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Acid stress activation of the σ^E stress response in *Salmonella enterica* serovar Typhimurium

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Summary

The alternative sigma factor σ^E is activated by unfolded outer membrane proteins (OMPs) and plays an essential role in *Salmonella* pathogenesis. The canonical pathway of σ^E activation in response to envelope stress involves sequential proteolysis of the anti-sigma factor RseA by the PDZ proteases DegS and RseP. Here we show that σ^E in *Salmonella enterica* sv. Typhimurium can also be activated by acid stress. A σ^E -deficient mutant exhibits increased susceptibility to acid pH and reduced survival in an acidified phagosomal vacuole. Acid activation of σ^E -dependent gene expression is independent of the unfolded OMP signal or the DegS protease but requires processing of RseA by RseP. The RseP PDZ domain is indispensable for acid induction, suggesting that acid stress may disrupt an inhibitory interaction between RseA and the RseP PDZ domain to allow RseA proteolysis in the absence of antecedent action of DegS. These observations demonstrate a novel environmental stimulus and activation pathway for the σ^E regulon that appear to be critically important during *Salmonella*–host cell interactions.

Introduction

Bacteria adapt to their environment by sensing conditions and responding through the co-ordinated expression of gene networks. The alternative sigma factor σ^E senses perturbations in the outer membrane and periplasm and activates a subset of genes in response to heat stress (Rouviere *et al.*, 1995; Missiakas and Raina, 1998). σ^E is required for *Escherichia coli*

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viability at all temperatures (De Las Penas *et al.*, 1997), implying that the σ^E regulon plays important roles in addition to thermal adaptation.

Under non-stress conditions, σ^E is sequestered by the inner membrane-spanning anti-sigma factor RseA (Missiakas *et al.*, 1997). The cytoplasmic domain of RseA interacts with the RNA Polymerase (RNAP) binding domain of σ^E , thereby inhibiting its assembly with core RNAP. Heat stress leads to the periplasmic accumulation of unfolded outer membrane proteins (OMPs) such as OmpC, which trigger the sequential proteolytic cleavage of RseA by the proteases DegS and RseP (YaeL) (Alba and Gross, 2004). DegS is an inner-membrane-anchored serine protease of the HtrA family (Clausen *et al.*, 2002) with proteolytic and PDZ domains positioned in the periplasm. RseP is a zinc metalloprotease possessing four transmembrane segments, a periplasmic PDZ domain and an inner membrane/cytoplasmic border proteolytic domain (Kanehara *et al.*, 2001; 2002; Koide *et al.*, 2007). The PDZ domains have been shown to bind in a sequence-specific manner to the C-termini of target proteins and inhibit protease activity (Kanehara *et al.*, 2003; Koide *et al.*, 2007).

Specifically, recognition of the C-termini of unfolded OMPs by the DegS PDZ domain allows DegS to cleave the periplasmic C-terminus (site-1) of RseA (Walsh *et al.*, 2003; Hasselblatt *et al.*, 2007). This converts RseA into a substrate for RseP, which cleaves the transmembrane segment (site-2) of RseA (Alba *et al.*, 2002; Kanehara *et al.*, 2002; Akiyama *et al.*, 2004). The glutamine (Gln)-rich region of RseA has been shown to interact with the RseP PDZ domain to inhibit RseP proteolytic activity. DegS-mediated processing removes the Gln-rich regions to relieve inhibition of RseP protease activity (Kanehara *et al.*, 2003). In the absence of a functional RseP PDZ domain, RseA proteolysis by RseP becomes DegS-independent (Ades *et al.*, 2003; Kanehara *et al.*, 2003; Bohn *et al.*, 2004). Following cleavage by RseP, the RseA/ σ^E complex is released from the membrane, and the cytoplasmic portion of RseA is rapidly degraded by proteases such as ClpXP (Alba *et al.*, 2002; Kanehara *et al.*, 2002; Chaba *et al.*, 2007).

Once released from the cytoplasmic membrane, free σ^E binds core RNAP and directs the resulting holoenzyme to a subset of promoters resulting in the expression of target genes, including *rpoE* itself, *rpoH*, *fkpA*, *surA* and *rybB* (Missiakas *et al.*, 1996; Dartigalongue *et al.*, 2001; Thompson *et al.*, 2007). The *rybB* gene encodes a small RNA that accelerates the global decay of OMP mRNAs (Papenfert *et al.*, 2006). RybB also appears to downregulate expression of *rpoE* (Papenfert *et al.*, 2006; Thompson *et al.*, 2007), probably by limiting OMP-dependent upstream signals that lead to σ^E activation. Additional regulation is provided by the periplasmic protein RseB, which increases RseA affinity for σ^E (Collinet *et al.*, 2000) and retards its cleavage by DegS (Cezairliyan and Sauer, 2007), and by the inner membrane protein RseC, which may promote σ^E activation (Missiakas *et al.*, 1997; Alba and Gross, 2004). It has been suggested that RseB may limit access of RseP to RseA until RseA is first cleaved by DegS (Grigorova *et al.*, 2004; Kim *et al.*, 2007).

