

Acid Shock Induction of RpoS Is Mediated by the Mouse Virulence Gene *mviA* of *Salmonella typhimurium*

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Salmonella typhimurium encounters a variety of acid stress situations during growth in host and nonhost environments. The organism can survive potentially lethal acid conditions (pH <4) if it is first able to adapt to mild or more moderate acid levels. The molecular events that occur during this adaptive process are collectively referred to as the acid tolerance response and vary depending on whether the cells are in log- or stationary-phase growth. The acid tolerance response of logarithmically growing cells includes the participation of an alternate sigma factor, σ^S (RpoS), commonly associated with stationary-phase physiology. Of 51 acid shock proteins (ASPs) induced during shifts to pH 4.4, 8 are clearly dependent on σ^S for production (I. S. Lee, J. Lin, H. K. Hall, B. Bearson, and J. W. Foster, Mol. Microbiol. 17:155–167, 1995). The acid shock induction of these proteins appears to be the result of an acid shock-induced increase in the level of σ^S itself. We have discovered that one component of a potential signal transduction system responsible for inducing *rpoS* expression is the product of the mouse virulence gene *mviA*⁺. MviA exhibits extensive homology to the regulatory components of certain two-component signal transduction systems (W. H. Benjamin, Jr., and P. D. Hall, abstr. B-67, p. 38, in Abstracts of the 93rd General Meeting of the American Society for Microbiology 1993, 1993). Mutations in *mviA* (*mviA*::Km) caused the overproduction of σ^S and σ^S -dependent ASPs in logarithmically growing cells, as well as increases in tolerances to acid, heat, osmolarity, and oxidative stresses and significant decreases in growth rate and colony size. Mutations in *rpoS* suppressed the *mviA*::Km-associated defects in growth rate, colony size, ASP production, and stress tolerance, suggesting that the effects of MviA on cell physiology occur via its control of σ^S levels. Western blot (immunoblot) analyses of σ^S produced from natural or arabinose-regulated promoters revealed that acid shock and MviA posttranscriptionally regulate σ^S levels. Turnover experiments suggest that MviA regulates the stability of σ^S protein rather than the translation of *rpoS* message. We propose a model in which MviA or its unknown signal transduction partner senses some consequence of acid shock, and probably other stresses, and signals the release of σ^S from proteolysis. The increased concentration of σ^S drives the elevated expression of the σ^S -dependent ASPs, resulting in an increase in stress tolerance. The avirulent nature of *mviA* insertion mutants, therefore, appears to result from inappropriate σ^S -dependent gene expression during pathogenesis.

Adaptations to changes in the environment allow microorganisms to survive and even flourish during confrontations with nature's extremes. One of the most common stress situations that bacteria encounter involves acid pH. Acidified ponds, degradative cellular organelles, and host digestive systems, as well as byproducts of the catabolic activities of the bacteria, contribute to microbial acid stress. Classically, pH homeostasis mechanisms involving a series of proton antiport systems have been viewed as the critical feature for surviving acid stress, at least among neutralophiles such as *Escherichia coli* and *Salmonella typhimurium* (10, 28, 58). However, these systems do not appear to be inducible by acid and protect the cell only within certain pH limits (20, 58). *S. typhimurium* has developed several low-pH-inducible acid defense systems, collectively referred to as the acid tolerance response (ATR), that push the limits of pH tolerance (19, 33, 34; reviewed in references 18 and 22).

The foundation of inducible acid tolerance is the induction of a set of acid shock proteins (ASPs); there are 51 ASPs for the log-phase ATR and 15 ASPs for the stationary-phase ATR.

At least some of these ASPs are important for protecting the cell against extreme acid pH (pH 3) in minimal or complex medium (16, 17, 33, 34). Two important objectives in the quest to understand the ATR are to identify which ASPs are essential for acid tolerance and to unravel the signal transduction pathways required for acid shock induction of those proteins. Answers to these questions may eventually prove useful in the design of novel antimicrobial therapies.

The alternate sigma factor σ^S , encoded by the *rpoS* (*katF*) locus, has been proven to be crucial for the induction of stationary-phase stress response systems in *E. coli* and *S. typhimurium* and is an essential virulence factor in *S. typhimurium* (15, 27, 44, 45). The cellular concentration of this transcription factor varies with growth phase from a very low level in logarithmically growing cells to a very high level in stationary-phase cultures (31, 32, 42, 46). The control of cellular σ^S levels occurs at the transcriptional and translational levels as well as through protein stability (32, 37). We have discovered that σ^S in *S. typhimurium* is also an ASP that is induced fourfold by transitions from normal to acidic conditions (pH <4.5) (33). This induction has been shown to be essential for *S. typhimurium* to mount what is referred to as sustained induction of the ATR (33). The ATR is a complex adaptive response that includes both a σ^S -independent transient ATR, which is maximally in-

