Supplemental material for:

Multiple envelope stress response pathways are activated in an *Escherichia coli* strain with mutations in two members of the DedA membrane protein family.

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Table S1: PCR Primers used in this study.

Name	Sequence (5'->3') [Restriction Sites Underlined]
pRS101fp	GCGCGTCGACTGCTTCCCTTGAAACCCTGAAACTGATCCCCATAA TAAGCGAAGTTAGCGAGATGAATGCGAAAAAAACGCGGAGAAATT CATGACCATGATTACGGATTCAC (SalI)
pRS101rp	GCGCGGATCCTTATTTTTGACACCAGACCAACTG (BamHI)
pRS102fp	GCGCGTCGACGAACTTCAGGCTATAAAACGAATCTGAAGAACACAG CAATTTTGCGTTATCTGTTAATCGAGACTGAAATACATGACCATGATT ACGGATTCACTG (SalI)
pRS102rp	GCGCGGATCCCTGATAAGCGCAGCGTATCAG (BamHI)
pRS103fp	GCGCGTCGACAAACTAATGAGACGAATCTGATCGACGCAAAAAGTC CGTATGCCTACTATTAGCTCATGACCATGATTACGGATTCACTG (SalI)
pRS104fp1	GCGCGTCGACACTAGTATCATTCCGCGAAACGTTTCAGGAAGAGAA ACTCTTAACGATGACCATGATTACGGATTCACTG (SalI)
pRS104fp2	GAGCGCGTCGACAGCTTATGACTAAGAGCACCACG (SalI)
pRS104rp	GCGCGCACTAGTGTAGCCAGCCAATAAGG (SpeI)
pBADMdfAfp	GCGCGCCTCGAGCAAAATAAATTAGCTTCCGGTGC (XhoI)
pBADMdfArp	GCGCGCAAGCTTTTACCCTTCGTGAGAATTTCCCATCTG (HindIII)
pRSpssAfp	GCGCCTCGAGTTGTCAAAATTTAAGC (XhoI)
pRSpssArp	GGGGAAGCTTACAGGATGCGGCTAATTAATCG (HindIII)
CATfp	GCGCGCGCATGCTGTGACGGAAGATCACTTCG (SphI)
CATrp	GCGCGCGTATACACCAGCAATAGACATAAGCG (AccI)

Supplemental Materials and Methods

Phospholipid Analysis: Overnight cultures of designated strains of *E. coli* were diluted 1:100 in fresh LB media supplemented with appropriate antibiotics and additives and were grown at 30°C to an $O.D_{600}$ ~ 1.0. Cells were diluted 1:3 into identical fresh LB medium pre-warmed to 44°C and grown in a shaking water bath for 30 min. $^{32}P_i$ (Perkin Elmer) was added to a final concentration of 10 µCi/ml, and growth was continued for 20 min. Following growth and labeling, phospholipids were directly extracted from *E. coli* cells in the culture media (1). Chloroform and methanol were added to cells to a final ratio of chloroform-methanol-water of 1:2:0.8. The extraction mixture was allowed to incubate for 1 hr at room temperature with occasional mixing. Insoluble material was removed by centrifugation in a micro-centrifuge for 15 min at 13,000 rpm at room temperature. The supernatant was transferred to a new tube, and chloroform and water were added to adjust the ratio of chloroform-methanol-water to 1:1:0.8, resulting in a two-phase mixture. The aqueous upper phase was discarded and the lower phase was washed with fresh pre-equilibrated upper phase. Lipid species were resolved by thin-layer chromatography (TLC) on Silica gel 60 plates (Merck) using the solvent chloroform-methanol-glacial acetic acid (65:25:10) and analyzed using a Phosphorimager equipped with IQMac software.

Reference:

1. **Thompkins, K., B. Chattopadhyay, Y. Xiao, M. C. Henk, and W. T. Doerrler.** 2008. Temperature sensitivity and cell division defects in an Escherichia coli strain with mutations in yghB and yqjA, encoding related and conserved inner membrane proteins. J Bacteriol **190:**4489-4500.

Figure S1.