The σ^E pathways of *Salmonella enterica* sv. Typhimurium (henceforth referred to as *Salmonella*) and *E. coli* are highly conserved with regard to organization and mechanism of activation (Rhodius *et al.*, 2006). However, σ^E is not essential in *Salmonella* (Humphreys *et*

al., 1999; Testerman *et al.*, 2002), allowing the construction of an *rpoE* null mutation and analysis of the contribution of σ^E to bacterial virulence. *S. enterica* is a globally important cause of food- and water-borne illness with clinical manifestations ranging from gastroenteritis to bacteremia and systemic infection (Ohl and Miller, 2001). Although it is not surprising that σ^E plays an essential role in *Salmonella* pathogenesis (Humphreys *et al.*, 1999; Testerman *et al.*, 2002; Onufryk *et al.*, 2005), an *rpoE* mutant strain is unexpectedly less virulent than a *degS* mutant (Rowley *et al.*, 2005), suggesting that σ^E might be activated by an alternative mechanism during the interaction of *Salmonella* with its host.

During passage through the gastrointestinal tract and replication in host phagocytic cells, *Salmonella* must withstand a number of stresses including nutrient deprivation, membrane damage, reactive oxygen and nitrogen species, and acid stress (Foster and Spector, 1995). *Salmonella* is exposed to acid stress during passage through the stomach and within the *Salmonella*-containing vacuole (SCV) of activated macrophages, which acidifies to pH 4–5 (Rathman *et al.*, 1996). Acid pH also represents a signal that triggers the expression of *Salmonella* virulence genes required for intracellular survival (Alpuche Aranda *et al.*, 1992; Rathman *et al.*, 1996) and the trans-location of virulence effectors into the cytosol of host macrophages (Beuzon *et al.*, 1999). The involvement of σ^E in the response of *Salmonella* to acid stress and the mechanism of σ^E activation was therefore investigated. We report here that acid pH activates σ^E in *Salmonella* via a non-canonical mechanism independent of DegS-mediated proteolysis of RseA.

Results

σ^E promotes *Salmonella* survival within the acidified vacuoles of RAW264.7 macrophages

To investigate if vacuolar acidification stimulates a σ^E -dependent *Salmonella* adaptive response in macrophages, bacterial survival was monitored following uptake by RAW264.7 murine macrophages with or without the addition of NH_4Cl to inhibit vacuolar acidification. Wild-type bacteria (WT) exhibited approximately 10-fold reduced survival in non-acidified vacuoles (Fig. 1), consistent with previous observations, demonstrating that acidification enhances the survival of intracellular *Salmonella* (Rathman *et al.*, 1996). A previous study showed that σ^E is required for the intracellular survival of *Salmonella* in macrophages (Humphreys *et al.*, 1999), so we examined whether σ^E participates in acidification-dependent *Salmonella* survival in the SCV. An isogenic *rpoE* mutant strain (Testerman *et al.*, 2002) exhibited reduced survival in the absence of NH_4Cl (Fig. 1). σ^E enhanced *Salmonella* survival only within acidified vacuoles, but interestingly, not in NH_4Cl -treated cells. These results implicate the σ^E regulon in the acid pH-triggered adaptation of *Salmonella* to the intracellular environment.

σ^E is necessary for *Salmonella* tolerance of acid pH

Reduced survival of *rpoE* mutant *Salmonella* in the acidified SCV might reflect increased susceptibility of σ^E -deficient bacteria to acid stress *per se*. We therefore measured the replication of *rpoE* mutant *Salmonella* in acidified medium (pH 5.0) and found substantially delayed growth compared with WT (Fig. 2A). In addition, the survival of an *rpoE* mutant was dramatically reduced under more extreme acid stress conditions (pH 3.0), and survival

was restored to WT levels when *rpoE* was provided on a complementing plasmid (Testerman *et al.*, 2002) (Fig. 2B). During the acid tolerance response (ATR) (Foster and Spector, 1995), *Salmonella* exposure to relatively mild levels of acid stress (pH > 4) induces the expression of genes that enable the bacteria to tolerate more extreme acidic conditions (Foster, 1999). We therefore examined whether σ^E participates in the ATR. An *rpoE* mutation dramatically reduced *Salmonella* survival at pH 3.0 and eliminated adaptive tolerance (Fig. 2C), demonstrating that the ATR requires σ^E . Collectively, these results indicate that the σ^E regulon makes an important contribution to the ability of *Salmonella* to adapt to and withstand acidic conditions.

Acid shock increases σ^E expression

The ATR requires *de novo* protein synthesis (Foster and Hall, 1991), so we hypothesized that σ^E itself might be acid-inducible. The expression of the *rpoE* gene was monitored in log- or stationary-phase cells submitted to acid stress. Quantitative real-time RT-PCR (qRT-PCR) revealed that *rpoE* mRNA levels begin to rise within 10 min at pH 4.5 and eventually increase 50- and 58-fold over basal levels after 60 min acid stress in log- and stationary-phase cells respectively (Fig. 3A).

