3929), the N-terminal domain (JF4470) was limited in its ability to facilitate σ^S-degradation, although some degradation did occur. This suggests the C-terminus is important for efficient ClpX interaction but that ClpX probably binds to residues within the N-terminal domain. *In toto*, these results suggest that an interaction between MviA(RssB) and σ^S occurs via the amino terminus of MviA but that this interaction alone (*i.e.* the interaction between σ^S and N-terminal-truncated MviA) does not efficiently target the σ^S protein for ClpXP-mediated degradation.

Table 1. *In vivo* Interaction Between σ^S and MviA(RssB) in *S. typhimurium* Measured as Growth in Minimal Mannitol Broth

	Strains				
Plasmids	JF4405	JF4275	JF4445	JF4446	JF4447
pT25Zip		+[a]			
pT18Zip		+			
pMF379			+		+
pMF383				+	+
OD_{600}[b] 24h	0.001	0.26	0.003	0.004	0.3
OD_{600}[b] 48h	0.005	1.7	0.006	0.005	1.9

[a] A (+) indicates presence of the indicated plasmid in the *cya clpP* mutant.
[b]Strains were grown at 30°C in NCE minimal media containing 0.2% mannitol.

Discussion

An important feature in the control of σ^S-dependent stress responses resides in the cell's ability to regulate σ^S turnover. The response regulator MviA(RssB) has a central role in that process. The two-hybrid results presented have significantly added to our understanding of how MviA(RssB) might function in facilitating σ^S degradation by the ClpXP protease and add physiological relevance to an earlier report that MviA(RssB) and σ^S interact *in vitro* (Becker, *et al.,* 1999). We have clearly demonstrated that MviA exhibits two types of *in vivo* interactions, one with σ^S and one with ClpX. Significantly, σ^S did not interact directly with ClpX under the conditions tested. The data suggests that MviA(RssB) will present σ^S to ClpX, possibly by forming a bridge between the two proteins with interaction between MviA(RssB) and σ^S requiring the amino terminal portion of MviA. However, the extremely poor degradation of σ^S by the N-terminal domain of MviA(RssB) suggests that efficient interaction between MviA(RssB) and ClpX requires the carboxyl terminus of MviA (Figure 7). In addition, it appears that phosphorylated MviA is the more active form *in vivo* since MviA lacking the phosphoacceptor residue D58 functions poorly to facilitate σ^S degradation. This result confirms earlier work indicating that *in vitro* interaction between RssB(MviA) and σ^S requires phosphorylated RssB(MviA) (Becker, *et al.,* 1999).

The results are consistent with the following model concerning the control of σ^S-stability in *S. typhimurium* and *E. coli*. Logarithmically growing, non-stressed cells likely maintain a steady level of MviA phosphorylated at residue D58. This phosphorylated form of MviA (MviA-P) will associate with σ^S *in vivo,* probably at σ^S lysine residue 173 as indicated by *in vitro* studies (Becker, *et al.,* 1999; Schweder, *et al.,* 1996). The MviA(RssB):σ^S complex may then bind ClpX bringing σ^S and ClpX in close proximity, although the order of binding may be reversed. In either case, ClpX would then present σ^S to the ClpP protease barrel for degradation. Since MviA(RssB) does not accumulate in a *clpP* mutant, MviA probably does not remain associated with σ^S as σ^S undergoes degradation.

The results presented also indicate that, as previously demonstrated with *E. coli* (Zhou and Gottesman, 1998), MviA(RssB) does not regulate σ^S stability in *S. typhimurium* by regulating the level of ClpXP protease.

It seems from these and other studies that the phosphorylation/dephosphorylation of MviA at residue D58 is an important factor in whether or not σ^S is degraded in log phase cells. Following the exposure of exponentially growing cells to an environmental stress such as an acid shock, overall energy levels may be depressed which in turn might limit the phosphorylation of MviA. Dephosphorylated MviA, present at low levels in the cell, is proposed to associate poorly with σ^S (Becker, *et al.,* 1999). Therefore, if σ^S is not bound to MviA, it is not recognized as a suitable substrate for ClpX. The result would be a rapid accumulation of σ^S and induction of σ^S-dependent genes.

A report utilizing *E. coli* suggests that cells depleted of acetyl phosphate do not degrade σ^S as efficiently as cells that can make acetyl phosphate leading to the hypothesis that acetyl phosphate is a phosphodonor for MviA (RssB) (Bouche, *et al.,* 1998). However, it was apparent from that study that acetyl phosphate in *E. coli* was not the primary signal to RssB(MviA) since σ^S stability in log phase only increased 2 to 3 fold in the *pta ack* mutant as opposed to the 8 to 10 fold increase normally observed in stationary phase or in log phase in an *rssB* (*mviA*) mutant. In contrast to the results with *E. coli*, an *ack* mutant of *S. typhimurium,* lacking both acetate kinase and phosphoacetyltransferase, did not appear to affect σ^S levels at all (Cunning and Elliott, 1999). Thus, if a specific sensor-kinase for RssB (MviA) exists, it remains unknown. The *lrh* gene identified in *E. coli* was proposed to regulate the expression of a putative sensor-kinase for the MviA(RssB) signal transduction pathway but the Lrh target remains unidentified (Gibson and Silhavy, 1999).