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TABLE 1. Strains used

Strain	Genotype	Source, reference, or description
UK1 (χ 3761)	Wild type	R. Curtiss III (13)
SF516	LT2 <i>mviA4185::Km</i>	B. Benjamin
SF619	LT2 <i>mviA::Cm</i>	B. Benjamin
JF2690	UK1 <i>rpoS::Ap</i>	33
JF2735	UK1 <i>rpoS^{UK1} zfi-5179::Tn10dTc</i>	33
JF2891	UK1 <i>mviA4185::Km rpoS::Ap</i>	P22(SF516) \times JF2690
JF2892	UK1 <i>mviA4185::Km</i>	P22(SF516) \times UK1
JF2980	UK1 <i>mviA::Cm</i>	P22(SF619) \times UK1
JF2982	UK1 <i>mviA::Km lcr-1</i>	JF2892 spontaneous large colony
JF2983	UK1 <i>mviA::Km lcr-2</i>	JF2892 spontaneous large colony
JF2984	UK1 <i>mviA::Km lcr-3</i>	JF2892 spontaneous large colony
JF2985	UK1 <i>mviA::Km lcr-4</i>	JF2892 spontaneous large colony
JF2986	UK1 <i>mviA::Km lcr-5</i>	JF2892 spontaneous large colony
JF2987	UK1 <i>mviA::Km lcr-6</i>	JF2892 spontaneous large colony
JF2988	UK1 <i>mviA::Km lcr-7</i>	JF2892 spontaneous large colony
JF3042	UK1 <i>rpoS10::MudJ mviA::Cm</i>	P22(SF619) \times JF2938
JF3057	UK1 <i>rpoS::Ap/pUBAD(rpoS⁺ Ap Tc)</i>	pUBAD \times JF2690
JF3058	UK1 <i>rpoS::Ap mviA::Km/pUBAD(rpoS⁺ Ap Tc)</i>	pUBAD \times JF2891

duced by 20 min of pH 4.4 acid shock but progressively lost during longer adaptations (17), and a σ^S -dependent sustained ATR, which can be observed during longer periods of adaptation (60 or 90 min) (33). Of the 51 ASPs identified when log-phase cells were subjected to acid shock, only 8 are dependent on σ^S for their induction (33). One or more of these eight proteins must be essential for the acid tolerance conferred by the sustained induction of the ATR.

We now report on a second regulator of the σ^S -dependent ASPs which was revealed while screening known mutations for effects on acid tolerance. The product of the mouse virulence regulatory gene *mviA*⁺ was found to negatively control the expression of the σ^S -dependent ASPs by modulating the levels of σ^S and so appears to form at least part of a potential acid shock signal transduction pathway. MviA is a 38-kDa protein which has significant homology to the response regulatory family of bacterial transcriptional regulatory proteins (8, 9, 56). The gene is required for *S. typhimurium* to infect genetically susceptible (*Ity*^S) strains of inbred mice (6, 7, 36). Virulent strains such as UK1 or SL1344 are *mviA*⁺ and so readily infect and kill *Ity*^S mice. Benjamin and colleagues have constructed *mviA* null mutants in which antibiotic cassettes have been inserted into *mviA*⁺ (7). These strains are avirulent and produce very small colonies on solid media; this phenotype is referred to as the small-colony morphology (Scm) phenotype (50). Sequence homology to other two-component signal transduction systems suggests that MviA or its signal transduction partner senses some aspect of the environment important for mouse virulence (9, 56). The relationship among MviA, σ^S -dependent ASPs, and acid tolerance suggests an intriguing connection between the ATR and virulence.

MATERIALS AND METHODS

Media and bacterial strains. Luria-Bertani (LB; 41) complex medium and Vogel and Bonner E minimal medium (53) supplemented with 0.4% glucose were prepared as liquid and solid (1.5% agar) media. The following antibiotics were used: ampicillin (60 μ g/ml), kanamycin (50 μ g/ml), chloramphenicol (30 μ g/ml), and tetracycline (10 or 20 μ g/ml for minimal or complex medium, respectively). The bacterial strains were derived from *S. typhimurium* and are listed in Table 1.

Two-dimensional SDS-PAGE. Two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Spector et al. (48) with cells labeled for 3 min with Tran³⁵S-label (40 μ Ci/ml; ICN Biomedical, Inc.). Approximately 5 μ g of protein was analyzed for each sample. In this procedure, basic and acidic proteins are situated to the left and right of the autoradiograph, respectively. The first dimension was a pH 5 to 7

isoelectric focusing gel containing 1.6% (pH 5 to 7) and 0.4% (pH 3 to 10) ampholytes (Bio-Rad), and the second dimension was an SDS-11.5% polyacrylamide gel. Coordinates given for individual proteins correspond to a standard two-dimensional map of *S. typhimurium* polypeptides (48).

ATR. ATR assays were conducted with cells that had been grown in E glucose (EG) medium overnight at 37°C. The overnight broth was diluted 1/100 in 3 ml of EG broth (pH 7.7) and grown to approximately 1×10^8 to 2×10^8 cells per ml. A 1/100 dilution was used rather than 1/1,000 because the carryover effect of stationary-phase-induced tolerance seen at pH 3.3 was eliminated by challenging cells with an exposure to pH 3.0 or 3.1 (33). Cultures destined for adaptation were adjusted to pH 4.4 and incubated for 30, 60, or 90 min. Acid challenge of all cultures was done by adjusting the pH of the medium to pH 3. Percent survival was calculated by dividing the CFUs at a given time by the CFUs at time zero and multiplying by 100. Results were reproducible to within 50% of the stated percent survival value (e.g., a stated value of 50% survival was reproducible to between 25 and 75%).

Tolerance to heat, oxidative, and osmotic stresses. Cross protection was assayed by using a 1:1,000 dilution of an overnight culture in 3.0 ml of minimal glucose medium. The diluted culture was grown to an optical density at 600 nm (OD_{600}) of 0.4, and cells were harvested by centrifugation and resuspended in minimal glucose medium (pH 7) which had been preheated to 52°C (thermotolerance) or which contained either 20 mM H₂O₂ (oxidative stress) or 2.5 M NaCl (osmotic stress). Survival was measured at various times depending upon the type of stress.