Transcription of *rpoE* is initiated from three promoters. Two of these, P1 and P2, are σ^{70} -dependent, while the P3 promoter is σ^E -dependent (Miticka *et al.*, 2003). P1_{*rpoE*}-*lacZ* and P3_{*rpoE*}-*lacZ* reporter fusions were used to determine the promoter involved in acid-induction (Testerman *et al.*, 2002). We observed that the P3_{*rpoE*}-*lacZ* fusion was induced by acid stress whereas the activity of P1_{*rpoE*}-*lacZ* was unchanged (Fig. 3B). These results indicate that increased *rpoE* transcription during acid stress results from activation of σ^E itself. Abrogation of acid-induced *rpoE* transcription in an *rpoE* mutant background supports this interpretation (Fig. 3A).

It should be noted that the fold-induction using *lacZ* transcriptional fusions was substantially lower than what was observed by qRT-PCR. This can be explained by high background expression from the plasmid-borne reporter.

Acid induction of σ^E is OMP- and DegS-independent in contrast to heat stress induction

The rate-limiting step in σ^E activation by heat stress is the sensing of unfolded OMPs by the DegS protease (Chaba *et al.*, 2007). To analyse the mechanism of σ^E activation by acid stress, relative levels of σ^E target gene (*rpoH*, *fkpA*, *surA*, *rpoE*) expression were monitored by qRT-PCR during acid stress and compared with pH 7.0. The *rpoH* gene encodes σ^{32} , which is involved in the cytoplasmic heat stress response. The FkpA and SurA proteins are peptidyl-prolyl isomerases, which function as protein folding catalysts in the periplasm. All of these σ^E -dependent loci were induced 20- to 80-fold at pH 4.5 in WT strain compared with the reference gene *gmk* (data not shown).

Papenfort *et al.* (2006) demonstrated that overexpression of the σ^E -regulated sRNA RybB promotes the decay of major OMP mRNAs by direct interaction, resulting in the rapid cessation of OMP synthesis. A plasmid (pBAD-RybB) expressing *rybB* under control of the arabinose-inducible P_{*araBAD*} promoter and pBAD vector control (Guzman *et al.*, 1995)

producing a short nonsense RNA (Papenfort *et al.*, 2006) were used to compare the effects of *rybB* overexpression on heat and acid stress induction of σ^E (Fig. 4). Thus, the effect of OMP presence (no induction of *rybB* expression) or absence (overproduction of *rybB*) on the σ^E activation pathway was studied. The σ^E -regulated genes *rpoH*, *fkpA*, *surA* and *rpoE* were induced by heat stress, and RybB overproduction substantially reduced this induction, as anticipated (Fig. 4A, WT pBAD versus WT pBAD-RybB). Inactivation of *rpoE* or deletion of the DegS PDZ domain eliminated heat stress induction of σ^E (Fig. 4A, WT pBAD versus *rpoE* pBAD versus *degS* PDZ pBAD). These data confirm the involvement of OMPs, DegS and σ^E itself in the canonical pathway of σ^E activation during heat stress.

In contrast, *rybB* overexpression had only a modest 1.3- to 2-fold effect on the expression of σ^E -regulated genes in response to acid stress (Fig. 4B, WT pBAD versus WT pBAD-RybB). Deletion of the DegS PDZ domain similarly had little or no effect on σ^E -regulated gene expression (Fig. 4B, WT pBAD versus *degS* PDZ pBAD). A *degS* null mutation produced effects on both heat and acid stress induction identical to those observed in the *degS* PDZ mutant strain (Fig. S1), with the exception that the *degS*-PDZ mutant exhibited a global increase in the expression of σ^E -regulated genes that may reflect loss of the inhibitory effect of the PDZ domain on DegS protease activity (Walsh *et al.*, 2003; Sohn *et al.*, 2007). qRT-PCR was used to confirm a reduction of *ompC* mRNA following *rybB* overexpression (data not shown). These observations indicate that acid stress induction of the σ^E regulon, unlike heat stress induction, does not require OMPs or the DegS protease.

Overproduction of OMPs leads to increased transcription at σ^E -dependent promoters (Mecbas *et al.*, 1993) by activating the DegS protease and triggering the degradation of RseA. We reproduced this condition by placing the *ompC* gene under control of the *P_{araBAD}* promoter (Bang *et al.*, 2005). OmpC overexpression at neutral pH induced the expression of *rpoE*, *rpoH*, *surA* and *fkpA* by 84-, 25-, 19- and 10-fold respectively (Fig. 4C, WT pBAD versus WT pBAD-*ompC*). OmpC-induction of the σ^E regulon was completely dependent on σ^E (Fig. 4C, *rpoE* pBAD versus *rpoE* pBAD-*ompC*) and the DegS PDZ domain (Fig. 4C). However, OmpC overproduction during acid stress only modestly augmented the response of σ^E (Fig. 4D, WT pBAD versus WT pBAD-*ompC*), and did not enhance σ^E target gene expression in *rpoE* and *degS* mutants (Fig. 4D), suggesting that σ^E activation by unfolded OMPs and acid stress occurs by distinct mechanisms.

