Stress sigma factor RpoS degradation and translation are sensitive to the state of central metabolism

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RpoS, the stationary phase/stress sigma factor of *Escherichia coli*, regulates a large cohort of genes important for the cell to deal with suboptimal conditions. Its level increases quickly in the cell in response to many stresses and returns to low levels when growth resumes. Increased RpoS results from increased translation and decreased RpoS degradation. Translation is positively regulated by small RNAs (sRNAs). Protein stability is positively regulated by anti-adaptors, which prevent the RssB adaptor-mediated degradation of RpoS by the ClpXP protease. Inactivation of *aceE*, a subunit of pyruvate dehydrogenase (PDH), was found to increase levels of RpoS by affecting both translation and protein degradation. The stabilization of RpoS in *aceE* mutants is dependent on increased transcription and translation of IraP and IraD, two known anti-adaptors. The *aceE* mutation also leads to a significant increase in *rpoS* translation. The sRNAs known to positively regulate RpoS are not responsible for the increased translation; sequences around the start codon are sufficient for the induction of translation. PDH synthesizes acetyl-CoA; acetate supplementation allows the cell to synthesize acetyl-CoA by an alternative, less favored pathway, in part dependent upon RpoS. Acetate addition suppressed the effects of the *aceE* mutant on induction of the anti-adaptors, RpoS stabilization, and *rpoS* translation. Thus, the bacterial cell responds to lowered levels of acetyl-CoA by inducing RpoS, allowing reprogramming of *E. coli* metabolism.

RssB | ClpXP | acetyl CoA | RpoS | pyruvate dehydrogenase

RpoS is a central sigma factor in *Escherichia coli* involved in gene expression in the stationary phase but also under a large number of stress conditions (1). As with many global transcriptional regulators, RpoS is highly regulated at the transcriptional, translational, and posttranslational levels. Under normal growth conditions, the translation of RpoS is blocked by a secondary structure in the 5′ UTR; this is relieved in response to various signals by the action of small RNAs (sRNAs) (reviewed in ref. 1). In addition, RpoS is rapidly degraded during exponential phase by the ClpXP protease. For RpoS proteolysis to occur, it must interact with RssB, an adaptor protein, which allows recognition of RpoS by the protease. Under stress conditions, RpoS is stabilized, allowing it to rapidly accumulate to activate the RpoS regulon.

Mechanisms leading to RpoS stabilization under stress conditions were not understood for many years. However, the discovery of three small proteins, IraP, IraD, and IraM, which are able to interact with RssB to stabilize RpoS, has provided a major insight into the regulatory pathways inhibiting RpoS proteolysis. These proteins, called anti-adaptor proteins, are each induced under a different stress condition (2). IraP, induced via an increase in ppGpp (3), is necessary for stabilization of RpoS during low phosphate growth. IraM, induced via activation of the PhoQ/PhoP two-component system, is similarly critical for RpoS stabilization during low Mg^{2+} growth (2). IraD induction is not fully understood, but it clearly plays a role both in the transition to stationary phase, via induction of a ppGpp-dependent promoter, and after DNA damage (4, 5). IraM and IraD are also negatively regulated by H-NS (6).

Regulation of RpoS is also mediated by three sRNAs that positively regulate translation. Each of these sRNAs requires the RNA chaperone Hfq for function, and each pairs with the 5′ UTR of RpoS to make the ribosome-binding site accessible. However, the sRNAs have very different induction pathways. DsrA is synthesized at low temperatures (7, 8). RprA is synthesized in response to activation of the Rcs phosphorelay under conditions of cell-surface stress (9). The third sRNA, ArcZ, is negatively regulated by the ArcB/ArcA two-component system under anaerobic conditions; therefore, ArcZ is more abundant under aerobic conditions (10).

Even with these recent advances, much remains to be discovered about when and how RpoS levels are regulated. For example, translational up-regulation at stationary phase has been reported, independent of the upstream hairpin that is the target for sRNAs (11). RpoS-dependent genes are known to help the cell to respond to a large number of stress conditions and metabolic shifts, suggesting a need for increased synthesis of RpoS and its stabilization under many conditions. Here we find that, in exponential phase cells, perturbing central metabolism by decreasing the pool of acetyl-CoA leads to dramatically increased levels of RpoS. This increase integrates two independent regulatory pathways: RpoS stabilization via the production of two of the known anti-adaptors and induction of RpoS translation. These results demonstrate both a previously unexplored signal for the RpoS general stress response and a unique coupling in signaling between translational regulation and protein stabilization.

Results

RpoS Levels Are Increased in an Δ*aceE* Mutant. Lycopene is a red pigment with antioxidative properties that can be produced in microorganisms. It is made in *E. coli* via the isoprenoid pathway,

Significance

Bacteria such as *Escherichia coli* encounter multiple environments and, as with all organisms, must balance growth and survival when conditions are suboptimal. *E. coli* uses the RpoS sigma factor, a specialized subunit of RNA polymerase, under conditions of stress or starvation to change the transcriptional program to one that promotes survival. We find that a block in central metabolism, via deletion of a gene encoding pyruvate dehydrogenase, leads to a dramatic increase in RpoS in exponential phase cells. This metabolic cue is likely via reduced amounts of acetyl-CoA. The increase in RpoS is mediated by stabilization of RpoS protein and by translational up-regulation of RpoS synthesis and links this stress response to the state of central metabolism.

