Supporting Information

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SI Materials and Methods

Strain construction is described briefly in Table S2. Recombineering (1) was used to create most of the fusions used here. For recombineering, we used strain NM1100 carrying a temperature-inducible miniλ-Red in the chromosome or strains transformed with the pSIM6 plasmid carrying the same genes as the miniλ. Cells were grown at 32 °C in LB to an OD_{600} of ∼0.4–0.6 and transferred to a shaking water bath at 42 °C for 15 min to induce expression of the lambda genes. The culture flasks were then quickly cooled by swirling continuously for 2 min in an ice-water slurry and then occasionally for another 10 min before washing four times with ice-cold sterile water in a refrigerated centrifuge at 2,600 × *g*. About 40 μL of electrocompetent cells were transferred to a chilled cuvette, and 100 ng of DNA [single-stranded or double-stranded (ds)] was added and electroporated with a GenePulser II (Bio-Rad). Cells were resuspended in 1 mL of LB and allowed to recover for 2–3 h before plating 10 and 100 μL on selective or counterselective plates. Leftover material was saved at room temperature and plated the next day if there were no colonies on the overnight plates. DNA materials used for recombineering were either PCR products or up to 1-kb-long dsDNA gBlocks from IDT. Primers and gBlock DNA fragments are listed in Table S3. The chloramphenicol (Cm) resistance cassette in BA761 was amplified from pKD3 (2) with the FRT sites. Two different kanamycin (Kan) gene replacements were used. In the first, a nonremovable Kan cassette lacking the transcription termination stem loop to allow read-through and prevent polarity on downstream genes (3); the mutation of *aceE* carrying this cassette is named *aceE*4 here. A second version used the removable Keio cassette flanked by FRT sites found in the Keio collection (2, 4). This latter cassette inserted in the *aceE* gene has the same boundaries as the Keio collection mutant and will be referred to as *aceE*10::*kan* or *aceE1* when unmarked, after removal of the Kan cassette. Removal of the Kan cassette was done using pCP20 as described by Datsenko and Wanner (2) and verified by PCR.

1. Sharan SK, Thomason LC, Kuznetsov SG, Court DL (2009) Recombineering: A homologous recombination-based method of genetic engineering. *Nat Protoc* 4(2):206–223.
2. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* 97(12):6640–6645.
3. Chaveroche M-K, Ghigo J-M, d'Enfert C (2000) A rapid method for efficient gene replacement in the filamentous fungus *Aspergillus nidulans*. *Nucleic Acids Res* 28(22):E97.
4. Baba T, et al. (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: The Keio collection. *Mol Syst Biol*, 10.1038/msb4100050.

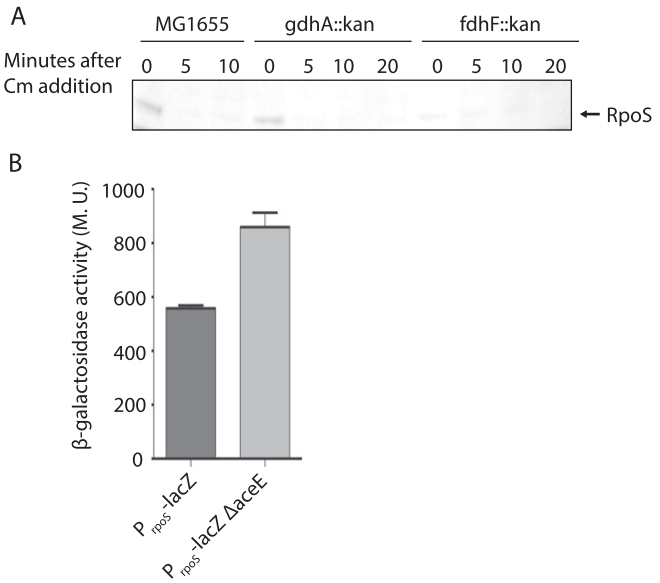

Fig. S1. Effects of metabolic mutants on RpoS. (*A*) *gdhA* and *fdhF* deletions have no effect on RpoS stability. Strains were grown at 37 °C in LB to an OD_{600} of ∼0.3, and protein synthesis was inhibited by Cm addition. Samples were then removed at the indicated time points, and RpoS levels were analyzed by Western blotting using an anti-RpoS antiserum. The following strains were used: WT (MG1655), *gdhA::kan* (BA513), and *fdhF::kan* (BA515). (*B*) *aceE* deletion only moderately affects the expression of an *rpoS-lacZ* transcriptional fusion. Strains containing *rpoS-lacZ* transcriptional fusions (P_{rpoS}-lacZ: NM6006; P_{rpoS}-lacZ Δ*aceE*: BA453) were grown at 37 °C in LB to an OD_{600} of ∼0.3. Samples were taken from each culture, and β-galactosidase activity was measured as described by Miller (1). The mean from three replicates is presented, and the SEM is indicated by the error bars. M.U., Miller units.