It should be noted that some acid induction of σ^E target genes was detected in the *rpoE* mutant strain (Fig. 4B and D, *rpoE* pBAD and *rpoE* pBAD-RybB/*ompC*), suggesting that some *rpoE*-dependent genes can be partially induced by acid stress in a σ^E -independent fashion.

In summary, the induction of σ^E during acid stress cannot be attributed to DegS-sensing of unfolded OMPs as occurs during heat stress or OmpC overproduction, because acid induction is largely indifferent to changes in OMP expression or the presence of the protease DegS.

Acid induction of σ^E requires RseP-dependent proteolysis of the anti- σ^E factor RseA

Degradation of RseA is the key control point in σ^E activation. In the canonical pathway, initial cleavage by DegS is required before subsequent proteolysis by RseP can occur (Grigorova *et al.*, 2004). However, our discovery that DegS is dispensable for σ^E activation during acid stress suggested that RseP might be able to cleave RseA in the absence of DegS under acidic conditions. To determine the contribution of regulatory proteins of the σ^E pathway, we measured σ^E target gene expression during acid stress by qRT-PCR. These experiments demonstrated that acid induction is abolished in an *rseA* or *rseP* mutant (Fig. 5). Elimination of RseA results in derepression and constitutive activation of σ^E , whereas elimination of RseP abrogates acid induction without affecting baseline activation (Fig. S2). These results eliminate the possibility that additional regulators can activate σ^E in response to acid stress and show that acid induction requires RseA and RseP. Possible involvement of RseB or RseC was also investigated. Interaction of RseB with the C-terminal domain of RseA modulates the affinity of the anti-sigma for σ^E (Collinet *et al.*, 2000). RseB and DegS independently repress RseP protease activity, and the elimination of RseB and DegS in *E. coli* promotes the cleavage of RseA by RseP and increases σ^E activity (Grigorova *et al.*, 2004), suggesting that acid stress might activate σ^E by disrupting RseB–RseA interactions. The elimination of RseB had a modest effect on baseline σ^E activity (Fig. S2). However, we observed that acid induction of σ^E -dependent gene expression is preserved in *Salmonella* mutant strains lacking RseB or RseC, as well as in an *rseB degS* double mutant (Fig. 5A).

To further investigate the role of RseP in the acid induction of σ^E , RseA proteolysis was monitored by immunoblot following heat or acid induction in WT and *degS* or *rseP* mutant backgrounds. An RseA derivative containing a N-terminal haemagglutinin epitope (HA-RseA) was constructed as described (Kanehara *et al.*, 2003) and expressed from a low-copy plasmid. A C-terminal truncated form of HA-RseA [HA-RseA(P)] resulting from site-1 cleavage by DegS has been shown to accumulate in an *E. coli rseP* mutant strain (Kanehara *et al.*, 2002), with lower levels of HA-RseA(P) detectable in an *rseP*- PDZ mutant (Kanehara *et al.*, 2003). Under non-stress conditions in *Salmonella*, intact RseA accumulated in WT, *degS* and *degS* PDZ strains (Fig. 6A, left panel). Both HA-RseA and HA-RseA(P) species were detected in an *rseP* mutant, reflecting partial degradation of RseA by DegS from basal activity of this protease. In an *rseP* PDZ strain, nearly all RseA was degraded due to disinhibition of RseP protease activity (Alba *et al.*, 2002; Kanehara *et al.*, 2003).

During heat stress, RseA was rapidly degraded in WT cells corresponding with activation of the σ^E regulon (Fig. 6A, centre panel). This degradation requires DegS, and RseA therefore persisted in heat-stressed *degS* and *degS* PDZ mutant cells. Loss of the inhibitory PDZ domain enhances RseA processing by DegS (Walsh *et al.*, 2003; Cezairliyan and Sauer, 2007; Hasselblatt *et al.*, 2007) and might have been predicted to reduce RseA levels. However, a *degS* PDZ allele only increased basal σ^E activity by 1.5- to 2-fold (Walsh *et al.*, 2003), suggesting the presence of other constraints on DegS –PDZ in intact cells. In an *rseP* mutant, RseA was degraded by OMP-activated DegS but could not be further processed so that the HA-RseA(P) product accumulated, whereas complete degradation of RseA was observed in an *rseP* PDZ mutant strain. These results confirm the σ^E activation model in

which DegS is activated by unfolded OMPs accumulating during heat shock and cleaves RseA to allow a second cleavage by RseP.

During acid stress, HA-RseA levels were reduced in WT, *degS* and *degS* PDZ strains (Fig. 6A, right panel), indicating that RseA degradation under these conditions is primarily DegS-independent. Basal levels of DegS-mediated cleavage were revealed by the presence of HA-RseA(P) in an *rseP* mutant strain, as observed under non-stress conditions. Again, an *rseP* PDZ mutant showed unregulated protease activity with no detectable HA-RseA. These results corroborate the qRT-PCR data, demonstrating that acid stress induction of σ^E differs from heat stress induction in its lack of a requirement for the DegS protease.