Measurement of σ^S levels. σ^S levels were estimated through Western blot (immunoblot) analysis. The method used was essentially as described earlier (33). Cultures of *mviA*⁺ (UK1) and *mviA::Km* (JF2892) strains were grown in minimal EG medium. Samples were removed at OD_{600} s of 0.1, 0.2, 0.3, 0.4, and 0.6. The cells were harvested and then lysed with 0.01% SDS. Five micrograms of total cellular protein from each sample was loaded onto an SDS-4.5% stacking-11.5% separating polyacrylamide gel. Following electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes (Immobilon; Millipore Corp.) with CAPS buffer (pH 11.0; Sigma Chemical Co.) and a Hoefer semidry transfer unit. The immunoblots were blocked with 1% bovine serum albumin in TBST buffer (150 mM NaCl, 10 mM Tris-HCl [pH 8], 0.05% [vol/vol] Tween 20) for 2 h and probed with a 1:2,000 dilution of anti- σ^S monoclonal antibody (43) in TBST for 2 h. The blots were rinsed with TBST and incubated for 1 h with a 1:2,000 dilution of goat anti-mouse immunoglobulin G coupled to alkaline phosphatase. The blots were rinsed three times with TBST and twice with TBS for 10 min each time, and reactive areas were visualized with an alkaline phosphatase color development solution (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 5 mM MgCl₂, 66- μ g/ml nitroblue tetrazolium, 82.5- μ g/ml 5-bromo-4-chloro-3-indolylphosphate). Antibody to σ^S was kindly provided by R. Burgess (43). Western blot analysis was also used to investigate σ^S expression in *mviA*⁺ and *mviA::Km Salmonella* strains containing an *rpoS::Ap* insertion and pUBAD, a plasmid expressing the full-length *rpoS* gene under the control of the arabinose operon promoter. Cultures were grown in minimal EG medium, and induction of the P_{BAD} promoter was accomplished by the addition of arabinose (15 mM) for one doubling before harvesting the cells. Samples were removed at OD_{600} s of 0.2 and 1.0 and prepared for Western blot analysis as described above. Acid shock cultures were first grown at pH 7.7 to an OD_{600} of 0.4 and then acid shocked at pH 4.4 for 20 min. Gels were digitized and densitometric analysis was performed with NIH Image version 1.55 from the National Institutes of Health.

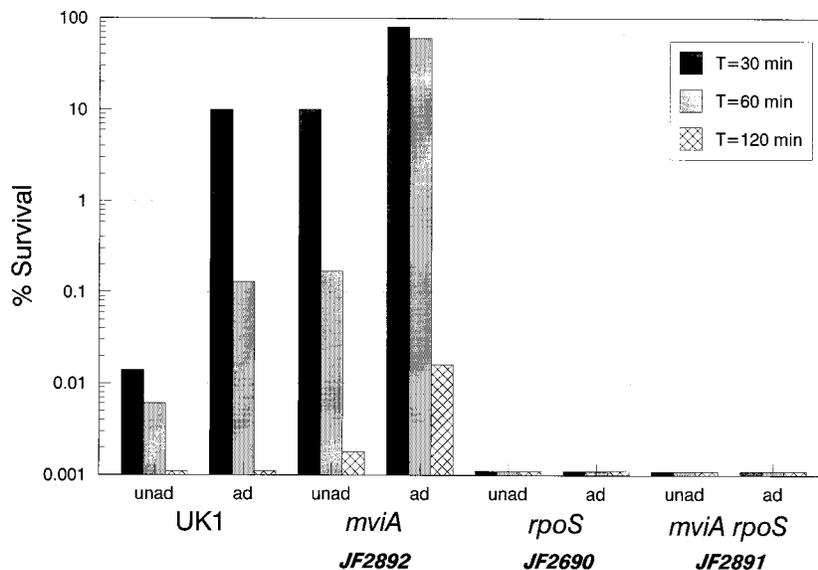


FIG. 1. Effect of an *mviA*::Km mutation on the ATR of strain UK1. Cultures were grown in minimal EG medium (pH 7.7) to an OD₆₀₀ of 0.4. Cultures to be adapted (ad) were shifted to pH 4.4 for 1 h and then acid challenged at pH 3.0. Unadapted (unad) cultures were shifted directly to pH 3.0. Viable counts were taken at 30, 60, and 120 min after acid challenge. One hundred percent viability was taken to be approximately 2×10^8 cells per ml and represents the viable counts obtained immediately before the challenge pH adjustment.

The effect of MviA on σ^S turnover was measured in *mviA*⁺ and *mviA* mutants. Cultures were grown to mid-log phase (OD₆₀₀ of 0.6), chloramphenicol was added to 100 μ g/ml, and 1.5-ml aliquots were removed at timed intervals. The relative levels of σ^S were measured by immunoblotting as described above.

Genetic procedures. Transductions were performed with P22 HT 105/1-*int* as described earlier (2, 25). *MudJ* insertions into *rpoS* were selected from a random pool of *MudJ* insertions generated in UK1 by the technique of transitory *cis*-complementation outlined by Hughes and Roth (26). Selection for *MudJ* insertions that would cotransduce with an *rpoS*::Ap insertion was made. Transformations were performed by following the procedure of Tasi et al. (52).

β -Galactosidase activity. β -Galactosidase activity was measured from an *rpoS*-*lacZ* operon fusion strain grown in LB and EG media. Mid-log phase (OD₆₀₀ of 0.2) and stationary-phase (OD₆₀₀ of 1.2) cells were assayed for β -galactosidase expression according to the method of Miller (41).