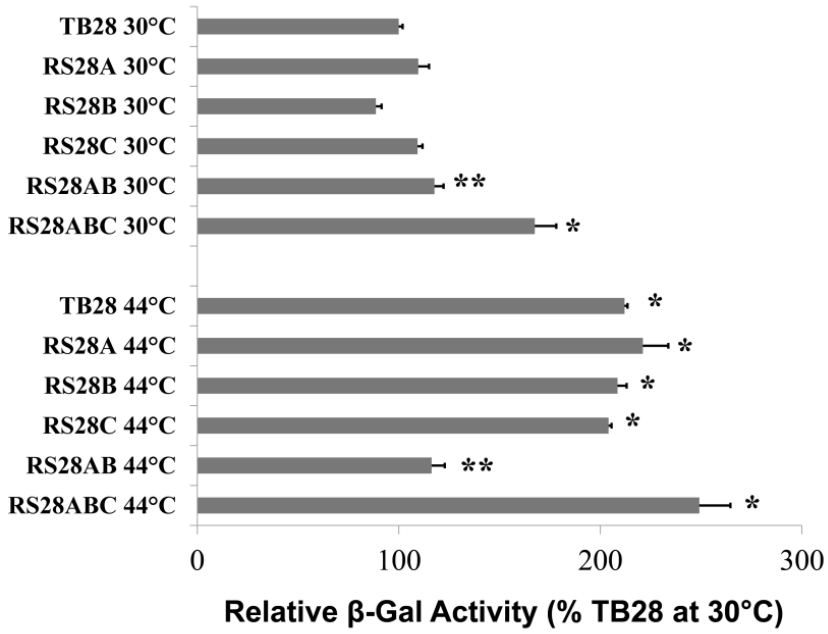

Figure S1: Repression of σ^E stress response in RS28AB by the activated Cpx pathway. RS28AB fails to induce the σ^E stress response at higher growth temperatures but regains this ability to do so upon removal of the *cpxR* gene in strain RS28ABC. Strains TB28, RS28A, RS28B, RS28C, RS28AB, and RS28ABC were transformed with plasmid pRS102 to measure induction of the σ^E stress response. Cells were grown to exponential phase (OD_{600} ~ 0.4 - 0.8) in rich media supplemented with appropriate antibiotics and assayed for β-galactosidase activity. All strains were grown at 30 °C as well as exposed to 44 °C for a period of 60 min. β-gal activity is expressed as percentage of that displayed by control wild-type strain TB28 grown at 30 °C. Each bar represents the average and standard deviation of three replicates of a representative experiment. Statistical significance was determined by Student's unpaired t-test. The significance values are represented as follows: (*) $p<0.001$ and (**) $p<0.01$ and unless pointed out, represent the significance of difference from the control experiment.

Figure S2.