As RseP is important for σ^E acid induction, and the PDZ domain regulates its protease activity, we hypothesized that this domain might play a role in the acid pH sensing. Measurement of σ^E -dependent gene expression by qRT-PCR revealed that the RseP PDZ domain is indispensable for the σ^E acid induction (Fig. 5B). The absence of acid induction is attributable to constitutive proteolysis of RseA by RseP PDZ protein; σ^E -dependent gene transcription is elevated in the *rseP* PDZ mutant at both neutral and acid pH (Fig. S2). The putative inhibitory interaction between the RseA periplasmic domain and the RseP PDZ domain may be destabilized at acid pH, to allow the activation of RseP with subsequent RseA cleavage and release of σ^E .

A *Salmonella* mutant strain with inactivation of both *degS* and *rseP* was constructed to exclude the presence of a novel site-1 protease that might be activated during acid pH stress. Western blot analysis of HA-RseA was repeated under conditions identical to those used in Fig. 6A, and demonstrated an absence of RseA cleavage in the double mutant (Fig. 6B). Thus, DegS-independent RseP-dependent proteolysis of RseA is induced by acid stress and does not appear to involve a novel site-1 protease.

Involvement of DegS and RseP in the survival within the acidified vacuoles of RAW264.7 macrophages

To validate our results concerning the role of the proteases DegS and RseP in σ^E regulon acid-induction and corroborate these *in vitro* observations with the acidification-dependent *Salmonella* survival in SCVs, we used the *Salmonella degS* and *rseP* mutants to infect macrophages treated or untreated with NH_4Cl . As shown in Fig. 7, in the absence of NH_4Cl and 6 h after infection, survival of *degS* and *rseP* mutants was decreased 10- and 100-fold compared with WT cells respectively. Thus, both proteases appear important for *Salmonella* survival in SCV. However, the *degS* mutant was more sensitive to the presence of NH_4Cl , whereas the *rseP* strain displayed the same behaviour as an *rpoE* mutant. Thus, these results confirm that σ^E , RseP and DegS are important for *Salmonella* pathogenesis, but only σ^E and RseP contribute to the acid stress response.

Discussion

Acid pH is an important environmental stress encountered by *Salmonella* in its interaction with the host. The SCV of infected macrophages acidify to pH 4–5 within an hour of bacterial uptake (Rathman *et al.*, 1996). Although previous studies have yielded conflicting

results regarding the effect of acidification on intracellular *Salmonella* replication (Rathman *et al.*, 1996; Steele-Mortimer *et al.*, 2000), our observations (Fig. 1) support the report of Rathman *et al.* (1996) that vacuolar acidification promotes survival and replication in RAW264.7 macrophages. This is consistent with the stimulation of *Salmonella* virulence gene expression (Alpuche Aranda *et al.*, 1992; Rathman *et al.*, 1996) and effector protein translocation (Beuzon *et al.*, 1999) by acid pH. Study of the survival of *degS* or *rseP* mutants in SCV revealed that both proteases are important for the basal survival of *Salmonella* in macrophages. However, neutralization of macrophage vacuolar pH with NH₄Cl revealed that survival of *Salmonella* within the acidified phagosome requires RseP but not DegS (Fig. 7); this requirement for RseP suggests that σ^E is involved in the acid pH-triggered adaptation of intracellular *Salmonella*.

The σ^E sigma factor is required for *Salmonella* virulence in mice (Humphreys *et al.*, 1999; Testerman *et al.*, 2002) and for resistance to two stress conditions imposed by host innate immunity, oxidative stress (Testerman *et al.*, 2002) and anti-microbial peptides (Onufryk *et al.*, 2005). We now further show that σ^E promotes *Salmonella* replication within the acidified SCV of macrophages (Figs 1 and 7), facilitates growth at pH 5.0 *in vitro*, and is essential for the ATR (Foster, 1999) (Fig. 2). These observations demonstrate for the first time that the σ^E regulon is involved in the maintenance of pH homeostasis.