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with the final steps carried out by enzymes cloned from other sources such as *Erwinia* (reviewed in refs. 12 and 13). Research on optimal conditions for lycopene expression in *E. coli* has included screening various gene knockouts and overexpression strains. Surprisingly, many regulators of RpoS degradation were identified using these screens. In one study, overexpression of the anti-adaptors IraD (*yjiD*) and IraM (*ycgW*), as well as AppY [also known to lead to RpoS stabilization (2)] and RpoS itself were each identified as improving lycopene formation, suggesting that increasing RpoS contributes to higher lycopene biosynthesis (14). In that study, as well as related ones, the deletion of the gene encoding RssB, the adaptor for RpoS degradation (called in ref. 15 *hnr*), also improved lycopene formation. Therefore, we began this study by asking if other mutants that increase lycopene formation also acted by increasing RpoS synthesis or stability. We focused on *gdhA*, *aceE*, and *fdhF* mutants (14–16). The deletion of *fdhF* and *gdhA* had no effect on RpoS stability, nor on the level of RpoS at 0′ (Fig. S1*A*). These gene deletions thus presumably act independently of increased RpoS in the lycopene synthesis pathway.

However, a deletion of *aceE*, encoding one of the subunits of pyruvate dehydrogenase, had a drastic effect on RpoS levels (Fig. 1*A*). RpoS was barely visible at $t = 0$ min in a WT strain at the low OD_{600} of 0.3 at which this experiment was done, but was abundant at 0′ and still present after a chase of 30 min in the *ΔaceE* background; the half-life of RpoS in midexponential phase changed from 3′ to more than 10′. The high level of RpoS at 0′ suggested that *ΔaceE* might also increase synthesis of RpoS. This was tested using *rpoS-lacZ* transcriptional and translational fusions, again measured in exponential phase. There were very modest effects on the transcriptional fusion (Fig. S1*B*). However, a translational fusion that is also subject to RssB-dependent degradation was almost undetectable in the wild-type background and was increased 140-fold in the *aceE* background (Fig. 1*B*). The same experiment was repeated in a strain in which RpoS degradation was blocked by inactivating *rssB*. Mutating *rssB* increased the expression of RpoS-Lac 12-fold; however, deleting *aceE* increased expression of the fusion protein another 15-fold (Fig. 1B). Therefore, there are two additive and independent effects on RpoS of mutating *aceE*: a 15-fold increase in synthesis and a further 10-fold increase due to stabilization. Because we saw very little increase for the transcriptional fusion, we conclude that the increase in synthesis reflects increased translation.

If the increased RpoS in the *aceE* mutant is active, it should be reflected in expression of RpoS-dependent RNAs; this was measured using the RpoS-dependent sRNA SdsR (17). RNA was isolated from wild-type, *aceE*, and *aceE rpoS* mutant cells grown to an OD_{600} of 0.3 and probed for SdsR, as well as for the sRNAs not under RpoS control, ChiX and SsrA (Fig. 1*C*). SdsR was undetectable in the wild-type cells at this OD, consistent with the expected very low levels of RpoS (Fig. 1*A*), but was expressed in the *aceE* mutant in an RpoS-dependent fashion. Therefore, the induced RpoS in the *ΔaceE* strain is active. As expected, ChiX and SsrA levels were not significantly affected by the *aceE* mutant (Fig. 1*C*).

RpoS Stabilization in the *aceE* Mutant Is Dependent upon Induction of Anti-Adaptors IraP and IraD. RpoS degradation by the ClpXP protease depends on the adaptor protein RssB. The major mechanism that leads to RpoS stabilization under different stress conditions is inactivation of RssB adaptor activity by anti-adaptor proteins IraP, IraD, and IraM (2, 18, 19). Thus, we investigated if the known anti-adaptors play a role in the stabilization of RpoS in the *aceE::kan* background. As shown in Fig. 2*A*, the deletion of all three genes coding for the anti-adaptors restored RpoS degradation in an *aceE*::*kan* mutant to the rate seen in the wild-type strain. Single and double mutants of the anti-adaptors in the *ΔaceE* background suggest that IraP and to a less extent IraD mediate stabilization of RpoS in the *ΔaceE* strain (Fig. 2*A* and Fig. S2*A*).