1. Miller JH (1992) *A Short Course in Bacterial Genetics* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

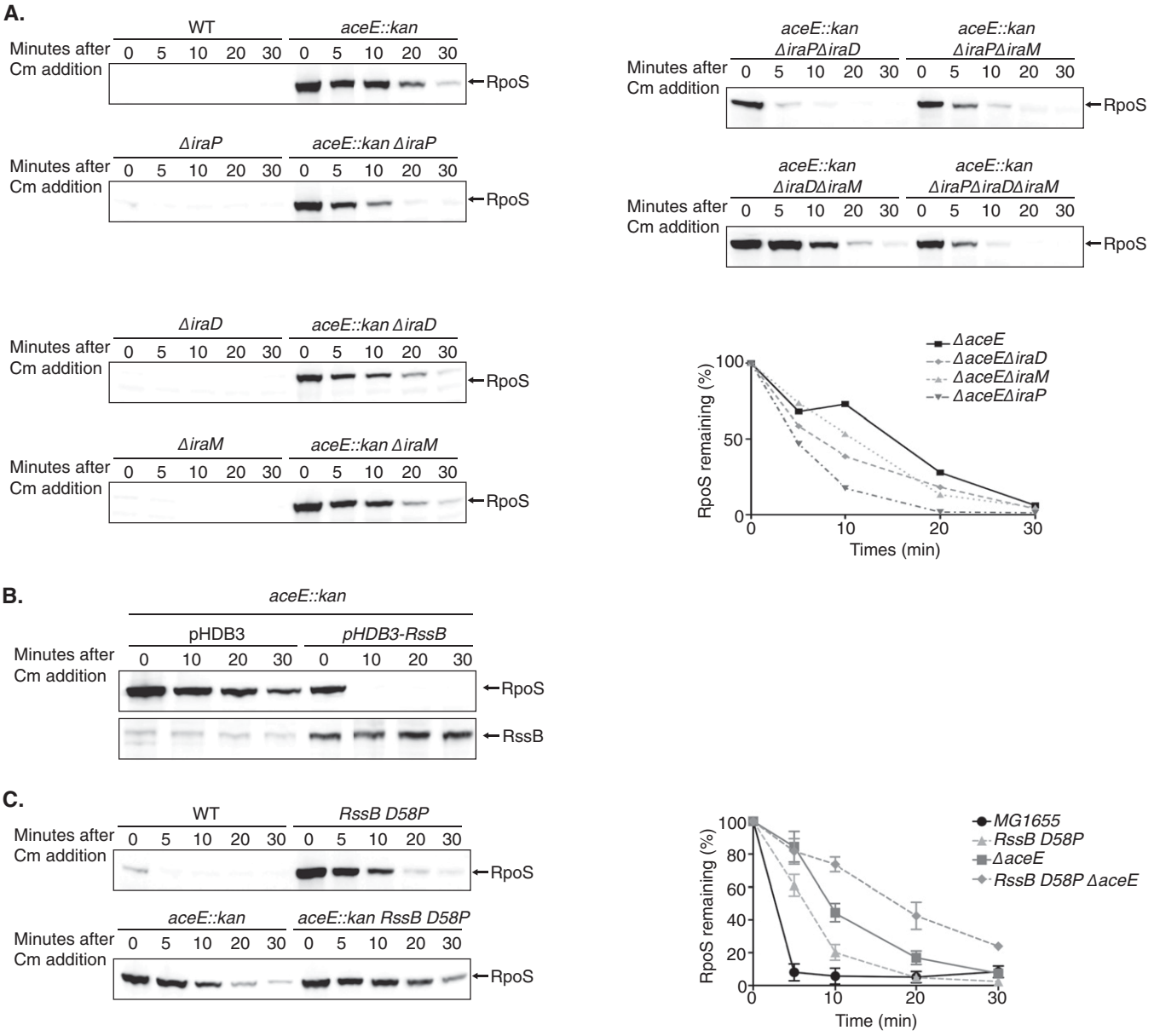

Fig. S2. RpoS stability in an *aceE* mutant is dependent on IraP and IraD, limiting RssB, and is independent of RssB phosphorylation. (*A*) Strains were grown at 37 °C in LB to an OD_{600} of ∼0.3, and protein synthesis was inhibited by Cm addition. Samples were then removed at the indicated time points, and RpoS levels were analyzed by Western blotting using an anti-RpoS antiserum. RpoS level was quantified in different genetic backgrounds. The following strains were used: WT (MG1655); Δ*aceE* (BA334); and the following isogenic derivatives: Δ*iraP* (AB047), Δ*iraP* Δ*aceE* (BA426), Δ*iraD* (AB046), Δ*iraD* Δ*aceE* (BA428), Δ*iraM* (DP003), Δ*iraM* Δ*aceE* (BA430), 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). For the graph, the intensity measured at time 0 for each strain was set at 100%. (*B*) Strains containing pW6 (pHDB3-RssB; pW6) or the vector control (pHDB3) were grown at 37 °C in LB to an OD_{600} of ∼0.3. Protein synthesis was inhibited by Cm addition. Samples were then removed at the indicated time points after Cm addition, and RpoS levels were analyzed by Western blotting using an anti-RpoS antiserum (*Upper*) and an anti-RssB antiserum (*Lower*). The strain used was Δ*aceE* (BA334). (*C*) The following strains were grown and assayed as in *A*: WT (MG1655), DJ480 $rssB_{D58P}$ (YN868), Δ*aceE* (BA334), and DJ480 $rssB_{D58P}$ Δ*aceE* (BA442). For the graph, the intensity measured at time 0 for each strain was set at 100%. The mean from three replicates is presented, and the error bars indicate the SEM.