RESULTS

The MviA protein is a potential component of an acid tolerance signal transduction pathway. A clue to the signal transduction puzzle arose from the results of an ongoing project in our laboratory to screen known mutations for effects on acid tolerance. We discovered that insertion mutations in the mouse virulence gene *mviA* had an intriguing effect on acid tolerance development in virulent strains of *S. typhimurium*. As shown in Fig. 1, *mviA*::Km insertions caused an increased tolerance to pH 3 in both adapted (pH 4.4) and unadapted (pH 7.7) cells. These results imply that MviA is an important factor in the development of acid tolerance. However, even though the overall level of tolerance was higher in these mutants, they still exhibited an adaptive response, suggesting that factors in addition to MviA are required for maximal acid tolerance. The homology between MviA and other two-component regulators immediately suggested a role for this protein in transmitting a signal generated by acid shock to the ATR system (8).

MviA regulates σ^S -dependent ASPs. If MviA is part of the ATR signal transduction system, then *mviA* insertion mutants should exhibit altered ASP expression. Also, since *mviA* insertion mutants show elevated acid tolerance in unadapted cells, we might expect to see elevated expression of certain ASPs at a noninducing pH (pH 7.7). The results presented in Fig. 2 confirmed this and revealed that the overexpressed proteins

constitute a specific subset of five σ^S -dependent ASPs. The protein profiles of UK1 at pH 7.7 and pH 4.4 are shown in the two-dimensional polyacrylamide gels of Fig. 2A and B, respectively. As shown in Fig. 2C, the σ^S -dependent ASPs are also present in an *mviA*::Km mutant at pH 7.7. Thus, MviA in some manner negatively regulates the expression of these ASPs at pH 7.7. These five proteins are probably not the only proteins required for full acid tolerance since the *mviA*::Km mutant still adapted to a low pH (Fig. 1).

MviA-mediated regulation of ASP synthesis is dependent on σ^S . There are two general ways in which MviA could regulate ASP synthesis. MviA-mediated control over ASP synthesis may require σ^S or might occur independently of σ^S . Consequently, we examined whether the overproduction of ASPs in an *mviA*::Km mutant required σ^S . If σ^S was required, then the ASPs would not be present in an *mviA*::Km *rpoS* mutant. In contrast, if σ^S was not required for MviA-mediated control of the ASPs, those ASPs would still be produced in the double mutant. The results of two-dimensional SDS-PAGE analysis indicated that σ^S was required. The ASP overexpression that was evident in an *mviA* insertion mutant was not observed in the *mviA*::Km *rpoS* mutant (Fig. 2D). The data support a model in which MviA normally prevents the σ^S -dependent expression of ASPs. Further indication of this relationship was obtained by examining whether the *mviA*::Km acid-tolerant phenotype would suppress the acid-sensitive phenotype of *rpoS* mutants. As predicted from the two-dimensional gel electrophoresis results, an *rpoS mviA*::Km double mutant retained the extreme acid-sensitive phenotype of *rpoS* mutants, confirming that the MviA effects on acid tolerance depend upon σ^S production (Fig. 1).

MviA controls the cellular levels of σ^S . One way MviA could affect the σ^S -dependent expression of ASP genes is by regulating the level of σ^S . MviA-mediated limitation of σ^S synthesis during log-phase growth would also effectively prevent the synthesis of σ^S -dependent ASPs. Figure 3 presents a Western blot analysis in which σ^S levels were measured in *mviA*⁺ and *mviA*::Km cells at various stages of growth. At each point

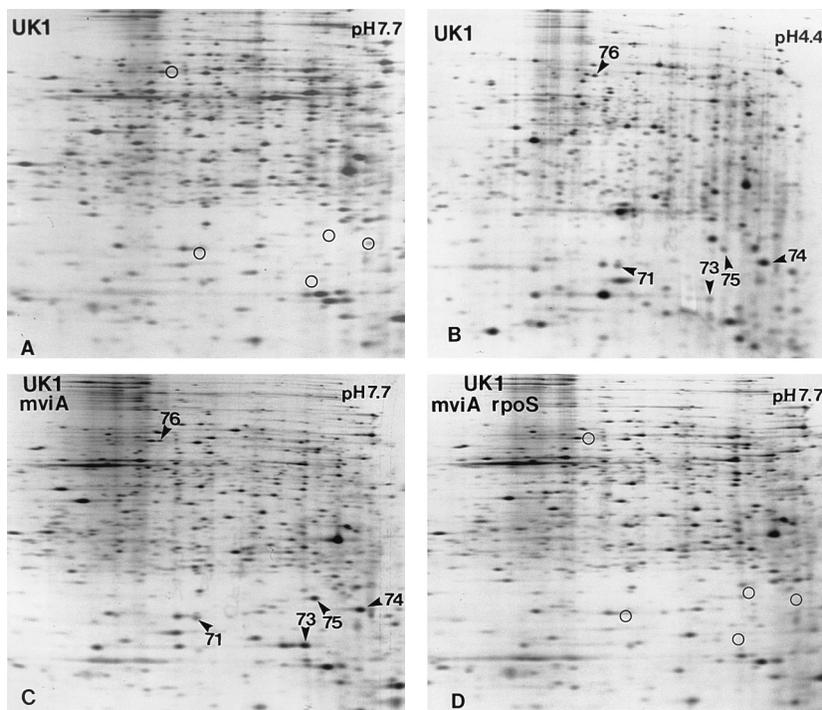


FIG. 2. Two-dimensional SDS-PAGE analysis of σ^S -dependent ASP synthesis. (A, C, and D) Cells were grown in minimal EG medium (pH 7.7) to an OD_{600} of 0.4 and immediately labeled for 3 min (40 μ Ci/ml). (B) Cells were acid shocked for 20 min before the 3-min labeling. (A and B) UK1; (C) JF2892 (*mviA::Km*); (D) JF2891 (*mviA::Km rpoS*). Arrowheads, σ^S -dependent ASPs induced at pH 4.4; circles, ASPs that are not present (or that are greatly diminished) at pH 7.7. Numbers in panels B and C denote specific ASP designations.

examined, the *mviA* insertion mutant possessed a four- to fivefold increase in σ^S levels compared with the *mviA*⁺ parent. This finding confirms that MviA negatively regulates some aspect of *rpoS* expression. Consequently, the loss of MviA would allow overexpression of σ^S and indirectly cause the overexpression of the σ^S -dependent ASPs described above.