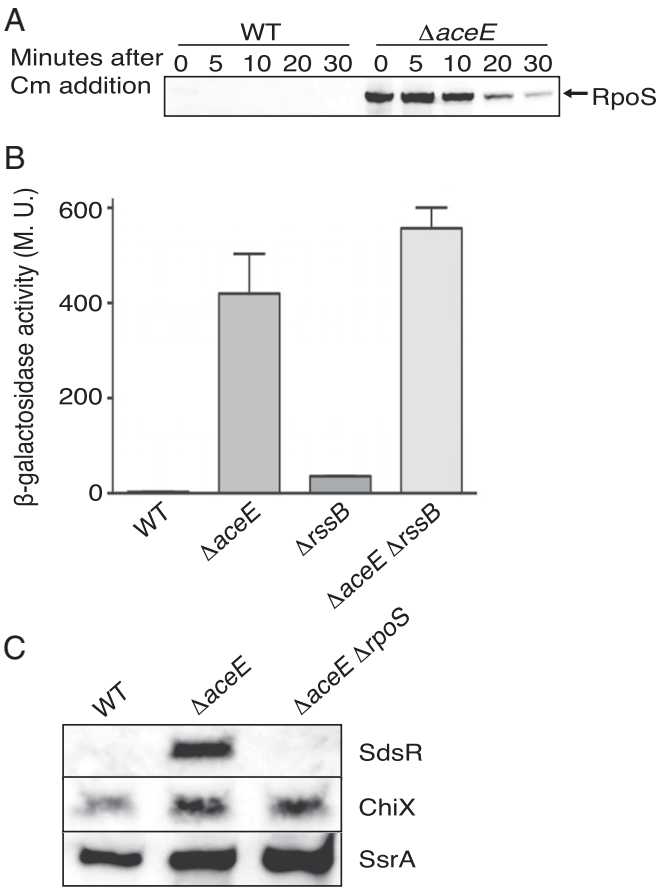

Fig. 1. Mutations in *aceE* lead to increased RpoS levels. (*A*) WT (MG1655) and Δ*aceE* (BA334) were grown to an OD_{600} of ∼0.3, protein synthesis was inhibited by chloramphenicol (Cm) addition, and RpoS levels were analyzed by Western blotting using an anti-RpoS antiserum. (*B*) Strains containing rpoS-lacZ translational fusions (WT, SG30013; Δ*aceE*, BA455; Δ*rssB,* SG30018*;* Δ*rssB* Δ*aceE*, BA679) were grown to an OD_{600} of ∼0.3 and assayed for β-galactosidase activity. This fusion has the promoters of *rpoS* and enough of the RpoS ORF to be subject to RssB-dependent degradation. The mean from three replicates is presented, and the standard error of the mean (SEM) is indicated by the error bars. M.U., Miller units. (*C*) Cells were grown to an OD_{600} of 0.2 in LB, and samples were removed for RNA analysis. Samples were probed with biotinylated probes for SdsR (*Top*), ChiX (*Middle*), or SsrA (*Bottom*). Strains: WT (NM1100), Δ*aceE*::kan (BA334), and *rpoS*::tet Δ*aceE*::kan (BA530).

Strains deleted for *iraD* and *iraP* or all three anti-adaptors still have a high level of RpoS at the initial time point (0 min) even though RpoS degradation was similar to that seen in the $aceE^+$ host (Fig. S2*A*), consistent with the increase in translation seen in the *aceE* mutant (Fig. 1*B*).

Anti-adaptors act by interacting with the limiting levels of RssB and preventing the interaction of RssB with RpoS (19). Therefore, a stabilization mechanism dependent on anti-adaptor titration of RssB can be bypassed by increasing the levels of RssB. Consistent with the *aceE* mutant acting via anti-adaptors, increasing the level of RssB also bypassed the *aceE* effect on stabilization (Fig. S2*B*).

RssB is an orphan response regulator in *E. coli* and can be phosphorylated on a conserved aspartate residue, D58. Mutating the D58 residue of RssB to A or P decreases but does not eliminate RpoS degradation in vivo and is still subject to stabilization by the anti-adaptors (2, 18–20). The small-molecule acetyl phosphate can phosphorylate RssB and stimulate degradation in vivo and in vitro (19, 20). Acetyl phosphate is synthesized from acetyl CoA, and one major pathway for acetyl CoA synthesis is dependent