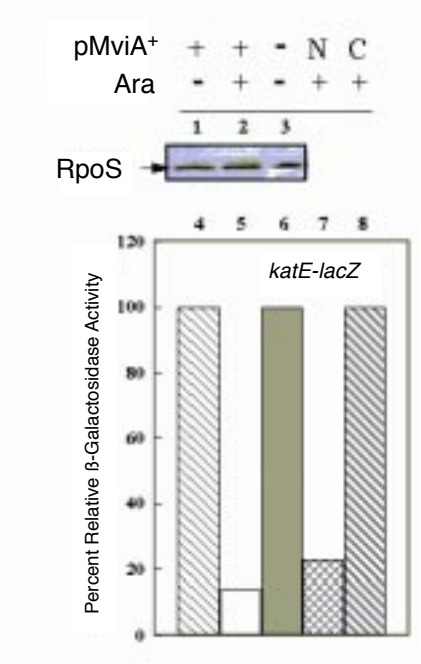

Figure 6. Inhibition of σ^S activity by MviA(RssB). Strains containing a *clpP*::Tn*10*dTc mutation and plasmids expressing *mviA*$^+$ were grown to stationary phase and tested for effects on σ^S levels (Western blot, top panel) and *katE-lacZ* expression (bottom panel). To avoid detecting a non-RpoS, cross-reacting band, *rpoS*::Ap derivatives were used to monitor and^S levels [lanes 1-3; Bearson *et al.,* 1996 #72] but *rpoS*$^+$ strains were necessary to monitor and^S activity (lanes 4-8). JF4028 (lanes 1 and 2); JF3964 (lane 3); JF4176 (lanes 4 and 5); JF4180 (lane 6). The strain in lanes 7 and 8 contain plasmids expressing the N-terminus (JF4453, pMF386) and C-terminus of MviA (JF4461, pMF390), respectively. Arabinose was added where indicated to 0.2%. Because of drug marker incompatibilities, different plasmids were tested for inhibition of σ^S activity in slightly different background strains. Consequently, values (average of triplicate experiments) for each plasmid were compared to the appropriate background strain and are reported as percent relative ß-galactosidase activity.

The impressive amount of control exerted over the synthesis and stability of σ^S reflects its importance to survival when the cell is under attack by a variety of harsh conditions. Proteolytic control provides for a particularly rapid response to sudden hazardous exposures, minimizing the amount of time the cell is vulnerable to stress-induced damage. However, one might question how the cell benefits from using MviA(RssB) as an intermediary protein to control the degradation of σ^S by ClpXP. Why not just regulate ClpX directly? The reason may be that ClpX is required to recognize other substrates that must be degraded during periods of stress when the cell needs σ^S. A protein designed to present σ^S to ClpX during rapid growth but not during stress would allow ClpXP to continue degrading other proteins during that stress while allowing σ^S to accumulate and drive the expression of survival genes. We are currently designing strategies to determine if MviA(RssB) truly cross-links σ^S and ClpX *in vivo* and to identify the specific residues involved.

Experimental Procedures

Bacterial Strains, Media and Cultural Conditions

The bacterial strains used in this study are listed in Table 2. LB complex medium and Vogel and Bonner E minimal medium supplemented with 0.4% glucose (EG) were prepared as liquid and solid (l.5% agar) media (Vogel and Bonner, 1956). Non-Citrate E medium (NCE) was used with 0.2% mannitol to test for use of this carbohydrate as a carbon source (Maloy and Roth, 1983). Casamino acids (CAA) supplementation, when used, was at 0.05%. The following antibiotics were used at the concentrations indicated; ampicillin (60 μg/ml), kanamycin (50 μg/ml), chloramphenicol (30 μg/ml) and tetracycline (10 or 20 μg/ml for minimal and rich medium, respectively). Unless otherwise stated, cultures were grown at 37°C with shaking. Cells grown to an OD_{600} of 0.4 were considered to be log phase cells; cells grown to an OD of 3.5 were considered early stationary phase cells.

General Genetic and Molecular Biology Techniques

Generalized transductions were performed using P22 HT 105/1-*int* (Aliabadi *et al.,* 1988; Holley and Foster, 1982). Transformations were performed following the procedure of Tsai *et al* (1989). Plasmids constructed in *E. coli* were passaged through the restriction-less *S. typhimurium* strain SF586 before introduction into other *Salmonella* strains. Likewise, plasmids derived from *S. typhimurium* were passaged through EK112 before introducing them into other *E. coli* strains. *Salmonella typhimurium* chromosomal DNA was purified as described by Maloy (1990). Restriction enzymes were purchased from BRL or Promega and digestions were performed following the manufacturer's suggestions. Purification of DNA fragments from gel slices was performed using the Geneclean II kit (BIO 101). T4 DNA ligase and Shrimp Alkaline Phosphatase were purchased from BRL. *Escherichia coli* INVαF', pCRII and pCR2.1 vectors were purchased from Invitrogen. Nucleotide sequencing by the dideoxy chain termination method of Sanger (1977) was performed using the Sequenase Version 2.0 DNA sequencing kit (USB).

Construction of a Chromosomal *mviA*::*lacZ* Operon Fusion

A fragment of *mviA* was generated by PCR using primer pair 84(5'GGGCTGATATCGATATTGCGG) and 184 (5'ACGGTGCTTTT GTTGGCCT). This 1195 base pair fragment contained 345 bases of the 3' end of *rssA* and 850 bases of the 5' end of *mviA*. This fragment was cloned into vector pCR2.1 forming plasmid pMF328 (TA Cloning Kit, Invitrogen). Plasmid pMF328 was digested with *Eco*RI and the gel-purified 1210 base pair fragment inserted into the suicide vector pVIK112 which possesses the *R6KoriV* origin of replication (Kalogeraki and Winans, 1997). The correct orientation of the fragment was determined by PCR using primer pair 84

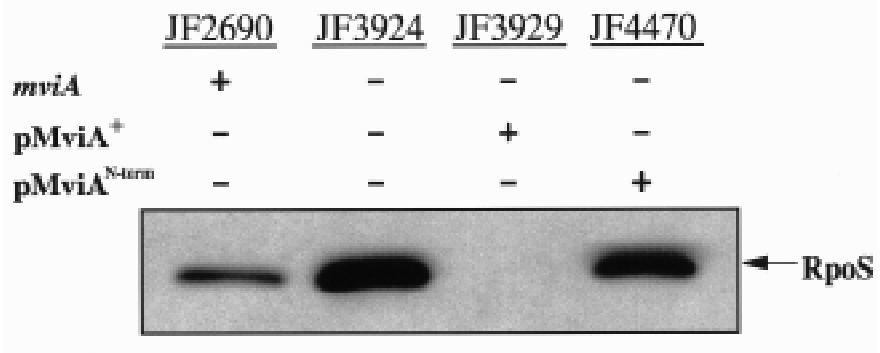

Figure 7. The N-terminus of MviA(RssB) is insufficient to mediate σ^S degradation. Cells were grown at 37°C in EG media containing 0.05% CAA and 0.2% arabinose. Where appropriate, chloramphenicol was added at 30 g/ml. Cells were harvested at mid log phase (OD_{600} 0.4). Protein samples (5 μg) were separated on a 10% Tris-HCl SDS PAGE gel and immunoblotted with RpoS monoclonal antibody. pMviA$^{N\text{-}term}$ (pMF386); pMviA$^+$(pMF298).