MviA exhibits elevated resistance to heat, osmolarity, and oxidative stresses. We have shown previously that acid shock will induce cross protection to other stresses, including heat, osmolarity, and oxidative damage (33). This cross protection was σ^S dependent. It seemed reasonable, therefore, to predict that an *mviA* insertion mutant with elevated levels of σ^S might also exhibit increased levels of cross protection. Figure 4 illustrates that the *mviA::Km* mutant JF2892 did exhibit increased resistance to heat, osmolarity, and oxidative stresses compared with a UK1 control.

Posttranscriptional control of *rpoS* by MviA. We next examined whether MviA controlled σ^S expression at the transcriptional or posttranscriptional level. For this purpose, Western blot analysis was performed on *mviA*⁺ and *mviA::Km* strains of *S. typhimurium* containing a truncated *rpoS* gene regulated by its natural promoters and a full-length *rpoS* gene regulated by the arabinose promoter/operator. The chromosomal *rpoS::Ap*

insertion produces the truncated RpoS, whereas plasmid pUBAD (54) contains the entire *rpoS* gene under the control of the arabinose operon promoter. One can differentiate transcriptional from posttranscriptional regulation by measuring the relative levels of truncated and full-length RpoS proteins in *mviA*⁺ and *mviA::Km* strains. If MviA regulates *rpoS* transcriptionally, then an *mviA* insertion mutation will not change the level of full-length RpoS protein, since the *rpoS* promoter/operator region is replaced with the arabinose operon promoter. However, since *rpoS::Ap* (truncated RpoS) is still under the control of the *rpoS* promoters, *mviA* insertion mutations will alter the level of truncated RpoS. On the other hand, if MviA regulates RpoS posttranscriptionally, higher levels of both forms of RpoS will be observed in the *mviA::Km* mutant. This presumes that the MviA interaction site on RpoS is not missing in the truncated message or protein. Results from our experiment suggest that σ^S is regulated posttranscriptionally by MviA. The *mviA*⁺ extracts in Fig. 5A exhibited typical stationary-phase, posttranscriptional control of RpoS. The levels of both truncated and full-length RpoS were low in log phase (OD_{600} of 0.2) but elevated at early stationary phase (OD_{600} of 1.0). However, comparing *mviA*⁺ and *mviA::Km* cells revealed that the loss of MviA caused the overproduction of both forms in log phase, implicating posttranscriptional control of RpoS by MviA. We confirmed that MviA did not affect the transcription of *rpoS* by measuring the β -galactosidase expression of an *rpoS-lacZ* operon fusion under different growth conditions. Although we could demonstrate stationary-phase induction of *rpoS* in complex (LB) medium, the *mviA::Km* insertion had no effect on the expression of *rpoS* in complex or minimal medium (Table 2). On the basis of similar reports for *E. coli* (32), the lack of *rpoS* transcriptional control in nonlimiting minimal medium was not unexpected.

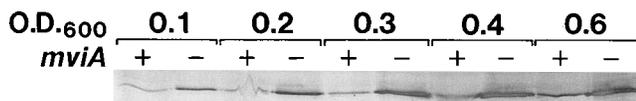


FIG. 3. *mviA* status affects σ^S levels. *mviA*⁺ (UK1) and *mviA::Km* (JF2892) cultures were grown in minimal EG medium, and samples were taken at OD_{600} s of 0.1, 0.2, 0.3, 0.4, and 0.6. The cells were harvested, and cell extracts were prepared via SDS boiling. Five-microgram samples of total cellular protein were loaded onto an SDS-4.5% stacking-11.5% separating polyacrylamide gel and processed for Western blot analysis with a monoclonal antibody to *E. coli* σ^S (43).

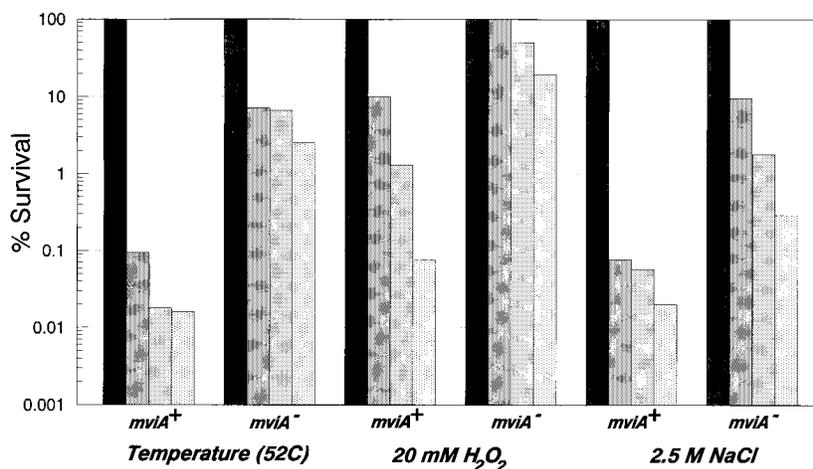


FIG. 4. Tolerance to heat, oxidative, and osmotic stresses in *mviA*⁺ and *mviA*::Km cells. Cells were collected at mid-exponential phase and tested as described in Materials and Methods. Time points for 52°C challenge were 0, 15, 30, and 45 min; time points for H₂O₂ challenge were 0, 5, 10, and 15 min; and time points for osmotic challenge (NaCl) were 0, 17, 28, and 45 h. The sequence of differently shaded bars (from darkest to lightest) corresponds to the respective time points for each challenge.