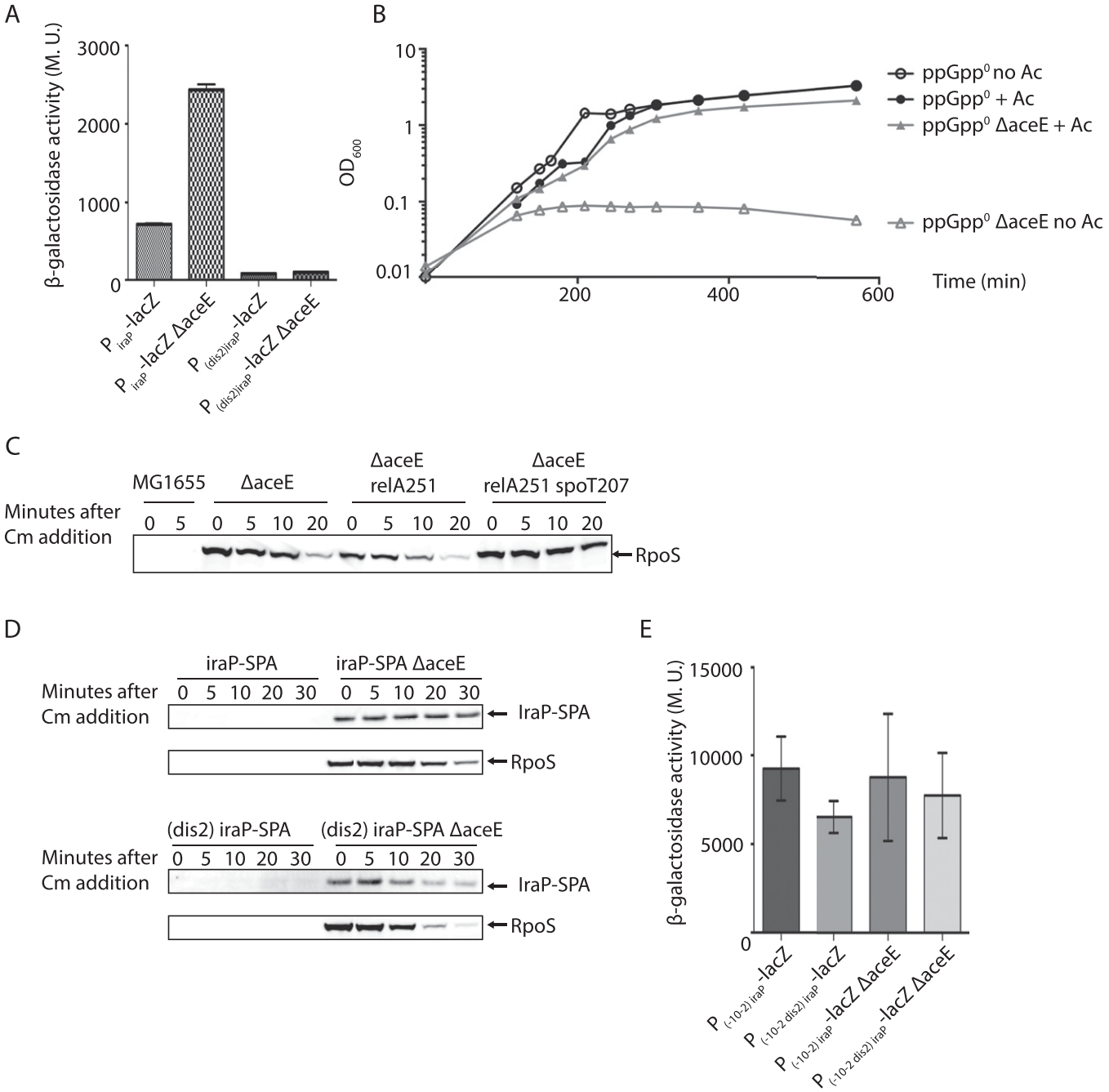

Fig. S3. Role of (p)ppGpp in anti-adaptor synthesis and RpoS stabilization and posttranscriptional induction of IraP. (*A*) Strains containing transcriptional fusions P_{iraP}-*lacZ* (WT, NM672; *ΔaceE*, BA592) or $P_{dis2iraP}$-*lacZ* (WT, NM674; *ΔaceE*, BA613) were grown at 37 °C in LB to OD_{600} of ∼0.3 and assayed for β-galactosidase activity as described by Miller (1). The mean from three replicates is presented, and the SEM is indicated by the error bars. M.U., Miller units. Note that these fusions contain −486 to +10 relative to the +1 of the *iraP* fusion and have a much higher basal level of expression than the fusion used in Fig. 2, which extends from −385 relative to the +1 through the 67-nt leader and the ORF to +325. (*B*) Strains were grown overnight at 37 °C in LB containing 30 mM sodium acetate (pH = 7) and were diluted into fresh LB with or without sodium acetate, and growth followed. Strains used were ppGpp^0 (BA785) and ppGpp^0 *ΔaceE* (BA787). (*C*) Strains were grown overnight at 37 °C in LB containing 30 mM sodium acetate (pH = 7), diluted in fresh LB without sodium acetate, and grown at 37 °C to an OD_{600} of ∼0.3. Protein synthesis was inhibited by Cm addition, and samples were removed and analyzed for RpoS by Western blotting. The following strains were used: MG1655, *ΔaceE* (BA334), *ΔaceE relA251* (BA434), and *ΔaceE relA251 spoT207* (BA600). (*D*) Strains were grown at 37 °C in LB to an OD_{600} of ∼0.3, protein synthesis was inhibited by Cm addition, and samples were analyzed for IraP-SPA, using anti-flag antiserum (*Upper*), and for RpoS (*Lower*). The following strains were used: *iraP*-SPA (BA424), *iraP*-SPA *ΔaceE* (BA460), (dis2)*iraP*-SPA (BA644), and (dis2) *iraP*-SPA *ΔaceE* (BA673). (*E*) Strains all carry transcriptional fusions of *iraP* with mutant derivatives of the promoter. Cells were grown to an OD_{600} of 0.3 and assayed. Strains were $p_{-10–2iraP}$-*lacZ*: (BA875), *aceE* derivative (BA878), $p_{-10–2dis2iraP}$-lacZ (BA876), and *aceE* derivative (BA880).

1. Miller JH (1992) *A Short Course in Bacterial Genetics* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

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24rpoS + 8rpoS:        CGTCAAGGGATCACGGGTAGGAGCCACCTT atg AGT CAG AAT ACG CTG AAA GTT GCC
24rpoS+lac             CGTCAAGGGATCACGGGTAGGAGCCACCTT atg ACC ATG ATT ACG GAT TCA CTG GCC
lac +8rpoS   AATTGTGAGCGGATAACAATTTCACACAGGAAACAGCT atg AGT CAG AAT ACG CTG AAA GTT GCC
lac:         AATTGTGAGCGGATAACAATTTCACACAGGAAACAGCT atg ACC ATG ATT ACG GAT TCA CTG GCC
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Fig. S4. Alignment of *rpoS* leader and initial sequence and *lac* chimeras. Sequences from *rpoS* are in black; sequences from *lac* are in red. Fusions are those used in Fig. 3*B*. Note that the *lac* leader also contains a binding site for the *lac* repressor. All assays reported here are in the absence of inducer.