and 194 (5'AGTATCGGCCTCAGGAAGATC, specific for *lacZ* in pVIK112). The subsequent plasmid pMF350 was $CaCl_2$ transformed into EK191. EK191 expresses the *R6K* protein *pir,* allowing for replication of the plasmid. Plasmid pMF350 was next conjugated into *S. typhimurium* strain JF3672, a non-permissive host lacking the *R6K* protein *pir*, and plated onto LB + Kanamycin agar. Consequently, stable Tet^RKan^R exconjugants arose *via* homologous recombination (JF4296). The *mviA-lacZ* (*kan*) fusion was moved from JF4296 into SF530 (UK1) via P22-mediated transduction. The proper chromosomal location of the integrated plasmid was confirmed using PCR with primer pair 186 (5'CTGTAATAAATTATCGGCG, specific for *rssA*, a gene immediately upstream from *mviA*) and 194. This technique was used to create the *mviA::lacZ* operon fusion strain JF4296.

Plasmid Constructions and Site-Directed Mutagenesis

The promoter-less *mviA* gene was PCR amplified from UK1 chromosomal DNA using oligonucleotide 66 (5'CATTCCGCAGACAACATCAA) and oligonucleotide 67 (5'GATTAAAGC**T**AG**C**CAGGGG, contains an engineered *Nhe*I site) and cloned into the TA cloning vector pCRII (Invitrogen, San Diego, CA) to make pDF219. A 1.1 kb *Nde*I/ *Hind*III fragment from pDF219 was subcloned into pBAD18 and pBAD18Cm forming pDF220 and pMF298, respectively, placing *mviA* under arabinose control. Site-directed mutagenesis was performed using the Promega Altered Sites II *in vitro* Mutagenesis System containing the mutagenesis phagemid, pAlter-1 vector. The *mviA* gene from pDF219 was cloned into the *Eco*R1 site of pAlter-1 forming pDF224. Plasmid pDF224 was subjected to site-directed mutagenesis as per manufacturers recommendations. "Mutagenic" oligonucleotide primers from nucleotide +109 to +135 of the *mviA* coding sequence (containing the putative D58 phosphorylation site) were designed. Oligo 74 (5'CTCATGATATGT**A**ATATCGCTATGCC) and Oligo 85 (5'CTCATGATATGTGA**G**ATCGCTATGCC) enabled the conversions D58N and D58E, respectively. A second mutagenic oligonucleotide primer designed to repair a mutation in the Ap^R gene present on the plasmid was included in the mutagenesis reaction. Site-directed mutagenesis involved hybridization of the mutagenic oligos to the single-stranded recombinant DNA and extension of the hybridized oligos by DNA polymerase. The mutagenized DNA was transformed into a repair minus strain of *E. coli* (BMH 71-18 *mutS*) and transformants were screened for those capable of growing in the presence of ampicillin. A second round of transformation in JM109 ensured proper segregation of mutant and wild type plasmids, resulting in a high proportion of mutants. Sequence analysis was used to confirm mutagenesis at the desired site. Recombinants containing the mutated site (pDF225a for D58N and pDF244 for D58E) were recovered and the mutated *mviA* fragments were cloned using *Nde*I and *Hind*III into expression vectors pBAD18 (Guzman *et al.,* 1995) and pBAD18Cm (Guzman, *et al.,* 1995) for further analysis. Oligonucleotides 51 (5'GACAAGATGTGGATCCACCTTAAC) and 162 (5'CATGATTTCC ATTGCCCCAG) were complementary to the inverted repeat of Tn*10* and to a sequence within *mviA*(*rssB*), respectively.

N-terminus and C-terminus truncations of *mviA* driven by the arabinose-inducible P_{BAD} promoter were constructed from pMF298 taking advantage of pre-existing, unique restriction enzyme sites. This plasmid (which expresses full length *mviA* under the control of P_{BAD}) was digested with *Hind*III and *Bgl*II to excise the carboxy terminus. Cohesive ends were filled in using the Novagen Perfectly Blunt kit (Novagen) and the plasmid re-ligated using T4 DNA ligase (New England Biolabs). A carboxy terminus expressing plasmid was constructed by blunt ending the the *Hind*III/*Bgl*II fragment of *mviA* (encoding the carboxy terminus) using the Novagen Perfectly Blunt kit and then ligating the fragment into the *Sma*I site of pBAD24 (Ap^R). The N-terminus- (pMF386) and the C-terminus- (pMF390) encoding plasmids were transformed by electroporation into strain JF4444 (*katE-lacZ clpP).*

Construction and Purification of His-Tagged MviA

An *mviA*-containing fragment was amplified from UK1 chromosomal DNA using oligonucleotides #77 (5'GGGAGAG**CAT**ATGACGCAGC, containing an engineered *Nde*I site) and #78 (5'GCAATTTC**G**G**A**TC**C**CTCTTCC, containing an engineered *Bam*HI site). The amplified fragment was cloned into pCRII forming pDF234. A 1.1 kb *Nde*I-*Bam*HI fragment was cloned from pDF234 into the pET15b His-Tag vector forming pDF236. His-Tagged MviA was induced as per manufacturers instructions and insoluble inclusion bodies containing the protein were purified. This form of purified His-tagged MviA was used by Caltag, Inc (Healdsberg, CA) to generate antibodies used in Western blot analyses.

Western Blot Analysis

Estimations of protein levels were determined through Western blot analysis. The methodology used was essentially as described earlier (Lee, *et al.,* 1995). Strains were grown in pH 7.7 minimal E glucose medium and samples were removed at the indicated time intervals. Cell extracts were prepared by harvesting the cells by centrifugation and suspending the pellets in 0.01% SDS. After determining the protein concentration of each sample, 5 μg of total cellular protein for each sample was loaded onto a 4.5% stacking/ 10% separating SDS polyacrylamide gel. Following electrophoresis, the proteins were transferred to a 0.45 μm pore size polyvinylidene difluoride (PVDF) membranes (Immobilon P transfer membranes, Millipore Corp) using CAPS buffer, pH 11.0, (Sigma Chemical Co) and a semi-dry transfer unit (Semi-Phor Transfer Unit, Hoeffer Scientific Instruments).