MviA controls the stability of σ^S . Defects in MviA could alter the translation of *rpoS* message or the proteolytic turnover of σ^S protein. The effect of *mviA* mutations on σ^S turnover was tested by growing *mviA*⁺ and *mviA* strains to mid-log phase, adding chloramphenicol to prevent further synthesis of σ^S , and then collecting samples for Western blot analysis at defined time intervals. The results presented in Fig. 5B indicated σ^S turnover rates of <3 min versus 30 min for *mviA*⁺ and *mviA* mutant strains, respectively. The data support a role for MviA in controlling the turnover of σ^S .

Acid shock posttranscriptionally increases σ^S levels. If MviA is involved in the acid shock-induced increase in σ^S , then

acid shock should have a posttranscriptional effect on *rpoS*. In addition, acid shock should not induce an increase in σ^S levels in an *mviA* mutant. JF3057, expressing both full-length σ^S regulated by the araBAD promoter and truncated σ^S controlled by its natural promoters, was used to examine whether acid shock posttranscriptionally regulated σ^S . The strain was grown to mid-log phase and acid shocked for 20 min. Acid shock increased the levels of both the full-length and truncated forms of σ^S , indicating that regulation was not transcriptional (Fig. 6, lanes 1 and 2). However, acid shock did not increase σ^S levels in an *mviA* mutant (Fig. 6, lanes 3 and 4). The results support a major role for the *mviA* signal transduction system in the acid shock induction of σ^S and σ^S -dependent ASPs.

The small-colony morphology of an *mviA* mutant requires σ^S -dependent proteins. Another interesting phenotype of *mviA* insertion mutants is a small-colony morphology (50). As shown in Fig. 7, an *mviA*⁺ cell (UK1 [Fig. 7A]) normally produces a large colony on LB agar; however, an *mviA*::Km mutant of UK1 (JF2892) yields an extremely small colony (Fig. 7B). This phenotype was evident in virulent strains of *S. typhimurium* such as UK1, SL1344, and 14028s. However, it is obvious from Fig. 7 that spontaneously arising large colonies appear during the growth of *mviA* insertion mutants. These large-colony revertants (*Scm*⁻) usually represent an extragenic mutation in one or more genes presumably regulated by MviA. The working model is that MviA regulates other genes that either directly or indirectly affect salmonella growth rate and possibly virulence. Several reports have correlated growth rate with salmonella virulence (1, 21, 35). We reasoned from the

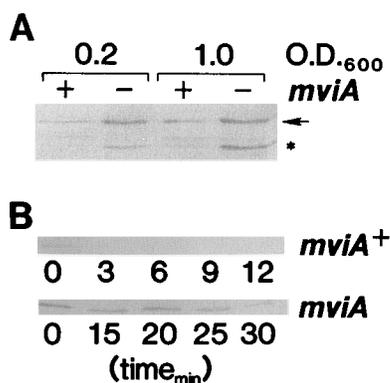


FIG. 5. MviA controls σ^S posttranscriptionally via protein stability. (A) *mviA*⁺ (JF3057) and *mviA*::Km (JF3058) strains containing an *rpoS*::Ap insertion and pUBAD, a plasmid expressing *rpoS* from the arabinose operon promoter, were grown in minimal medium. The cultures were induced for one doubling with 15 mM arabinose before samples were taken at OD₆₀₀s of 0.2 and 1.0. Cell extracts were prepared by the SDS boiling method. Five-microgram samples of total cellular protein were loaded onto an SDS-11.5% separating polyacrylamide gel and processed for Western blot analysis with a monoclonal antibody to *E. coli* σ^S . The arrow denotes the full-length RpoS protein driven by the arabinose promoter, and the asterisk marks the truncated RpoS under its natural promoters. (B) σ^S turnover. JF2690 (*rpoS*::Ap; upper row) and JF2891 (*rpoS*::Ap *mviA*::Km; lower row) were grown in EG medium (pH 7.7) to an OD₆₀₀ of 0.6, chloramphenicol was added to 100 μ g/ml, and aliquots were removed at timed intervals. Extracts were prepared and separated by SDS-PAGE. σ^S was detected by immunoblotting as described in Materials and Methods. The full-length σ^S was not used for this study because of a comigrating immunoreactive band. Turnover was more accurately depicted by the genetically truncated form of σ^S .

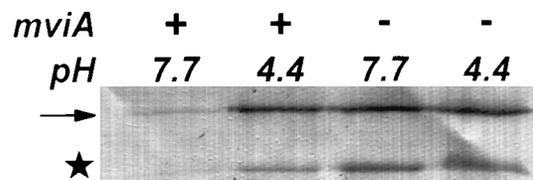


FIG. 6. Acid shock posttranscriptionally regulates σ^S levels via MviA. JF3057 (*mviA*⁺ *rpoS*::Ap/pUBAD [lanes 1 and 2]) and JF3058 (*mviA* *rpoS*::Ap/pUBAD [lanes 3 and 4]) were grown in EG medium (pH 7.7) to an OD₆₀₀ of 0.4. Extracts (5 μ g of protein) from unadapted (lanes 1 and 3) and acid shock-adapted (pH 4.4, 20 min [lanes 2 and 4]) cultures were separated on an SDS-11.5% PAGE gel and processed by immunoblotting to detect σ^S .