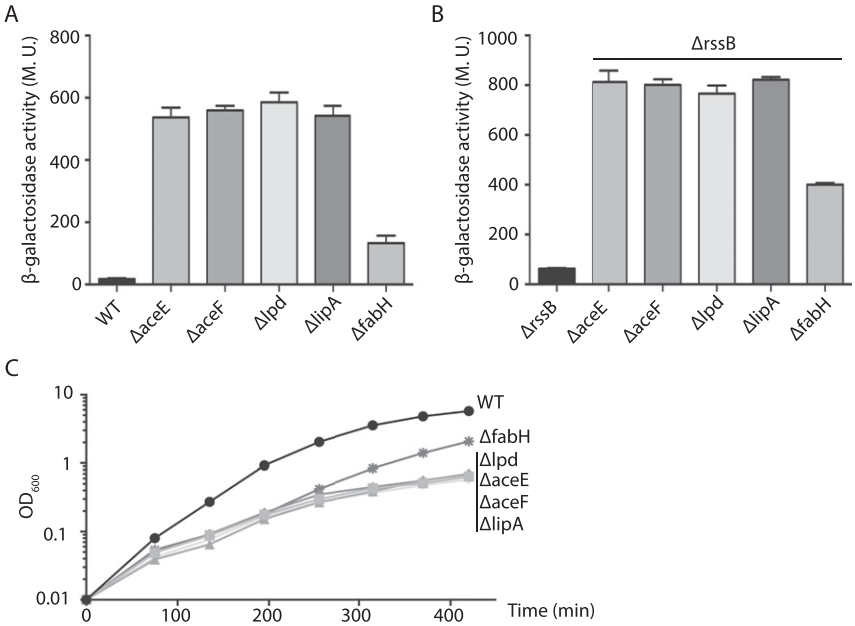

Fig. S5. Mutations that inactivate any subunit of PDH induce RpoS-lacZ. (*A*) Strains all carry the *imm*21 *rpoS-lacZ* translational fusion as in Fig. 1*B* and were grown and assayed as in Fig. 1*B*. The fusion protein is subject to degradation. Strains used were WT (SG30013); Δ*aceE* (BA811); Δ*aceF* (BA813); Δ*lpd* (BA815); Δ*lipA* (BA817); and Δ*fabH* (BA731). (*B*) Strains carry the same fusion as in *A*, but are all deleted for *rssB*; therefore, there is no degradation of the fusion. Strains were grown and assayed as for Fig. 1*B*. Strains used were *rssB* (SG30018); Δ*aceE* (BA810); Δ*aceF* (BA812); Δ*lpd* (BA814); Δ*lipA* (BA816); and Δ*fabH* (BA733). (*C*) Growth of strains used in *A* was determined in LB at 37 °C.