For immunodetection, the blots were blocked with 5% powdered milk in TBST buffer [150 mM NaCl; 10 mM Tris-HCl (pH 8); 0.05% v/v Tween 20] for two hours and probed with a 1:2000 dilution of antiserum in TBST for two hours. The blots were rinsed with TBST and incubated for one hour with a 1:2000 dilution of goat anti-mouse or anti-rabbit IgG coupled to horse-radish peroxidase. The blots were rinsed three times with TBST and twice with TBS for 10 minutes each and reactive areas were visualized using the ECL detection system (Amersham). Antibody to σ^S was kindly provided by R. Burgess (Nguyen *et al.,* 1993). The ClpX antibody was a gift from S. Gottesman.

RNA Extraction, Radiolabeled Probe Preparation and Northern blot Analysis

Bacterial cultures of wild type (UK1) and pMviA$^+$ (JF3929) were grown overnight in LB media supplemented with the appropriate antibiotics and subcultured 1:100 into fresh LB. Strain JF3929 was subcultured and grown in the presence of chloramphenicol and 0.2% arabinose. Cultures were incubated at 37°C with aeration (200 rpm) and samples were removed in exponential phase (at a OD_{600} 0.4) and stationary phase (at a OD_{600} 3.0). Bacteria were collected by centrifugaiton in a Beckman (Model J2-21) centrifuge at 5000 x*g* for 10 min at 4°C. The supernatant was removed and the bacterial pellets were stored at -80°C until processed. Total bacterial RNA was extracted using the hot phenol method (Miller, 1972), quantified by measuring absorbency at 260 nm, and stored at -80°C. Integrity of the RNA was verified by separating the RNA on a non-denaturing, 0.8% agarose

Table 2. Strains and Plasmids Used

Strain	Genotype	Source
Salmonella typhimurium		
SF36	LT2 *srl*::Tn*10 recA1 rpsL*	K. Sanderson
SF530 (03761)	UK1	Curtiss *et al.,* 1981
SF516 (WB4188)	LT2 *mviA*4185::km	Benjamin *et al.,* 1991
SF586	*hsdSA29 hsdSB121 hsdL6 metA22 metE551 trpC2 ilv-452 rpsL120 xyl-404 galE719 H1-6* h2 -e, n, x *nml* (Fels2)$^-$ *fla-66*	Bullas and Ryu, 1983
SF619	(WB4227)	LT2 *mviA*4185::Cm Benjamin *et al.,* 1991
SF680 (TE6153)	LT2 *putPA1303*::km-*katE-lac*(op)	Brown and Elliott, 1996
JF2690	UK1 *rpoS*::Ap	Bearson *et al.* 1996
JF2891	UK1 *rpoS*::Ap *mviA*4185::km	Bearson *et al.* 1996
JF2980	UK1 *mviA*4185::Cm	Bearson *et al.* 1996
JF2891	UK1 *mviA*4185::km *rpoS*::Ap	Bearson *et al.* 1996
JF2892	UK1 *mviA*4185::km	Bearson *et al.* 1996
JF2997	UK1 *cya*::Tn*10*	
JF3144	UK1 *mviA*4185::km/ pDF220 (*mviA$^+$)*	JF2892 X pDF220
JF3183-5	UK1 *mviA*4185::km/ pDF228a-c	JF2892 X pDF228a-c
JF3266	UK1 *putPA1303*::km-*katE-lac*(op)	SF680 X SF530
JF3277	UK1 *mviA*4185::Cm *putPA1303*::km-*katE-lac*(op)	JF3266 X SF619
JF3424	UK1 *putPA1303*::Km^R -*katE-lac*(op) *lcr-7*(*rpoS*) Δopp	Webb *et al.,* 1999
JF3464	UK1 *mviA*4185::Cm *putPA1303*::km-*katE-lac*(op) / pDF220 (*mviA$^+$)*	JF3144 X JF3277
JF3485	UK1 *clpP1*::Tn*10*dTc *lcr-7* (*rpoS*) *putPA1303*::Km^R -*katE-lac*(op)	Webb *et al.,* 1999
JF3487	UK1 *clpP1*::Tn*10*dTc	Webb *et al.,* 1999
JF3644	UK1 *clpP2*::Tn*10*dTc	Webb *et al.,* 1999
JF3672	UK1 *hns*::Tn*10*dTc (95% to *mviA*$^+$)	(JF3424 X Tn*10*dTc pool) X SF530
JF3924	UK1 *rpoS*::Ap *mviA*4185::Km *srl*::Tn*10 recA1*	SF36 X JF2891
JF3929	UK1 *rpoS*::Ap *mviA*4185::Km *srl-202*::Tn*10 recA1* /pMF298 *mviA*$^+$	JF3924 X pMF298
JF3930	UK1 *rpoS*::Ap *mviA*4185::Km *srl-202*::Tn*10 recA1* /pMF299 *mviA*D58N	JF3924 X pMF299
JF3947	UK1 rpoS::Ap *mviA*4185::Km *srl-202*::Tn*10 recA1* / pBAD18-Cm	JF3924 X pBAD18-Cm
JF3950	UK1 *rpoS*::Ap *mviA*4185::Km *srl-202*::Tn*10 recA1* /pMF309 *mviA*D58E	JF3924 X pMF309
JF3964	UK1*rpoS*::Ap *mviA*4185::*Km clpP1*::Tn*10d*Tc	JF2891 X JF3485
JF4028	UK1*rpoS*::Ap *mviA*4185::*Km clpP1*::Tn*10d*Tc / pMF298 (*mviA$^+$)*	JF3964 X pMF298
JF4176	UK1 *mviA*4185::Cm *putPA1303*::Km-*katE-lac*(op) *clpP1*::Tn*10*dTc/ pDF220 *mviA*$^+$ Ap^R	JF3485 X JF3464
JF4180	UK1 *mviA*4185::Cm *putPA1303*::Km-*katE-lac*(op) *clpP1*::Tn*10*dTc	JF3485 X JF3277
JF4263	UK1 Δ *cya*	JF2997 Tc^S
JF4275	UK1 Δ *cya* / pT18-Zip Ap^R / pT25-Zip Cm^R	JF4263 X pT18-Zip X pT25-Zip
JF4296	UK1 *mviA-lacZY*-kan(pVIK112)	[EF563 X JF3672] X SF530
JF4405	UK1 Δ *cya clpP1*::Tn*10*	JF3644 X JF4263
JF4444	UK1 *clpP2*::Tn*10*dTc *putPA1303*::Km^R -*katE-lac*(op)	JF3644 X JF3266
JF4445	UK1 Δ *cya clpP1*::Tn*10*dTc / pMF379A [*rpoS*-'*cyaA*(pT18) Ap]	JF4405 X pMF379A
JF4446	UK1 Δ *cya clpP1*::Tn*10*dTc / pMF383 [(T25) *cyaA*'-*mviA* Cm]	JF4405 X pMF383
JF4447	UK1 Δ *cya clpP1*::Tn*10*dTc / pMF383/ pMF379A	JF4405 X pMF383 X pMF379A
JF4448	UK1 Δ *cya clpP1*::Tn*10*dTc / pMF379B / pMF383	JF4405 X pMF383 X pMF379B
JF4453	UK1 *clpP2*::Tn*10*dTc *putPA1303*::Km^R -*katE-lac*(op) / pMF386 [MviA$^{N\text{-terminus}}$ in pBAD18Cm]	JF4444 X pMF386
JF4461	UK1 *clpP2*::Tn*10*dTc *putPA1303*::Km^R -*katE-lac*(op) / pMF390 [MviA$^{C\text{-terminus}}$ in pBAD24Ap]	JF4444 X pMF390
JF4470	UK1 *rpoS*::Ap *mviA4185*::Cm *srl*::Tn*10 recA1* /pMF386 [MviA$^{N\text{-terminus}}$ in pBADCm]	JF3924 X pMF386
Escherichia coli		
EK78[JM109]	Δ (*pro-lac*) *thi-1 gyrA96 endA1 hsdR17 relA1 supE44 recA1* / F' *traD36 proA$^+$B$^+$ lacI^q lacZ* Δ(M15)	Yanisch-Perron *et al.,* 1985
EK112[XL1-Blue]	*recA1 lac$^-$ endA1 gyrA96 thi hsdR17 supE44 relA1* / F' *proAB$^+$lacI^q lacZ*Δ(M15)	Stratagene
EK191[S17-1 λ pir]	*recA thi pro hsdRM* RP4:2-Tc:Mu:Km:Tn7 λ *pir*, Tp^R Sm^R	De Lorenzo and Timmis, 1994
EK193 [HMS174 (DE3)]	F$^-$ r$_{K12}$ m$_{K12}^+$ *recA* Rif^R λ lysogen (DE3) [T7 RNA polymerase]	Novagen
EK395	Hfr D Δ*cya1400*::Km λ - e14- *relA1 spoT1 thi-1*	J. Kaper
EF330	EK193 / pDF236 (*mviA* in pET15b, His-Tag-MviA)	pDF236 X EK193
EF563	EK191 / pMF350 (*mviA-lacZ)*	pMF350 X EK191
EF644	Δ*cya-1400*::Km / pMF379A / pMF383	EF395 X pMF379A X pMF383
EF646	Δ*cya-1400*::Km / pT18-Zip/ pT25-Zip	EF395 X pT18-Zip X pT25-Zip
EF682	Δ*cya-1400*::Km / pAF404 / pAF406	EF395 X pAF404 X pAF406
EF686	Δ*cya-1400*::Km / pMF383 [*cyaA*(T25)- *mviA*]	EF395 X pMF383
EF687	Δ*cya-1400*::Km / pAF404 [*clpX*^{K12}- *cyaA* (T18)]	EF395 X pAF404
EF688	Δ*cya-1400*::Km / pAF404 / pT25	EF395 X pAF404 X pT25
EF689	Δ*cya-1400*::Km / pMF379A / pAF406	EF395 X pMF379A X pAF406