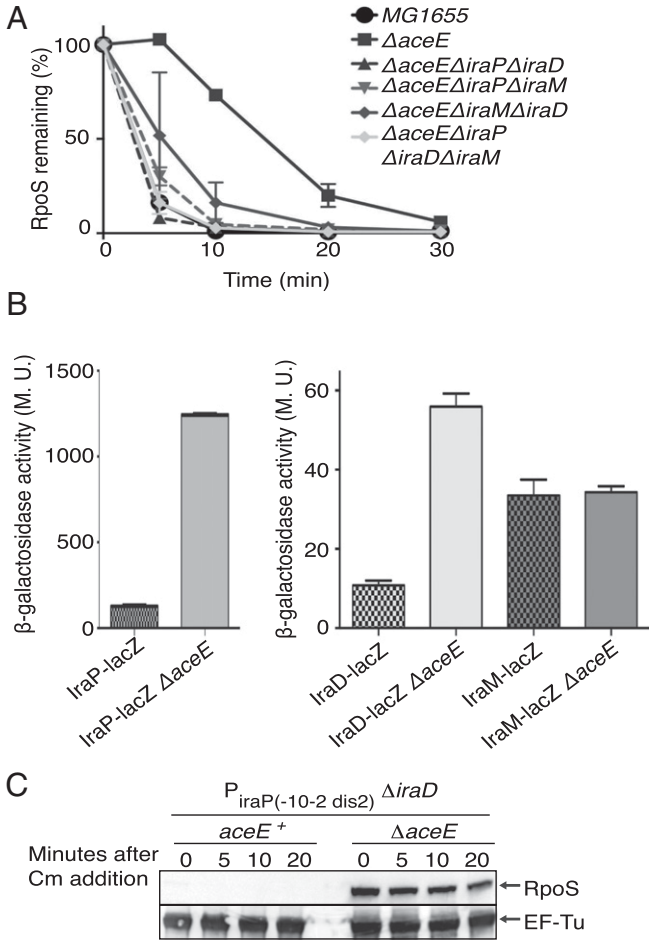

Fig. 2. RpoS stabilization in an *aceE* mutant is dependent on the anti-adaptors IraP and IraD. (*A*) Strains were grown and RpoS levels analyzed by Western blotting as in Fig. 1*A*. The RpoS level was quantified, with intensity measured at time 0 for each strain set at 100%. The mean from three replicates is presented, and the error bars indicate the SEM. The following strains were used: WT (MG1655); Δ*aceE* (BA334); and the isogenic derivatives Δ*aceE* Δ*iraP* Δ*iraD* (BA449), Δ*aceE* Δ*iraP* Δ*iraM* (BA447), Δ*aceE* Δ*iraD* Δ*iraM* (BA467), and Δ*aceE* Δ*iraP* Δ*iraD* Δ*iraM* (BA385). The gels and other mutants are in Fig. S2*A*. (*B*) Strains containing transcriptional fusions *iraP-lacZ* (WT, AB060; Δ*aceE*, BA407), *iraD-lacZ* (WT, AB050; Δ*aceE*, BA411), or *iraM-lacZ* (WT, AB042; Δ*aceE*, BA409) were grown in LB to an OD_{600} of ∼0.3 and assayed for β-galactosidase activity. The mean from three replicates is presented; the SEM is indicated by the error bars. M.U., Miller units. The level of expression of the *iraP* fusion is significantly higher than that for the other two fusions. (*C*) Strains were grown as in Fig. 1*A*, and RpoS levels were analyzed by Western blotting using anti-RpoS antiserum (*Upper*) or anti–EF-Tu antiserum (*Lower*). The following strains were used: −10–2 dis2 *iraP* Δ*iraD*::*tet* (BA946) and the isogenic *aceE* derivative (BA948).

upon the product of the *aceE* gene. Therefore, we examined the dependence of the stabilization by the *aceE* mutation on RssB phosphorylation. This was tested by comparing RpoS stability in a strain expressing *rssBD58P*, mutated at the site of phosphorylation, to stability in an *rssB*D58P Δ*aceE* double mutant. As expected, the strain with the RssBD58P mutant was not fully functional; the half-life of RpoS was significantly longer than that of the wild-type strain (half-life of 8′, Fig. S2*C*). However, RpoS was more stable in the *aceE*::*kan* strain than in the *rssB*D58P mutant strain, and the effects of *rssB*D58P and *aceE*::kan were additive (half-life in double mutant of 19′). Therefore, the major effect of an *aceE* mutant in stabilizing RpoS is via the anti-adaptors, and not due to loss of RssB phosphorylation.

IraP and IraD are normally present in the cell at low levels; their synthesis increases enough to stabilize RpoS only under specific stress conditions (2, 4, 18). The fact that IraP and IraD stabilize RpoS in an *aceE*::*kan* mutant suggests that their expression is induced in this background.

Induction of the promoters for these anti-adaptors was followed by using transcriptional fusions. The *aceE* deletion increased the expression of *iraP* 10-fold and the expression of *iraD* 5-fold; *iraM* expression was unchanged by the *aceE* mutant (Fig. 2*B*). Both *iraP* and *iraD* are positively regulated by ppGpp (3, 5), suggesting that an increase in this alarmone might explain the induction of these anti-adaptors. Direct measurement of ppGpp in the *aceE* host was not possible because the strain grew very poorly in the 3-(*N*-morpholino)propansulfonic acid medium needed for the experiment. However, the promoter of *iraP* itself acts as a measure of ppGpp. It is strongly positively regulated by ppGpp, and mutations in the *iraP* discriminator abolish the response to ppGpp (3). Consistent with an increase in ppGpp in the *aceE* mutant, the induction of the *iraP* transcriptional fusion in an *aceE* mutant was abolished in a fusion carrying the ppGpp-insensitive discriminator mutant *dis2* (changed from AT-rich to GC-rich) (3) (Fig. S3*A*). The *dis2* mutant reduces the basal level of *iraP* expression significantly, as previously seen (3), but this reduced level was not increased at all in the *aceE* mutant. Therefore, these results suggest that ppGpp increases in the *aceE* mutant and in turn induces the promoters of *iraP* and *iraD*.

The contribution of ppGpp was further examined in a strain devoid of ppGpp ($ppGpp^0$). In *E. coli*, RelA mediates ppGpp synthesis during amino acid starvation; SpoT synthesizes but also degrades ppGpp and has been implicated in ppGpp synthesis after various stress treatments (21, 22). The triple mutant (*aceE relA spoT*) was unable to grow without the addition of acetate (Fig. S3*B*). The strain was grown in the presence of acetate, acetate washed out, and the stability of RpoS was then examined. Unexpectedly, RpoS was still stable in this strain in the absence of ppGpp (Fig. S3*C*).