In conjunction with the Cpx system, σ^E plays a critical role in activating the expression of genes encoding membrane proteins, enzymes involved in phospholipid, lipopolysaccharide and outer membrane biosynthesis, and primary metabolic and signal transduction pathways (Dartigalongue *et al.*, 2001) that help to maintain cellular integrity under stress conditions. Studies in *E. coli* have demonstrated that the σ^E regulon can be activated by the presence of unfolded OMPs in the periplasm, which are recognized by the DegS PDZ domain and lead to sequential regulated proteolysis of the RseA anti- σ (Alba *et al.*, 2002; Kanehara *et al.*, 2002; Walsh *et al.*, 2003; Chaba *et al.*, 2007). The present study demonstrates that σ^E can also be activated by acid stress (Fig. 3). However, the well-characterized mechanism of DegS-dependent σ^E activation in response to heat shock and unfolded OMPs does not appear to be responsible for activation at acid pH. In contrast to the heat stress induction of *rpoE* and the σ^E -dependent genes *rpoH*, *fkpA* and *surA*, acid stress induction of the σ^E regulon is not abolished by *rybB* over-expression (i.e. is not OMP-dependent) and is not dependent on the PDZ domain of DegS (Fig. 4). The cleavage of RseA by DegS is normally rate-limiting for σ^E activation (Chaba *et al.*, 2007), so that RseA degradation and the release of free σ^E during heat stress are determined by the OMP signal. Activation by acid stress is independent of DegS (or RseB and RseC) but remains dependent on RseP and RseA (Fig. 5), and RseP but not DegS is necessary for RseA degradation under this condition (Fig. 6). Moreover, RseP appears sufficient to trigger the σ^E -mediated response because qRT-PCR assays demonstrate acid pH-induction of σ^E target genes even in a *degS* mutant (Figs 4 and 5, Fig. S1). Thus, σ^E activation by acid stress does not even require basal levels of RseA processing by DegS (Fig. 6) that may result from low steady-state levels of unfolded OMPs in the periplasm (Sohn *et al.*, 2007). No RseA proteolysis was observed in a *degS rseP* double mutant, thus excluding the possibility that an acid-inducible protease is responsible for site-1 processing in the absence of DegS.

The precise molecular mechanism by which acid stress is sensed in the σ^E activation cascade awaits further investigation. An *rseP* PDZ mutation allows RseP-mediated proteolysis of RseA to take place in a DegS-independent fashion (Ades *et al.*, 2003; Kanehara *et al.*, 2003; Bohn *et al.*, 2004). We have observed that an *rseP*-PDZ mutation abolishes acid induction of σ^E -dependent gene expression (Fig. 5B), which suggests that the putative interaction between RseA and the RseP PDZ domain might be sensitive to changes in pH. Reversible protonation of His ($pK_a \approx 6$) serves as a pH sensor in some regulatory proteins. However, we replaced the single His residue (His188) in the RseA periplasmic domain with Ala and observed no effect on acid induction of σ^E -dependent target gene expression (data not shown). Alternatively, interaction between the two Gln-rich regions of RseA with the RseP PDZ domain might be sensitive to changes in pH and responsible of the RseP protease activation. Indeed, removal of the Gln-rich regions by DegS is responsible for relieving inhibition of RseP by its PDZ domain in response to unfolded OMPs (Kanehara *et al.*, 2003; Akiyama *et al.*, 2004). Further studies will be required to establish the mechanism of RseP activation in response to acid stress.

In conclusion, the expression of σ^E -dependent genes can be triggered by heat stress sensed by the PDZ domain of the DegS protease, or by acid stress, possibly sensed by the PDZ domain of the RseP protease (Fig. 8). Both heat stress and acid stress are important stress conditions encountered by bacteria in the environment or during interactions with mammalian hosts. The complex regulation of σ^E activation by multiple proteases permits the integration of diverse environmental signals to trigger a common stress response, an emergent theme in gene regulation by alternative sigma factors (Fang, 2005).

Experimental procedures

Construction of *Salmonella* mutants

In this study, *Salmonella* 14028s (ATCC) was used as WT. Mutations were constructed by the λ -Red recombinase method (Datsenko and Wanner, 2000). DNA primers used for *rseB*, *rseC*, *degS* or *rseP* mutant constructions are listed in Table S1, and strains are listed in Table 1. After construction, all mutations were transduced into WT using bacteriophage P22HT105/int and non-lysogenic colonies selected by sensitivity to lytic P22 H5 (Maloy, 1990). The *degS* PDZ and *rseP* PDZ mutants were constructed as described (Karlinsky, 2007): PCR amplification of the *tetRA* element was performed using primer pairs CM1/CM2 for *degS* and CM7/CM8 for *rseP* (Table S1), and allelic replacement of *tetRA* was achieved with the double-stranded oligomers CM3 and CM43 respectively. For construction of N-terminal HA-tagged RseA, an HA duplex was created with primers HA1/HA2 and ligated into *NcoI*–*SmaI*-digested pBAD24 (Guzman *et al.*, 1995) to produce plasmid pBAD24-HA. The *rseA* gene was amplified with primers HA3/HA4 and cloned into pBAD24-HA. For constitutive expression, an HA-*rseA* DNA fragment was amplified from pBAD24-HA-*rseA* with the primers HA5/HA6 and cloned into pACYC177 (Chang and Cohen, 1978).