gel and subsequently staining the nucleic acid with ethidium bromide. Northern blot analysis was performed using 5 μg of total bacterial RNA per well as previously described (Seldon, 1989). RNA was transferred, overnight, onto Nytran (Super Charge) nylon transfer membranes (Schleicher and Schuell) by capillary action transfer and nucleic acid was fixed by baking at 80°C for 30 min and then by ultraviolet irradiation in a UVP Ultraviolet Cross-linker(CL1000). Pre-hybridization and hybridization were carried out at 42°C with gentle agitation. Oligonucleotide primers specific to the *mviA* ORF (oligos 84 and 184) were used to generate specific, PCR-amplified probes to detect the *mviA* mRNA. PCR products were then radiolabelled with [α-^{32}P]-dCTP (NEN Life Science Prods, Inc.) by random-primed labeling.

Bacterial Two-Hybrid System

The two hybrid system used to demonstrate *in vivo* interaction between MviA(RssB) and σ^S is based upon the reconstitution of adenylate cyclase activity of *Bordetella pertussis* CyaA protein (Karimova, *et al.,* 1998). Plasmid pT25 is a derivative of pACYC184 that encodes the N-terminus T25 fragment of CyaA (amino acids 1-224, CyaA') with a multicloning site at the C-terminus. Expression is controlled by the *lacUV5* promoter. Plasmid pT18 is a derivative of pBluescript II KS (Stratagene), compatible with pT25, that encodes the C-terminal T18 fragment of CyaA (amino acids 225-339, 'CyaA) with the multicloning site of pBluescript II KS located at the N-terminus. Functional reconstitution of adenylate cyclase activity is achieved if each Cya fragment is fused in frame to one of two interacting proteins. Adenylate cyclase activity was monitored in *E. coli* and *Salmonella* strains deficient in endogenous adenylate cyclase (*cya*). The *rpoS* gene was cloned into pT18

Table 2. Strains and Plasmids Used (continued)