We considered the possibility that, in addition to induction of ppGpp-dependent promoters, there was posttranscriptional induction of the anti-adaptors. This was examined for IraP. Induction of IraP tagged at its C terminus with SPA was examined in wild-type and *aceE* mutants in both a strain carrying a wild-type *iraP* promoter and a strain carrying the *dis2 iraP* promoter mutant, rendering it no longer positively regulated by ppGpp (3). IraP-SPA levels were induced in the *aceE* mutants even with the dis2 *iraP* promoter (Fig. S3*D*). RpoS was partially stabilized even in the dis2 mutant (Fig. S3*D*). To bypass the low expression of IraP from the *dis2* promoter, an additional experiment was done with an up mutation in the −10 region (−10-2), combined with the *dis2* mutant (3). In a transcriptional fusion, neither the −10-2 mutant nor the −10-2 *dis2* mutant showed further induction in the *aceE* mutant, confirming the absence of transcriptional induction of IraP from these promoters (Fig. S3*E*). This same −10-2 dis2 promoter was then used to drive native IraP in isogenic $aceE^+$ and *aceE* strains; strains were also mutated for *iraD* so that any stabilization would depend on IraP (Fig. 2*C*). RpoS was stable in the *aceE* mutant, but not in the $aceE^+$ parent. Therefore, we conclude that the *aceE* mutant leads both to ppGpp induction of the *iraP* promoter and to posttranscriptional induction of IraP, probably by increased translation. This additional induction likely occurs in the $ppGpp^0$ strain as well because RpoS is still stable in that strain.

Translational Up-Regulation of *rpoS* in *aceE* Mutants Is Not via the Known sRNA-Dependent Pathway and Is Independent of Most of the *rpoS* Leader. RpoS accumulates in the *aceE* mutant due to increased translation in addition to stabilization of the protein (Fig. 1*B*, *rssB*::tet strains, and Fig. S2*A*, 0′ point). *rpoS* has a long untranslated leader, and RpoS translation is positively regulated in response to different environmental signals via the action of

three different sRNAs; each requires the RNA chaperone Hfq for function (reviewed in ref. 1). Initially, an *rpoS-lacZ* translational fusion was evaluated for the effects of each of the sRNAs on expression in a Δ*aceE* mutant (Table S1); an *rssB* mutant derivative was used to stabilize the fusion protein. Our expectation was that if one or more of the sRNAs were responsible for increased translation in the *aceE* mutant, deleting the sRNA(s) should abolish the induction. As in previous experiments (10), deletion of *dsrA* from the $aceE^+$ parent had the biggest effect under these growth conditions, reducing RpoS-LacZ expression significantly. Single mutations in either *arcZ* or *rprA* had very little effect. A triple mutant was somewhat more defective than the *dsrA* deletion alone. However, none of these deletions, including the *arcZ dsrA rprA* triple mutant, abolished the effect of an *aceE* mutant. In these experiments, the ratio of $aceE^-/aceE^+$ was 4 for the wild-type strain; in the triple mutant the ratio was 20, reflecting the low level of the $aceE^+$ strain. Therefore, the induction of translation of RpoS by *aceE* is apparently not by the action of any of these Hfq-dependent sRNAs.

For further analysis of how *aceE* induces RpoS synthesis, a set of fusions, all expressed from the constitutive Cp17 promoter (23), were constructed and assayed in $aceE^+$ and $aceE^-$ backgrounds. The parent fusion carries the full-length leader and 477 nt of the *rpoS*-coding region, deleting the region necessary for RpoS degradation (24). Thus, levels of RpoS-lac should reflect changes only in mRNA stability and translation. In this strain, an *aceE* mutant increases expression more than fivefold at OD_{600} 0.3 (Fig. 3*A*). A control fusion of the promoter to the *lac* leader and *lacZ* had a ratio of $aceE^-/aceE^+$ of 0.7 (Fig. 3*A*). A mutation, C125T, which disrupts the inhibitory hairpin and renders translation independent of Hfq (25) (Fig. 3*A*), increased expression in the wild-type strain by fourfold. An *aceE* mutant increased expression by an additional threefold. Therefore, by this test the *aceE* effect is independent of the hairpin, and the hairpin opening is additive with the effect of the *aceE* mutant (Fig. 3*A*). We also looked at the effect of an *hfq* deletion on the Cp17 fusions. The *aceE* deletion increased expression in the *hfq* mutant by 14-fold (Fig. 3*A*). The C125T *hfq* fusion was increased twofold in the *aceE* mutant; why this is somewhat less than the C125T fusion is not clear. Overall, these experiments confirm the results in Table S1, ruling out a critical role for sRNAs, Hfq, and the *rpoS* inhibitory hairpin in the stimulation of RpoS in an *aceE* mutant.

Further fusions were examined to identify the region necessary for the response to the *aceE* mutant. A fusion carrying the same Cp17 promoter but only 24 nt of the leader and 24 nt of the translated *rpoS* gene (first eight codons) lacks the inhibitory hairpin and therefore had a higher basal level of expression. The *aceE* mutant increased this similarly to the open hairpin derivative (Fig. 3*B*, 24rpoS + 8rpoS, 2.8-fold). These results suggest that the major effect of the *aceE* mutant on translation/mRNA stability is likely specific to sequences near the sites for the initiation of translation. As a comparison, parallel fusions were constructed expressing the 24 nt of the *rpoS* leader fused to *lacZ* at the ATG (24rpoS +lac) and a reciprocal fusion containing the *lac* leader and eight codons of *rpoS* (lac + 8rpoS) (Fig. S4). The 24 nt of the *rpoS* leader were sufficient to give 2.5-fold induction in the *aceE* mutant, whereas the reciprocal fusion gave no increase (1.3-fold) in the *aceE* strain (Fig. 3*B*). Therefore, the region immediately before the ATG is necessary for the effect of *ace*, and the codons within the ORF do not contain essential information. Because the *aceE* induction in the full-length fusion (Fig. 3*A*) was considerably higher (7× rather than 3×), additional information may be contained elsewhere.