Culture conditions and physiological assays

Bacterial cells were grown in Luria–Bertani (LB) or E minimal medium (Vogel and Bonner, 1956) containing 0.4% glucose at 30°C, 37°C or 42°C with agitation, or on solid LB

medium containing 15 mg ml⁻¹ agar. As necessary, media were supplemented with penicillin (250 µg ml⁻¹) or chloramphenicol (40 µg ml⁻¹). Acidic LB medium was supplemented with 100 mM MES [2-(N-morpholino)ethanesulphonic acid] buffer for pH < 5. The pH of E medium was adjusted with HCl. Bacterial growth rates were measured by determining the optical density at 600 nm (OD₆₀₀) at 37°C with agitation on a BioScreen C Microbiology Microplate reader (Growth Curves USA). For acid pH survival assays, cells were grown in E medium 0.4% glucose at 37°C to OD₆₀₀ ~1.5, and incubated at 37°C in fresh medium adjusted to pH 7.0 or pH 3.0. To measure the ATR, bacteria were pre-adapted in mildly acidic medium (pH 5.0) for 2 h before challenge at pH 3.0. For each time point colonies were enumerated on LB agar plates. Per cent survival represents the number of viable cells after exposure to acid stress as a proportion of the number of cells prior to challenge. To measure β-galactosidase activity of P1_{rpoE}-lacZ and P3_{rpoE}-lacZ (Testerman *et al.*, 2002), bacterial cells collected from overnight cultures were divided and re-suspended in neutral (pH 7.0) or acidic (pH 4.5) E medium. At timed intervals, samples were collected for β-galactosidase assays (Maloy, 1990).

Macrophage killing assay

RAW264.7 macrophages were infected with mouse serum-opsonized *Salmonella* at a multiplicity of infection of 10:1 and allowed to internalize bacteria for 15 min. After phagocytosis, cells were washed with PBS and incubated in RPMI1640 medium containing 6 mg ml⁻¹ gentamicin. To neutralize vacuolar pH, 20 mM ammonium chloride (NH₄Cl) was added. At timed intervals, infected macrophages were lysed with 1% Triton X-100 and surviving bacteria enumerated on LB agar plates. Results were expressed as per cent survival.

Real-time reverse transcription PCR (qRT-PCR)

Total RNA was isolated using an RNeasy Mini kit (Qiagen) according to manufacturer's instructions. For acid stress, cells were grown in E medium until OD₆₀₀ ~0.4 and incubated at 37°C for 1 h in E medium at pH 7.0 or pH 4.5. Heat stress was administered in E medium at 50°C. Each RNA extraction was conducted on three independent biological samples and qRT-PCR assays performed on cDNA synthesized from those RNA. The housekeeping gene *gmk* (guanylate mono-phosphate kinase) was used as reference after checking that *gmk* expression does not change under conditions of heat or acid stress. Expression of the σ^E-dependent *rpoE*, *rpoH*, *fkpA* and *surA* genes was quantitatively assessed by qRT-PCR in a Rotor-Gene 3000 (Corbett Research), with *gmk* from the same sample as reference. Forward (F) and reverse (R) primers are listed in Table S1. For the *rpoE* insertion mutant, primers used to quantify the *rpoE* transcript were designed upstream the mutation (Bang *et al.*, 2005). For each reaction, 8 µl of RNA sample (50 ng per reaction) were mixed with 1.8 µl of primer sets (0.9 µM final) and 10 µl of SYBR Green mix (Qiagen) containing 0.2 µl of reverse transcriptase. Each sample was assayed in triplicate for each run. Control RNA from WT cells was used to construct a standard curve. Reaction conditions were: 20 min at 50°C, 15 min at 95°C and 40 cycles at 94°C for 15 s, 52°C for 25 s, 72°C for 20 s.

Immunoblotting

Culture samples (1 ml) were harvested and re-suspended in 200 ml sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 25% glycerol, 0.01% Bromophenol Blue, 350 mM DTT). After electrophoresis and protein transfer onto a PVDF membrane (Millipore), blots were blocked in 5% non-fat dry milk (NFT) PBST (Phosphate-buffered saline, 0.05% Tween 20). Primary and secondary incubations used anti-HA mouse antibody (Sigma) and goat peroxidase-conjugated anti-mouse HRP (Bio-Rad), respectively, in 5% NFT/PBST. Blots were developed with the ECL Plus Western Blotting Detection System (Amersham).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
 σ^E -dependent survival of *Salmonella* in acidified macrophage vacuoles. RAW264.7 macrophages, treated (filled symbols) or untreated (open symbols) with NH_4Cl , were infected with complement-opsonized WT (diamonds) or *rpoE* mutant (squares) *Salmonella*.

Fig. 2.
 σ^E -dependent acid resistance of *Salmonella*.

A. Growth of WT (triangles) and *rpoE* mutant (squares) *Salmonella* was monitored at pH 7.0 (open symbols) or pH 5.0 (filled symbols).

B. Survival of stationary-phase WT (diamonds), *rpoE* mutant (squares) or *rpoE* mutant complemented with pBR3-*rpoE* (triangles) was determined following a 2 h acid challenge (pH 3.0).

C. The ATR was compared by adapting WT (grey bars) or *rpoE* mutant (white bars) at pH 5.0 for 2 h before challenge at pH 3.0.

Fig. 3.

Acid induction of σ^E expression.

A. Levels of *rpoE* mRNA were measured by qRT-PCR in log-phase ($OD_{600} \sim 0.4$) and stationary-phase (overnight) cells of the WT (grey bars) and *rpoE* (white bars) strains, after 10, 30 and 60 min of an acid stress (pH 4.5) compared with pH 7.0 and normalized to reference *gmk* mRNA.