Plasmid	Description	Source
pAF404	*cyaA*(T18)'-*clpX*^{K12} in pT18, Ap^R	This study
pAF406	*clpX*^{K12}-'*cyaA*(T25) in pT25, Cm^R	This study
pBAD18	expression vector containing the arabinose operon P_{BAD} promoter, Ap^R	Guzman *et al.,* 1995
pBAD18cm	expression vector containing the arabinose operon P_{BAD} promoter, Cm^R	Guzman *et al.,* 1995
pBAD24	expression vector containing the arabinose operon P_{BAD} promoter, Ap^R	Guzman *et al.,* 1995
pCR2.1	TA cloning vector	Invitrogen
pDF219	pCRII containing *mviA*$^+$	This study
pDF220	pBAD18 containing *S. typhimurium mviA* gene	(Ap^R) This study
pDF228a-c	pBAD18 containing site-directed mutant MviA^{D58N} (Ap^R)	This study
pDF236	*mviA* in pET15b, produces His Tagged-MviA	This study
pDF269	pBAD18 containing site-directed mutant *mviA*^{D58E} (Ap^R)	This study
pDF270	pBAD18 containing site-directed mutant *mviA*^{D58H} (Ap^R)	This study
pDF271	pBAD18 containing site-directed mutant *mviA*^{D58Y} (Ap^R)	This study
pET15b	His-Tag vector	Novagen, Madison, WI
pMF298	pBAD18cm *mviA*$^+$	This study
pMF299	pBAD18 containing site-directed mutant MviA^{D58N}	This study
pMF309	pBADcm containing site-directed mutant MviA^{D58E}	This study
pNF327	*'rssA-mviA'* (oligo 51 and 162 PCR fragment in pCR2.1)	This study
pMF328	pCR2.1 containing *mviA*$^+$ (Ap^{R}Km^R)	This study
pMF350	*mviA-lacZ in* pVIK112, Km^R	This study
pMF379	*rpoS*^{UK1} -'*cyaA*(T18) in pT18, Ap^R	This study
pMF383	*cyaA*(T25)'- *mviA* in pT25, Cm^R	This study
pMF386	pBAD18Cm containing N-terminus of *mviA*$^+$	This study
pMF390	pBAD24 containing C-terminus of *mviA*$^+$ (Ap^R)	This study
pT18	pBluescript II KS containing T18 C-terminus of *B. pertussis cyaA*	Karimova *et al.,* 1998
pT25	pACYC184 containing T25 N-terminus of *B. pertussis cyaA*	Karimova *et al.,* 1998
pT18-Zip	pT18 containing leucine zipper domain	Karimova *et al.,* 1998
pT25-Zip	pT25 containing leucine zipper domain	Karimova *et al.,* 1998
pUBAD18	pBAD18 containing *S. typhimurium rpoS* gene	(Tet^R) Volkert *et al.,* 1994
pVIK112	Km^R suicide vector, to construct *lacZ* operon fusions	Kalogeraki and Winnans, 1997

utilizing Vent polymerase to PCR amplify *rpoS* from UK1 with oligo 223 (5'ACTTGCTCGCGGAACA) and oligo 224 (5'GAGTCAGAATACGCTG). This blunt ended PCR product was then ligated into pT18 cut with *Hinc*II. Oligo 223 was designed so the N-terminus of σ^S would be in frame with the αLacZ in pT18. This LacZ fragment is upstream of '*cyaA* in the plasmid. Oligo 224 was designed so σ^S would be fused in frame with 'CyaA. The orientation of the insert was determined by *Hinc*II(in pT18) and *Kpn*I (in *rpoS*)restriction mapping. Plasmid pMF379 (*rpoS-'cyaA*) was transformed from EF600 into the restriction-less *S. typhimurium* strain SF586 and then into an *S. typhimurium cya clpP* mutant (JF4405) resulting in strain JF4445. The *mviA* gene was PCR amplified (Taq polymerase) from UK1 using oligo 242 (5'GAGAGAAGGATCCGCAGCCATTGGTCG), which has a *BamH*I site (underlined), and oligo 241 (5'GGGGTACC**TCA**TTCCGCAGACAACATC), which has an engineered *Kpn*I site (underlined). The *BamH*I and *Kpn*I sites were used to directionally clone the insert into pT25. The oligonucleotides were designed so that the insert would be in frame with CyaA' and contain a stop codon after *mviA* (bold bases). The correct orientation of the insert was determined by *EcoR*V restriction enzyme mapping. Plasmid pMF383 (CyaA'-MviA)was transformed into JF4405 (*cya clpP*) resulting in strain JF4446. The plasmids were then combined in JF4405 resulting in strain JF4448. Strains were then plated onto MacConkey mannitol (1%) agar and incubated for 48 hours at 30°C to determine if adenylate cyclase activity resulted from the interactions of hybrid proteins.

The *clpX* open reading frame (ORF) was cloned, in-frame, into both the pT18 and pT25 vectors as follows. The *clpX* gene was PCR amplified (Taq polymerase) using oligo 262 (5'GGGGCCCCATGACAGAT AAACGCAAAGAT)and oligo 264 (5'CCCAAGCTTGGTTCACCA GATGCCTGTTGCGC) engineered to contain, respectively, *Apa*I and *Hind*III sites (underlined). *E. coli* K12 chromosomal DNA was used as the template. The PCR product was gel-purified (Geneclean II kit, BIO 101), digested with *Apa*I and *Hind*III and ligated into similarly digested pT18 using T4 ligase. This created a plasmid in which the N-terminus of the *clpX* ORF was fused, in-frame, with the Lac fragment of pT18. The *clpX* stop codon was omitted from oligo 264 to allow read-through into the T18 fragment of *cya* to generate a ClpX-'Cya(T18) hybrid protein(pAF404). The orientation of the insert was verified by restriction enzyme digestion of the resulting plasmid.

The *clpX* gene was also amplified from *E. coli* K12 chromosomal DNA in a second PCR (Taq polymerase) using oligo 267 (5'AACTGCAGCAATGACAGATAAACGCAAAGAT) and oligo 268 (5'CGGGATCCTTATTCACCAGATGCCTGTTGCGC) designed to incorporate, respectively, a *Pst*I recognition site (underlined) and a *BamH*I recognition site (underlined). Gel purified PCR product and pT25 were digested with *Pst*I and *Bam*HI and ligated using T4 ligase. This created a plasmid in which the *clpX* ORF was fused, in-frame, downstream of the pT25 *cya'* gene resulting in a Cya(T25)'-ClpX hybrid protein (pAF406).

Various combinations of these two hybrid plasmids were introduced into EK395 and the expression of chromosomal *lacZ* monitored as an indicator of cAMP production that might result from the interaction between pairs of Cya fusion proteins. All cultures were grown in LB broth in the presence of chloramphenicol, ampicillin, and 0.5 mM IPTG at 30°C with aeration (250 rpm). Aliquots were removed as indicated in Figure legends and ß-galactosidase activity was measured as described by Miller (Miller, 1992).