Thus, both IraP and RpoS are subject to translational up-regulation in the *aceE* mutant. This effect is specific; *lacZ* translation was not similarly up-regulated. The sequences that allow this up-regulation are close to the translational initiation site, at least for RpoS (Fig. 3*B*).

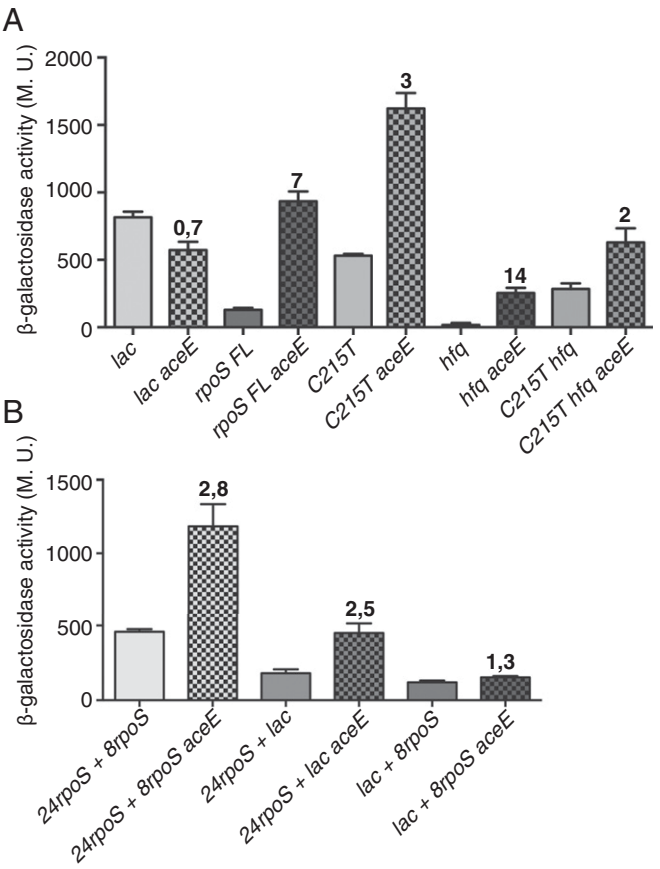

Fig. 3. Regions in *rpoS* needed for translational induction in the *aceE* mutant. All strains carried an RpoS-LacZ translational fusion driven by a Cp17 promoter; the portion of RpoS present does not include the region necessary for RssB-dependent degradation. Without the promoter there was no expression of *lacZ*. (*A*) Cells were grown to an OD_{600} of 0.3 and were assayed for β-galactosidase. Isogenic pairs of $aceE^+$ and *aceE*::kan strains were assayed. Strains used were the following: lac (control fusion, with 38-nt leader of *lac* and *lacZ*: BA926, BA928); rpoS FL (full-length 567-nt leader of *rpoS* and 477 nt of *rpoS* ORF: BA938, BA940); C125T (derivative of rpoS FL with leader mutation C125T, disrupting hairpin: BA942, BA944); hfq (Δ*hfq* derivatives of rpoS FL: BA960, BA984); C125T hfq (Δ*hfq* derivatives of C125T rpoS FL: BA962, 986). (*B*) Cells were grown and assayed as for *A*. Strains used were the following: 24rpoS + 8rpoS (BA964, BA966); 24rpoS + lac (BA992, BA996); and lac + 8rpoS (BA994, BA998).

Metabolic Defects in the *aceE* Mutant. *aceE* encodes the E1 component of pyruvate dehydrogenase (PDH), an enzyme complex that converts pyruvate to acetyl-CoA, critical for the TCA cycle, for fatty acid synthesis and for synthesis of the signaling molecule acetyl-phosphate. The other two subunits of PDH are encoded by *aceF* and *lpd*; the lipoamide dehydrogenase (LPD) subunit of PDH, encoded by *lpd*, is also part of the 2-oxogluatarate dehydrogenase and glycine cleavage system complexes. To examine whether it is the absence of AceE itself or loss of PDH that leads to increased RpoS, mutations in *aceF* and *lpd*, as well as a mutation in *lipA* encoding lipoate synthetase, necessary for all LPD activities, were compared with the *aceE* mutation for effects on the *rpoS-lacZ* fusions. Each of these mutations increased the expression of the *rpoS-lacZ* fusion similarly to that seen for *aceE* (Fig. S5 *A* and *B*), suggesting that loss of PDH activity and thus loss of acetyl-CoA or a downstream product of acetyl-CoA leads to RpoS induction.