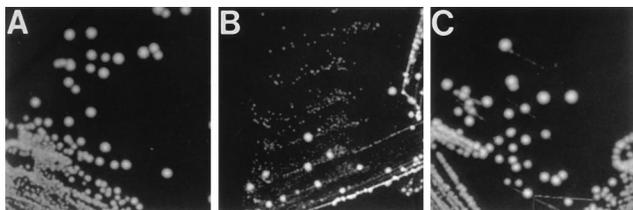


FIG. 7. Colony morphology of *mviA::Km* and *mviA::Km rpoS* mutants. UK1 (A), JF2892 (*mviA::Km*) (B), and JF2891 (*mviA::Km rpoS*) (C) mutants on an LB agar plate after 18 h of incubation at 37°C.

relationship among MviA, RpoS, and the σ^S -dependent ASPs that overexpression of these ASPs may be the cause of the *mviA::Km* small-colony phenotype. If true, then *rpoS* mutations should suppress the small-colony morphology of *mviA::Km* mutants because *rpoS* mutants will not make these proteins regardless of *mviA* status. Alternatively, MviA could regulate another set of proteins that do not require σ^S . If overexpressing these hypothetical proteins actually causes the small-colony phenotype, then *rpoS::Ap* mutations should have no effect on *mviA::Km* mutant colony size. As shown in Fig. 7C, *rpoS::Ap* did suppress the small-colony morphology of an *mviA::Km* mutation. The *mviA::Km rpoS::Ap* double mutant (JF2891) exhibited a normal colony morphology compared with the *mviA::Km* mutant (JF2892). This suggests that the small-colony morphology is due to the overproduction of σ^S and the subsequent overexpression of σ^S -dependent ASPs.

Effect of spontaneous suppressor mutations on acid tolerance of an *mviA::Km* mutant. Plating *mviA::Km* mutants on LB medium generally results in small colonies as noted above. However, spontaneous large-colony revertants are frequently seen (Fig. 7B) (50). We predicted that the *Scm*⁻ mutations occur in genes regulated by MviA either directly or indirectly through RpoS. We further predicted that some, but not all, of these mutations would also affect the ATR either by restoring a normal ATR on *mviA* insertion mutants or by making cells acid sensitive. Several *Scm*⁻ mutants were selected to study their effects on acid tolerance. As shown in Fig. 8, two of the strains tested did affect the ATR. One restored a normal ATR (JF2988), while one (JF2982) conferred a more acid-sensitive

TABLE 2. Effect of *mviA* on transcription of *rpoS*

Strain	Genotype	β -Galactosidase activity ^a			
		Minimal medium		Complex medium	
		Log phase	Stationary phase	Log phase	Stationary phase
JF2938	<i>mviA</i> ⁺ <i>rpoS::MudJ</i>	220	225	130	315
JF3042	<i>mviA::Cm rpoS::MudJ</i>	170	210	155	320

^a β -Galactosidase activity was measured in Miller units (41). Cells were grown in minimal EG or LB medium to an OD₆₀₀ of 0.2 (log phase) or 1.2 (stationary phase).

phenotype. Neither of these secondary mutations occurred within *rpoS* as determined by catalase production and the inability of a *Tn10* insertion near *rpoS*⁺ to repair the *Scm*⁻ phenotype. The *Scm*⁻ mutations were not in or near *mviA*, since replacing *mviA::Km* in an *mviA::Km Scm*⁻ mutant with *mviA::Cm* did not restore the small-colony phenotype. Western blot analysis did not detect any dramatic differences in the levels of σ^S in the *lcr* mutants compared with the parental *mviA* strain (data not shown). It appears that the *lcr* mutations affect growth by suppressing the effect of high σ^S levels. We do not, at present, know why the *lcr* mutation in JF2982 made acid tolerance *mviA* independent. The suppressor mutation could be in a gene whose product collaborates with σ^S . The lower activity level of that protein could be perceived by the cell as having less σ^S . A lower level of σ^S activity might lower uninduced acid tolerance, as seen in JF2982, but acid shock induction of the other 42 ASPs could increase tolerance even without further increases in σ^S . Alternatively, the suppressor mutation might have affected a protein that, when overexpressed in the *mviA* mutant, aids unadapted acid tolerance but becomes redundant once the other ASPs are induced. A loss of this protein appears to restore normal inducible acid tolerance. These possibilities are under investigation.

DISCUSSION

Acid encounters are frequent during the pathogenesis of *S. typhimurium*. In response, the organism has developed several adaptive strategies to counteract lethal acid stress. Some of

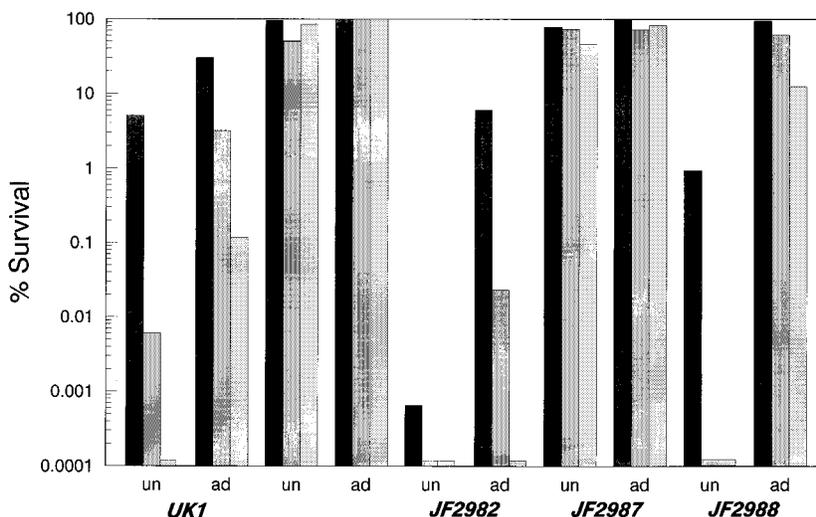


FIG. 8. ATR of large-colony revertant suppressors of *mviA::Km* mutations. Acid shock adaptation was performed at pH 4.4 for 1 h. Acid challenge was at pH 3.1 for 30, 60, and 90 min (darkest to lightest bars, respectively). un, unadapted; ad, adapted.