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References

Aliabadi, Z., Park, Y.K., Slonczewski, S.L., and Foster, J.W. 1988. Novel regulatory loci controlling oxygen and pH-regulated gene expression in *Salmonella typhimurium.* J. Bacteriol. 170: 842-851.

Barth, M., Marschall, C., Muffler, A., Fischer, D., and Hengge-Aronis, R. 1995. Role for the histone-like protein H-NS in growth phase-dependent and osmotic regulation of σ^S and many σ^S-dependent genes in *Escherichia coli.* J. Bacteriol. 177: 3455-3464.

Bearson, S.M.D., Benjamin, W.H., Jr., Swords, W.E., and Foster, J.W. 1996. Acid shock induction of *rpoS* is mediated by the mouse virulence gene *mviA* of *Salmonella typhimurium.* J. Bacteriol. 178: 2572-2579.

Becker, G., Klauck, E., and Hengge-Aronis, R. 1999. Regulation of RpoS proteolysis in *Escherichia coli*: the response regulator RssB is a recognition factor that interacts with the turnover element in RpoS. Proc. Natl. Acad. Sci. USA. 96: 6439-44.

Benjamin, W.H., Jr., Wu, X., and Swords, W.E. 1996. The predicted amino acid sequence of the *Salmonella typhimurium* virulence gene *mviA*$^+$ strongly indicates that MviA is a regulator protein of a previously unknown *S. typhimurium* response regulator family. Infect. Immun. 64: 2365-2367.

Benjamin, W.H., Jr., Yother, J., Hall, P., and Briles, D.E. 1991. The *Salmonella typhimurium* locus *mviA* regulates virulence in *Ity*^{s} but not *Ity*^{r} mice: functional *mviA* results in avirulence; mutant nonfunctional *mviA* results in virulence. J. Exp. Med. 174: 1073-1083.

Bohringer, J., Fisher, D., Mosler, G., and Hengge-Aronis, R. 1995. UDP-Glucose is a potential intracellular signal molecule in the control of expression of σ^S and σ^S-dependent genes in *Escherichia coli.* J. Bacteriol. 177: 413-422.

Bouche, S., Klauck, E., Fischer, D., Lucassen, M., Jung, K., and Hengge-Aronis, R. 1998. Regulation of RssB-dependent proteolysis in *Escherichia coli*: a role for acetyl phosphate in a response-control process. Mol. Microbiol. 27: 787-795.

Brown, L., and Elliott, T. 1996. Efficient translation of the RpoS sigma factor in *Salmonella typhimurium* requires host factor I, an RNA-binding protein