B. β -Galactosidase expression was measured in stationary-phase cells harbouring $P3_{rpoE}$ - $lacZ$ (grey bars) or $P1_{rpoE}$ - $lacZ$ (white bars) fusions after re-suspension in pH 7.0 or pH 4.5 medium and incubation at the indicated time intervals.

Fig. 4.

Role of OMPs and DegS in σ^E induction. Expression of the σ^E -dependent *rpoE*, *rpoH*, *fkpA* and *surA* genes was measured by qRT-PCR. Fold induction represents relative gene expression during stress in comparison to control conditions after normalization to reference gene (*gmk*) mRNA levels. Cells were obtained at OD₆₀₀ ~0.4, re-suspended in stress media with 0.2% arabinose for induction of RybB or OmpC expression, and incubated for 1 h. WT and mutants *Salmonella* were subjected to heat stress (50°C) (A), acid stress (pH 4.5) (B, D) or control conditions (37°C, pH 7.0) (C).

Fig. 5.

Effects of *rseA*, *rseB*, *rseC* and *rseP* mutations on acid induction of σ^E . Expression of the σ^E -dependent *rpoE*, *rpoH*, *fkpA* and *surA* genes was measured by qRT-PCR in WT, *rseA*, *rseB*, *rseB degS* *rseC* (A) or *rseP* and *rseP* PDZ (B) mutant *Salmonella* subjected to 1 h acid stress (pH 4.5) during early stationary phase and compared with expression at pH 7.0. Steady-state mRNA levels were normalized to levels of reference mRNA (*gmk*) under the same conditions.

Fig. 6.

Effects of heat and acid stress on RseA degradation. The pACYC177-HA-RseA plasmid was introduced into WT, *degS*, *degS* PDZ (A), *rseP*, *rseP*ΔPDZ (A), or *degS rseP* (B) strains. Cells at OD₆₀₀ ~1.5 were subjected to heat (centre) or acid stress (right) and compared with baseline (left). HA-tagged RseA accumulation was measured by anti-HA immunoblotting.

Fig. 7. Survival of WT and *rpoE*, *degS* or *rseP* mutant *Salmonella* in the SCV. RAW264.7 macrophages treated (dotted lines) or untreated (continuous lines) with NH_4Cl were infected with complement-opsonized WT (diamonds), *rpoE* (squares), *degS* (crosses) or *rseP* (triangles) mutant *Salmonella*.

Fig. 8.

Pathways of σ^E activation by heat and acid stress. σ^E can be activated by the accumulation of unfolded OMPs during heat stress, which leads to activation of DegS and the sequential proteolysis of the RseA anti- σ by DegS and RseP. Alternatively, acid stress can trigger RseA proteolysis by RseP in an OMP- and DegS-independent fashion.

Table 1

Strains and plasmids used in this study.

Strains/plasmids	Relevant characteristics	Source
Strains		
14028s	Wild-type <i>Salmonella enterica</i> sv. Typhimurium	ATCC
IB2	14028s <i>rpoE</i> : <i>cat</i>	Bang <i>et al.</i> (2005)
IB355	14028s <i>rseC</i> : <i>cat</i>	This study
IB408	14028s <i>degS</i> : <i>cat</i>	This study
IB412	14028s <i>rseP</i> : <i>cat</i>	This study
IB480	14028s <i>rseB</i> : <i>cat</i>	This study
JK628	14028s <i>degS</i> : <i>aph</i>	This study
JK629	14028s <i>degS</i> : <i>aph rseP</i> : <i>cat</i>	This study
CM10	14028s with deletion of the <i>degS</i> PDZ domain coding sequence	This study
CM35	14028s with deletion of the <i>rseP</i> PDZ domain coding sequence	This study
Plasmid		
pACYC177	p15A <i>ori bla aph</i>	Chang and Cohen (1978)
pBAD24	ColE1 <i>ori P_{araBAD} bla</i>	Guzman <i>et al.</i> (1995)
pBAD30	p15A <i>ori P_{araBAD} bla</i>	Guzman <i>et al.</i> (1995)
pFB175	pBAD30: <i>ompC</i> , <i>ompC</i> under the control of <i>P_{araBAD}</i>	Bang <i>et al.</i> (2005)
pFB188	pACYC177 carrying the HA-RseA DNA sequence	This study
pKD46	<i>oriR101 repA101ts P_{araBAD}-λRed bla</i>	Datsenko and Wanner (2000)
pKP17-1	pBAD-RybB, <i>rybB</i> under the control of <i>P_{ara}</i>	Papenfort <i>et al.</i> (2006)
pKP8-35	pBAD control plasmid, produces short nonsense RNA	Papenfort <i>et al.</i> (2006)
pRB983	pBR3- <i>rpoE</i> , <i>rpoE</i> complementation	Testerman <i>et al.</i> (2002)
pTFP1	pRS1274: <i>P1_{rpoE}-lacZ</i>	Testerman <i>et al.</i> (2002)
pTFP2	pRS1274: <i>P3_{rpoE}-lacZ</i>	Testerman <i>et al.</i> (2002)