One of the major consequences of reduced acetyl-CoA is reduced fatty acid synthesis. It has previously been shown that starvation for fatty acids leads to elevated ppGpp via an interaction of the Acyl carrier protein with SpoT, demonstrating the global regulatory effect of this metabolic change (26). In addition, *fabH* is synthetically lethal in strains unable to synthesize ppGpp (27), as is *aceE* (Fig. S3*B*). Thus, it seemed possible that the inducing signal in the *aceE* mutant was decreased fatty acid synthesis. A mutation in *fabH* will block use of acetyl-CoA to make acetoacetyl-ACP, an early step in fatty acid synthesis. The *fabH* mutation also induced the fusion, although not to the same extent as the mutations in PDH (Fig. S5 *A* and *B*). All of the mutations that block PDH activity also grew very slowly; *fabH* growth was intermediate between the PDH mutants and wild type, correlating with the less dramatic induction of RpoS (Fig. S5*C*). Thus, a defect in fatty acid synthesis partially mimics the PDH mutant.

Alternative pathways for synthesis of acetyl-CoA from acetate exist in *E. coli*. One uses acetyl-CoA synthetase (Acs); a parallel pathway uses Pta and AckA when external acetate levels are high. If lowered levels of acetyl-CoA are responsible for the signals leading to RpoS induction, adding acetate to the medium should improve acetyl-CoA levels and reverse induction of RpoS. As seen in Fig. S6*A*, acetate improved growth. Addition of acetate also led to reduced stability of RpoS (Fig. 4*A*) and reduced expression of both *iraP* and *iraD* transcriptional fusions (Fig. S6*B*). In addition, the translational induction of RpoS was partially reversed (Fig. 4*B*). Addition of other carbon sources that do not directly improve acetyl-CoA synthesis (succinate and oleate) did not mimic the effect of acetate in reversing RpoS stability (Fig. S6 *C* and *D*) (28). These results are consistent with a depletion of acetyl-CoA or its products as the major defect in the *aceE* mutant.

Discussion

RpoS is used by bacteria to respond to a variety of starvation conditions and stresses in what has been termed a "general stress response." Under these conditions, the level of RpoS increases, reflecting stabilization of the protein and increased translation. RpoS-dependent genes encode functions that help the cell repair damage and await better times. Because of the range of RpoS-dependent functions, one RpoS-inducing stress can provide cross-resistance to many others (reviewed in ref. 1). As cells enter stationary phase, some combination of changes (starvation, accumulation of toxic by-products, other changes) lead to higher levels of RpoS. The pathways for inducing RpoS during stationary phase in complex medium have not been fully defined, but include increased translation and decreased degradation.

Here, we find that a mutation in the gene encoding a subunit of PDH leads to induction of RpoS in exponential phase; our studies were primarily in cells mutant for *aceE*, encoding the E1a subunit of PDH. This increase resulted from decreased RpoS degradation and increased translation. RpoS was stabilized via two anti-adaptor proteins, IraP and IraD. Induction of these anti-adaptors is in part likely due to increased levels of ppGpp, a positive regulator of both the *iraP* and *iraD* promoters (3, 5). However, even in the absence of ppGpp (Fig. S3*C*) or in a cell carrying a ppGpp-independent mutation in the *iraP* promoter (Fig. S3*D*), RpoS is stabilized. IraP translation is apparently also up-regulated in the *aceE* mutant sufficiently to stabilize RpoS, although the basis for this up-regulation is not yet understood.

The second component of induction is high-level translation of RpoS. Surprisingly, the activation of translation was independent of the well-studied small regulatory RNAs that are responsible for translational induction of RpoS translation under other growth conditions (Table S1). Activation of translation was also independent of the long *rpoS* leader where these sRNAs act; a fusion that contains only the final 24 nt of the *rpoS* leader was still induced in an *aceE* mutant (Fig. 3*B*). Hirsch and Elliott used a similarly short *rpoS* translational fusion (24 nt of leader and eight codons of RpoS) and observed stationary phase induction (11).

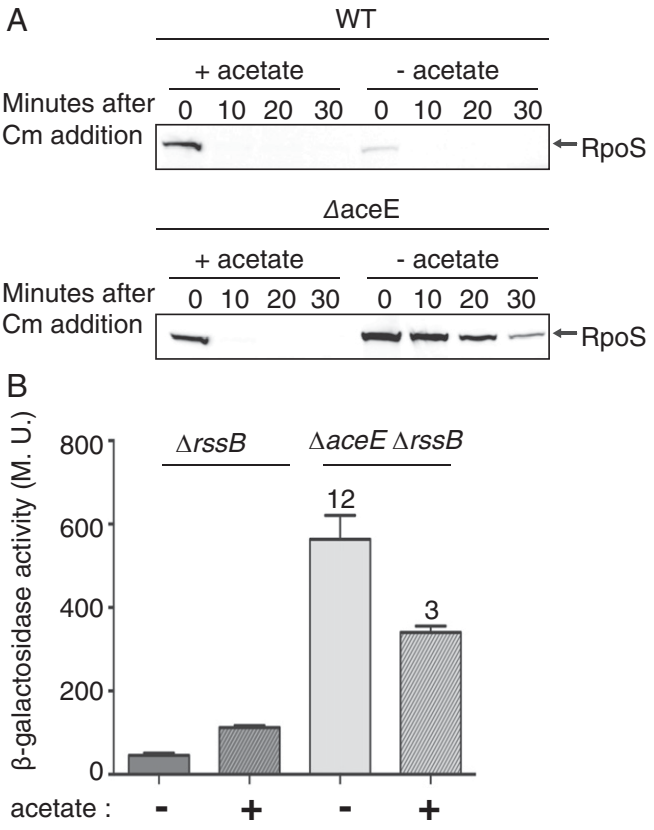

Fig. 4. Acetate overcomes effect of *aceE* mutant. (*A*) WT (MG1655) and *ΔaceE* (BA334) strains were grown and RpoS levels were analyzed as for Fig. 1*A*. (*B*) Strains contain the *rpoS-lacZ* translational fusion used in Fig. 1*B* (under the control of *rpoS* promoters and subject to RssB-dependent degradation). Δ*rssB* (SG30018) and *ΔaceE* Δ*rssB* (BA679) strains were grown at 37° in LB in the absence or presence of acetate (30 mM) and assayed as for Fig. 1*B*.