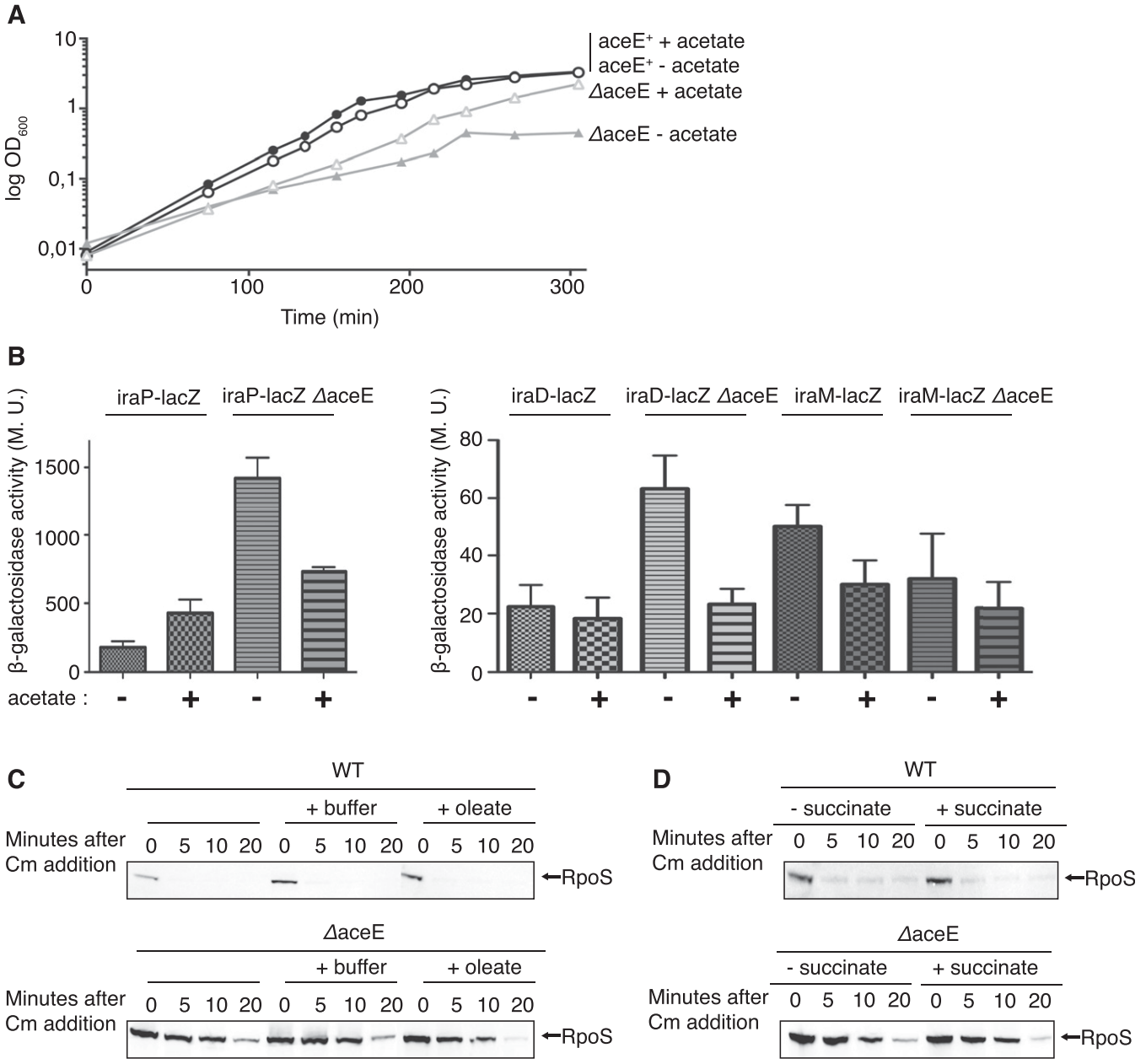

Fig. S6. Effects of acetate, oleate, and succinate addition on RpoS expression in an *aceE* mutant. (*A*) Cultures were inoculated from overnight cultures grown with 30 mM sodium acetate (pH = 7) into LB with or without sodium acetate and followed over time. The following strains were used: *aceE*$^+$ (*rssB*::tet *rpoS*-lacZ translational fusion; SG30018) and isogenic *aceE*$^-$ derivative (BA679). (*B*) Strains containing transcriptional fusions *iraP-lacZ* (WT, AB060; *ΔaceE*, BA407), *iraD-lacZ* (WT, AB050; *ΔaceE*, BA411), or *iraM-lacZ* (WT, AB042; *ΔaceE*, BA409) were grown at 37 °C in LB in absence or presence of acetate (30 mM) to an OD_{600} of ~0.3. Samples were taken from each culture, and β-galactosidase activity was measured as described by Miller (1). The mean from three replicates is presented, and the SEM is indicated by the error bars. M.U., Miller units. (*C*) Strains were grown at 37 °C in LB in absence or presence of oleate (200 mg·ml^{-1}) to an OD_{600} of ~0.3, and protein synthesis was inhibited by addition of Cm. Samples were then removed at the indicated time points, and RpoS levels were analyzed by Western blotting using an anti-RpoS antiserum. The following strains were used: WT (MG1655) and *ΔaceE* (BA334). (*D*) Strains were grown at 37 °C in LB in absence or presence of succinate (0.2%) to an OD_{600} of ~0.3, and protein synthesis was inhibited by addition of Cm. Samples were then removed at the indicated time points, and RpoS levels were analyzed by Western blotting using an anti-RpoS antiserum. The following strains were used: WT (MG1655) and *ΔaceE* (BA334).

1. Miller JH (1992) *A Short Course in Bacterial Genetics* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

Other Supporting Information Files

Table S1 (DOC)
Table S2 (DOC)
Table S3 (DOC)