encoded by the *hfq* gene. J. Bacteriol. 178: 3763-3770.
Bullas, L.R., and Ryu, J.-I. 1983. *Salmonella typhimurium* LT2 strains which are r^- m^+ for all three chromosomally located systems of DNA restriction and modification. J. Bacteriol. 156: 471-474.
Cunning, C., and Elliott, T. 1999. RpoS synthesis is growth rate regulated in *Salmonella typhimurium*, but its turnover is not dependent on acetyl phosphate synthesis or PTS function. J. Bacteriol. 181: 4853-62.
Curtiss, R., III, Porter, S.B., Munson, M., Tinge, S.A., Hassan, J.O., Gentry-Weeks, C., and Kelly, S.M. 1991. Nonrecombinant and recombinant avirulent *Salmonella* vaccines for poultry. In: Colonization control of human bacterial enteropathogens in poultry. L. C. Blankenship, J. H. S. Bailey, N. A. Cox, N. J. Stern and R. J. Meinersmann, eds. Academic Press, New York. p. 169-198.
De Lorenzo, V.C., and Timmis, K.M. 1994. Analysis and construction of stable phenotypes in gram-negative bacteria with Tn*5*- and Tn*10*-derived minitransponsons. Meth. Enzymol. 235: 386-405.
Fang, F.C., Libby, S.J., Buchmeier, N.A., Loewen, P.C., Switala, J., Harwood, J., and Guiney, D.C. 1992. The alternative and factor RpoS regulates *Salmonella* virulence. Proc. Natl. Acad. Sci. USA. 89: 11978-11982.
Foster, J.W. 1991. *Salmonella* acid shock proteins are required for the adaptive acid tolerance response. J. Bacteriol. 173: 6896-6902.
Foster, J.W. 1993. The acid tolerance response of *Salmonella typhimurium* involves transient synthesis of key acid shock proteins. J. Bacteriol. 175: 1981-1987.
Gibson, K.E., and Silhavy, T.J. 1999. The LysR homolog LrhA promotes *rpoS* degradation by modulating activity of the response regulator *sprE*. J. Bacteriol. 181: 563-71.
Guzman, L.-M., Belin, D., Carson, M.J., and Beckwith, J. 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. J. Bacteriol. 177: 4121-4130.
Hengge-Aronis, R. 1993. Survival of hunger and stress: The role of *rpoS* in early stationary phase gene regulation in *E. coli*. Cell. 72: 165-168.
Hengge-Aronis, R. 1996. Back to log phase: σ^S as a global regulator in the osmotic control of gene expression in *Escherichia coli*. Mol. Microbiol. 21: 887-893.
Hengge-Aronis, R., Lange, R., Henneberg, N., and Fischer, D. 1993. Ostomic regulation of *rpoS*-dependent genes in *Escherichia coli*. J. Bacteriol. 175: 259-265.
Holley, E.A., and Foster, J.W. 1982. Bacteriophage P22 as a vector for Mu mutagenesis in *Salmonella typhimurium*: Isolation of *nad-lac* and *pnc-lac* gene fusions. J. Bacteriol. 152: 959-962.
Kalogeraki, V.S., and Winans, S.C. 1997. Suicide plasmids containing promoterless reporter genes can simultaneously disrupt and create fusions to target genes of diverse bacteria. Gene. 188: 69-75.
Karimova, G., Pidoux, J., Ullmann, A., and Ladant, D. 1998. A bacterial two-hybrid system based on a reconstituted signal transduction pathway. Proc. Natl. Acad. Sci. USA. 95: 5752-6.
Klauck, E., Böhringer, J., and Hengge-Aronis, R. 1997. The LysR-like regulator LeuO in *Escherichia coli* is involved in the translational regulation of *rpoS* by affecting the expression of the small regulatory DsrA-RNA. Mol. Microbiol. 25: 559-563.
Lange, R., Barth, M., and Hengge-Aronis, R. 1993. Complex transcriptional control of the σ^S-dependent stationary-phase-induced and osmotically regulated *osmY csi-5* gene suggests novel roles for Lrp, cyclic AMP cAMP receptor protein-cAMP complex, and integration host factor in the stationary-phase response of *Escherichia coli*. J. Bacteriol. 175: 7910-7917.
Lange, R., and Hengge-Aronis, R. 1994. The cellular concentration of the σ^S subunit of RNA polymerase in *Escherichia coli* is controlled at the levels of transcription, translation, and protein stability. Genes Dev. 8: 1600-1612.
Lee, I.S., Lin, J., Hall, H.K., Bearson, B., and Foster, J.W. 1995. The stationary-phase sigma factor σ^SRpoS is required for a sustained acid tolerance response in virulent *Salmonella typhimurium*. Mol. Microbiol. 17: 155-167.
Loewen, P.C., and Hengge-Aronis, R. 1994. The role of the sigma factor σ^S KatF in bacterial global regulation. Annu. Rev. Microbiol. 48: 53-80.
Loewen, P.C., Hu, B., Strutinsky, J., and Sparling, R. 1998. Regulation in the *rpoS* regulon of *Escherichia coli*. Can. J. Microbiol. 44: 707-17.
Maloy, S.R. 1990. Experimental techniques in bacterial genetics. Jones and Bartlett Publishers, Boston.
Maloy, S.R., and Roth, J.R. 1983. Regulation of proline utilization in *Salmonella typhimurium*: Characterization of *put*:Mu*d*Aplac operon fusions. J. Bacteriol. 154: 561-568.
McCann, M.P., Fraley, C.D., and Matin, A. 1993. The putative and Factor KatF is regulated posttranscriptionally during carbon starvation. J. Bacteriol. 175: 2143-2149.
Miller, J.H. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
Miller, J.H. 1992. A short course in bacterial genetics. A laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
Muffler, A., Fischer, D., Altuvia, S., Storz, G., and Hengge-Aronis, R. 1996. The response regulator RssB controls stability of the sigma S subunit of RNA polymerase in *Escherichia coli*. EMBO J. 15: 1333-1339.
Muffler, A., Fischer, D., and Hengge-Aronis, R. 1996. The RNA-binding protein HF-I, known as a host factor for phage Q RNA replication, is essential for *rpoS* translation in *Escherichia coli*. Genes Dev. 10: 1143-1151.
Muffler, A., Traulsen, D.D., Lange, R., and Hengge-Aronis, R. 1996. Posttranscriptional osmotic regulation of the σ^S subunit of RNA polymerase in *Escherichia coli*. J. Bacteriol. 178: 1607-1613.
Nguyen, L.H., Jensen, D.B., Thompson, N.E., Gentry, D.R., and Burgess, R.R. 1993. *In vitro* functional characterization of overproduced *Escherichia coli katF/rpoS* gene product. J. Biochem. 32: 11112-11117.
Parkinson, J.S., and Kofoid, E.C. 1992. Communication modules in bacterial signaling proteins. Annu. Rev. Genet. 26: 71-112.
Pratt, L., and Silhavy, T. 1996. The response regulator, SprE, controls the stability of RpoS. Proc. Natl. Acad. Sci. USA. 93: 2488-2492.
Prince, R.W., Xu, Y., Libby, S.J., and Fang, F.C. 1994. Cloning and sequencing of the gene encoding the RpoSKatF and factor from *Salmonella typhimurium* 14028s. Biochim. Biophys. Acta. 1219: 198-200.
Rathman, M., Sjaastad, M.D., and Falkow, S. 1996. Acidification of phagosomes containing *Salmonella typhimurium* in murine macrophages. Infect. Immun. 64: 2765-2773.
Sanger, F., Nicklen, S., and Coulson, A.R. 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA. 74: 5463-5467.
Schweder, T., Lee, K.-H., Lomovskaya, O., and Matin, A. 1996. Regulation of *Escherichia coli* starvation sigma factor σ^S by ClpXP Protease. J. Bacteriol. 178: 470-476.
Seldon, R.F. 1989. Analysis of RNA by Northern hybridization. Current Protocols in Molecular Biology FM Ausubel R. Brent, R.E. Kingston, D.D. Moore, J.D. Siedman, J.A. Smith and K. Struhl, eds.
Siegele, D.A., and Hu, J.C. 1997. Gene expression from plasmids containing the araBAD promoter at subsaturating inducer concentrations represents mixed populations. Proc. Natl. Acad. Sci. USA. 94: 8168-72.
Swords, W.E., Cannon, B.M., and Benjamin, W.H., Jr. 1997. Avirulence of LT2 strains of *Salmonella typhimurium* results from a defective *rpoS* gene. Infect Immun 65: 2451-2453.
Tsai, S.P., Hartin, R.J., and Ryu, J.-I. 1989. Transformation in restriction-deficient *Salmonella typhimurium* LT2. J. Gen. Microbiol. 135: 2561-2567.
Vogel, H.J., and Bonner, D.M. 1956. Acetylornithase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. 218: 97-106.
Volkert, M.R., Hajec, L. I., Matijasevic, Z., Fang, F.L., and Prince, R. 1994. Induction of the *Escherichia coli aidB* gene under oxygen-limiting conditions requires a functional *rpoS katF* gene. J. Bacteriol. 176: 7638-7645.
Webb, C., Moreno, M., Wilmes-Riesenberg, M., Curtiss, I.R., and Foster, J.W. 1999. Effects of DksA and ClpP protease on sigma S production and virulence in *Salmonella typhimurium*. Mol. Microbiol. 34: 112-123.
Wilmes-Riesenberg, M.R., Foster, J.W., and Curtiss, R., III 1997. An altered *rpoS* allele contributes to the avirulence of *Salmonella typhimurium* LT2. Infect. Immun. 65: 203-210.
Yanisch-Perron, C., Vieira, J., and Messing, J. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene. 33: 103-119.
Zgurskaya, H.I., Keyhan, M., and Matin, A. 1997. The σ^S level in starving *Escherichia coli* cells increases solely as a result of its increased stability, despite decreased synthesis. Mol. Microbiol. 24: 643-651.
Zhou, Y., and Gottesman, S. 1998. Regulation of proteolysis of stationary-phase sigma factor RpoS. J. Bacteriol. 180: 1154-1158.