these strategies are σ^S dependent and others are σ^S independent. Acid tolerance systems that require σ^S can be induced by entry into stationary phase or by an acid shock of log-phase cells. In either circumstance, cross protection to a variety of unrelated stresses will result (33, 39). Consequently, acid shock, as it occurs in the host environment, may be an important trigger for the cell to resist the broad arsenal of antimicrobial stresses confronted during its travels through a host. Mechanisms designed to modulate levels of σ^S in response to various host environments could contribute to the pathogenesis of the organism by allowing rapid growth of the organism in body sites presenting minimal stress but programming slow growth in sites presenting potentially lethal stress.

The control of *rpoS* expression has been examined most extensively in *E. coli*. Induction of σ^S levels has been shown to occur in response to stationary phase (31, 32, 37, 40, 46) and increased osmolarity (32). Under both conditions, the control of cellular σ^S levels operates at the transcriptional and post-transcriptional levels but also involves changes in σ^S stability (32, 40, 51). We have shown in *S. typhimurium* that acid shock conditions cause an elevation in σ^S levels and an increased cross protection to a variety of environmental stress conditions (33).

Within the large *E. coli* σ^S regulon, differential regulation has been demonstrated for subsets of genes in response to anaerobiosis (4, 12, 14), osmolarity (24), and oxidative stress (3, 38). In addition, Crp, IHF, Lrp, and H-NS (5, 23, 29, 30, 44, 49, 55, 57) have been identified as modulating factors in the control of various σ^S -dependent genes (39). Our studies further indicate that differential regulation of σ^S -dependent genes occurs during acid shock and that the virulence gene *mviA*⁺ participates by posttranscriptionally regulating RpoS expression through increased σ^S stability. MviA may, in fact, modulate the activity of ClpX, a protease recently shown to cleave σ^S (47). We propose that within the host environment, acid induction constitutes a significant means for modulating σ^S levels during pathogenesis. MviA appears to be a key component in a putative signal transduction pathway linking acid and probably other pertinent stresses to the production of σ^S .

Recent evidence obtained in studies using *E. coli* has implicated the histone-like protein H-NS in the posttranscriptional control of *rpoS* (5, 57). Many of the phenotypes associated with the *E. coli* *hns* mutations are similar to what we see for *mviA* insertion mutations in *S. typhimurium*. These include increased expression of σ^S -dependent genes, reduced growth rate, the appearance of secondary-site suppressor mutations, and post-transcriptional control of σ^S . However, *hns* mutations appear to act differently in *S. typhimurium*. For example, they do not exhibit the small-colony morphology noted in *E. coli* (5a). One possible explanation for this apparent discrepancy is that an *E. coli* *mviA*⁺ gene homolog is closely linked to *hns*. The *E. coli* homolog of *mviA*⁺ is called *hnr* (GenBank accession number X66003) or ORF-37 and resides approximately 1 kb from *hns* (11). It seems that either the H-NS effects on σ^S production differ in the two organisms or perhaps there were second-site *hnr* (*mviA*) mutations present in the *E. coli* strains used. The strain used by Yamashino et al. (57), for example, had an *hns* deletion that could have included *hnr* (*mviA*). Even the *hns* mutants constructed by Barth et al. (5) were made by cotransducing *hns* mutations to a "clean" MC4100 strain. The close linkage of the two genes might have made separating them difficult.

In addition to their roles in acid tolerance, the identification of specific σ^S -dependent ASPs may lead to the discovery of proteins and genes important for stress cross protection and for slowing growth during stress. One question is prevalent

throughout the literature: what triggers the induction of RpoS? Is it the slowing of cell division due to the stress or does the induction of RpoS itself limit cell division? It seems from these studies that at least part of the function of σ^S is to slow cell division. Our working model is that MviA or its unknown signal transduction partner senses some consequence of low pH, and perhaps other stresses, and signals the release of RpoS from posttranscriptional control. Subsequent elevations in σ^S levels would cause increased transcription of genes whose products slow cell division and increase resistance to a variety of stresses. For example, the morphogene *bolA* is regulated by σ^S and mediates the reduction in cell size associated with stationary phase (30). Increased stress resistance could itself be a partial consequence of properly programmed growth arrest.

It may seem counterintuitive that *rpoS* mutants producing no σ^S and *mviA* insertion mutants producing excess σ^S are both avirulent. However, as noted above, the need for σ^S -dependent stress proteins will vary during pathogenesis. The decreased virulence of *mviA* insertion mutants, therefore, could be explained by inappropriate σ^S -dependent gene expression in sites requiring rapid growth.

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ADDENDUM IN PROOF

Since completion of this work, the *mviA* homolog of *E. coli* (*hnr*) has also been shown to affect proteolytic turnover of σ^S . The *hnr* gene has been renamed *rssB* (A. Muffler, D. Fischer, S. Altuvia, G. Storz, and R. Hengge-Aronis, EMBO J., in press).

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