Thus, perturbing the metabolism of pyruvate to acetyl-CoA has uncovered a previously unrecognized signaling pathway for high level induction of RpoS. PDH uses pyruvate, CoASH, and NAD to synthesize acetyl-CoA, a precursor for a variety of cellular functions, including the TCA cycle and fatty acid synthesis. Acetyl-CoA also is the precursor for the signaling molecule acetyl-phosphate, which can also be metabolized to acetate, thus providing a pathway from pyruvate, via PDH, to acetate. An alternative pathway from pyruvate to acetate uses the enzyme PoxB, which is expressed in an RpoS-dependent fashion (29). Although PoxB does not itself synthesize acetyl-CoA, AMP-forming Acs can use the acetate formed by PoxB and synthesize acetyl-CoA (30, 31). Thus, one consequence of the induction of RpoS in PDH-deficient cells is likely to be expression of higher levels of PoxB, increasing the alternative route for acetate and acetyl-CoA synthesis. Another consequence may be that PoxB, unlike PDH, does not convert NAD to NADH.

Although our results are consistent with lack of acetyl-CoA as the primary signal for RpoS induction, inhibition of the fatty acid synthesis pathway in a *fabH* mutation also leads to an intermediate level of RpoS induction (Fig. S5 *A* and *B*). A *fabH* mutation will block use of acetyl-CoA to make acetoacetyl-ACP, an early step in fatty acid synthesis; therefore, a product beyond FabH may be what is sensed in these mutants.

The role of ppGpp in the *aceE* stress response is not entirely clear. We, as well as others (R. Harinarayanan and M. Cashel), see synthetic lethality of a strain unable to synthesize ppGpp in the *aceE* mutant. It has also been reported that *fabH* is synthetically lethal with a ppGpp null strain (27). Therefore, interference with synthesis of acetyl-CoA or the resulting defect in fatty acid synthesis is likely to induce ppGpp synthesis and may be the lethal event in ppGpp-null cells. Although ppGpp certainly contributes to RpoS induction via induction of the anti-adaptors IraP and IraD in the *aceE* mutant, our results suggest that additional levels of regulation allow the cell to induce RpoS independently of ppGpp.

As noted above, cells mutant in *aceE* grow slowly (Fig. S5*C*), consistent with a general decrease in protein synthesis. If translation is limited in these cells, and RpoS (and IraP) escape this limitation, this would explain the rapid increase in their relative abundance. There are a number of other examples of such selective translation. After cold shock, specific genes escape the general translation slowdown (32). Some ribonuclease toxins encoded by toxin/antitoxin pairs cut at specific sequences in mRNAs, degrading most but not all mRNAs. We suggest that, in the absence of acetyl-CoA or one of its downstream products, RpoS translation is designed to escape, allowing the cell to partially deal with the metabolic defect. The mechanistic basis of this selectivity will be the subject of future research.

Materials and Methods

Media and Growth Conditions. Cells were grown in Lennox broth (LB) at 37 °C with aeration. When indicated, sodium acetate was added to a final concentration of 30 mM (adjusted to pH 7.0).

Strains and Plasmids. All strains used are derivatives of MG1655 or DJ480 (MG1655 Δ*lac*X74) (33); strains were made either by P1 transduction, selecting for the appropriate antibiotic resistance marker, or by lambda Red recombineering (34) as outlined in Table S2 and *SI Materials and Methods*. Primers are listed in Table S3.

The Cp17 synthetic promoter sequence is from Jensen and Hammer (23); the *iraP* promoter mutations were described previously (3). The p_{iraD2}-lacZ fusion starts at the P2 promoter of *iraD* at −137 and extends through the entire ORF into the 10th codon of lacZ.

Assay for in Vivo RpoS Degradation. Cells were grown overnight in LB medium, diluted into fresh LB medium at an OD_{600} of ∼0.01, and grown to midlogarithmic phase (OD_{600} of ∼0.3) at 37 °C. Chloramphenicol (200 μg/mL) or tetracylcline (100 μg/mL) was added. Samples were analyzed as described by Battesti et al. (19). Values presented are the mean of at least three independent assays.

Northern Blots. Cultures were grown to an OD_{600} of ∼0.3, and RNA was extracted by the hot phenol method (35). RNAs were separated on an acrylamide gel, transferred to a nylon membrane, probed, and developed as described (36). Biotinylated probe sequences are listed in Table S3.

β-Galactosidase Assay. Triplicate cultures were grown in LB medium at 37 °C and assayed using the assay described by Miller (37). Averaged values are presented.

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