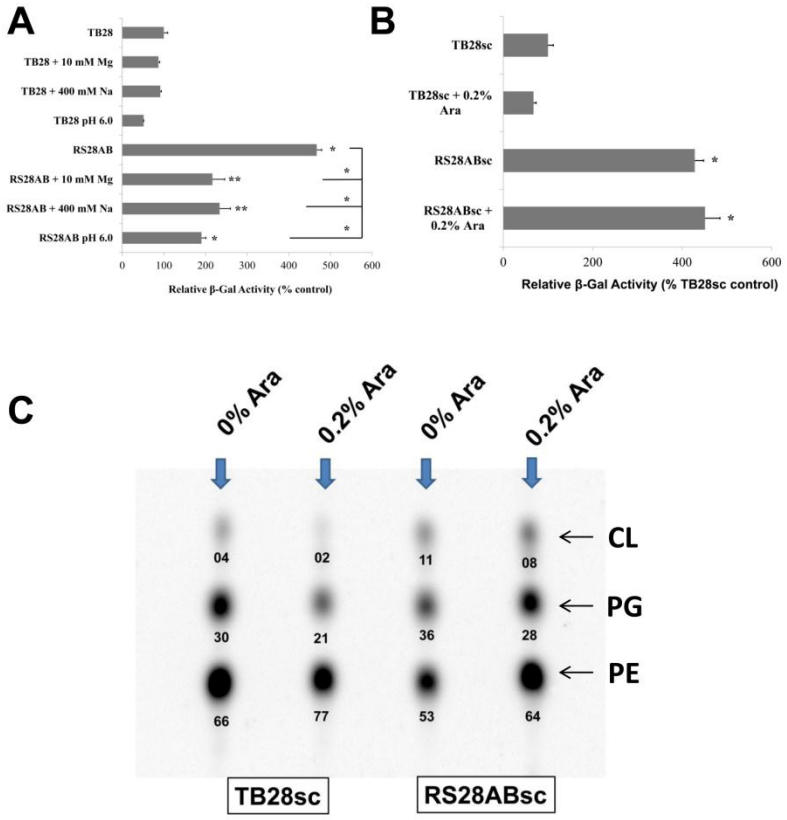

Figure S2: Addition of 10 mM Mg^{2+} and 400 mM NaCl or lowering the pH of the growth media partially attenuates the Cpx activation in RS28AB while restoration of PE level in RS28AB fails to attenuate its Cpx activation. (A) Strains TB28 and RS28AB transformed with plasmid pSH10 (Table 1) were grown in rich media at 30 °C with appropriate antibiotics and with or without 10 mM $MgCl_2$ and 400 mM NaCl. The pH of the growth media was lowered in indicated cases by buffering with 100 mM MES at pH 6.0. Cells were harvested during exponential growth and assayed for β-galactosidase activity. Addition of 10 mM $MgCl_2$ and 400 mM NaCl or growing the cells at pH 6.0 attenuates the Cpx activation in RS28AB by about 2-fold but the Cpx stress response pathway still remains significantly activated when compared to the wild type RS28AB. Activity is expressed as percentage of control strain TB28. Each bar is the average ± standard deviation of three replicates of a representative experiment. Statistical significance was determined by Student's unpaired t-test. The significance values are represented as follows: (*) $p<0.001$ and (**) $p<0.01$ and unless pointed out, represent the significance of difference from the control experiment normalized to 100%. (B, C) Strains TB28 and RS28AB, transformed with plasmids pRSpssAC and pSH10, are designated as TB28sc and RS28ABsc respectively (Table 1). TB28sc and RS28ABsc were grown in rich media at 30 °C supplemented with appropriate antibiotics and with or without the addition of 0.2% arabinose for overexpression of PssA. Cells were harvested during exponential growth and assayed for β-galactosidase activity (B) or for determining the phospholipid composition (C). Overexpression of PssA at 30 °C in RS28ABsc increases production of PE and restores its phospholipid levels closer to that found in wild-type TB28sc under uninduced conditions (C), but the activation of the Cpx stress response remains unaltered (B). Resolved phospholipids are designated on the TLC autoradiograph (C): Phosphatidylethanolamine (PE), phosphatidylglycerol (PG), cardiolipin (CL).

Figure S3.

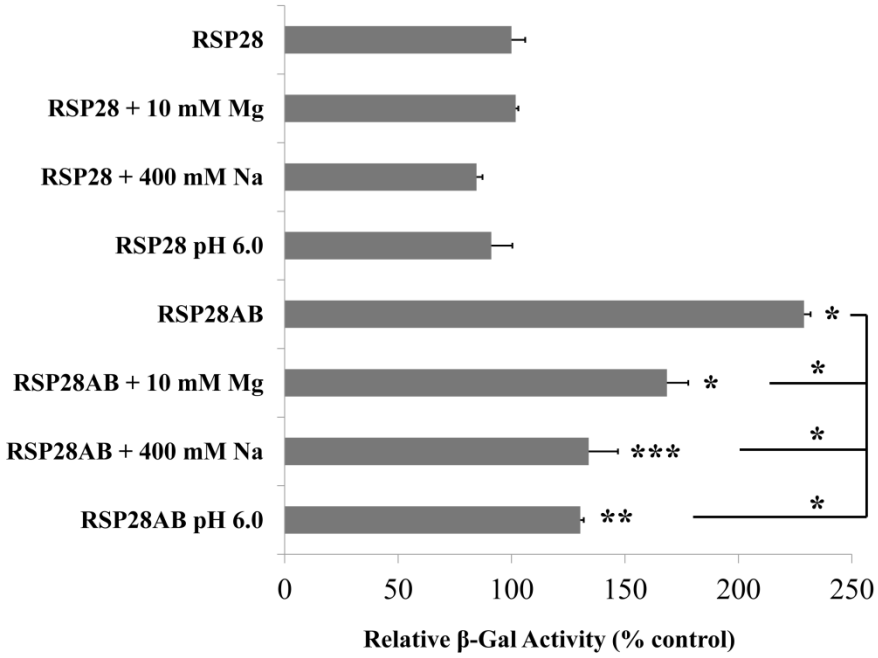

Figure S3: Partial attenuation of Psp activation in RS28AB is observed when either 10 mM Mg^{2+} or 400 mM NaCl is added to the growth media or when the cells are grown in rich media of pH 6.0. Strains RSP28 and RSP28AB (Table 1) were grown in rich media at 30 °C supplemented with appropriate antibiotics with and without 10 mM $MgCl_2$ or 400 mM NaCl. The pH of the growth media was lowered in indicated cases by buffering with 100 mM MES at pH 6.0. Cells were harvested during exponential growth and assayed for β-galactosidase activity. Addition of 10 mM $MgCl_2$ or 400 mM NaCl to the growth media or lowering its pH to 6.0 partially but significantly attenuates the Psp activation in RSP28AB. Standard deviation and statistical significance were determined as in Figure S1. The significance values are represented as follows: (*) $p<0.001$, (**) $p<0.01$ and (***) $p<0.05$ and, unless pointed out, represent the significance of difference from the control experiment.

Figure S4.

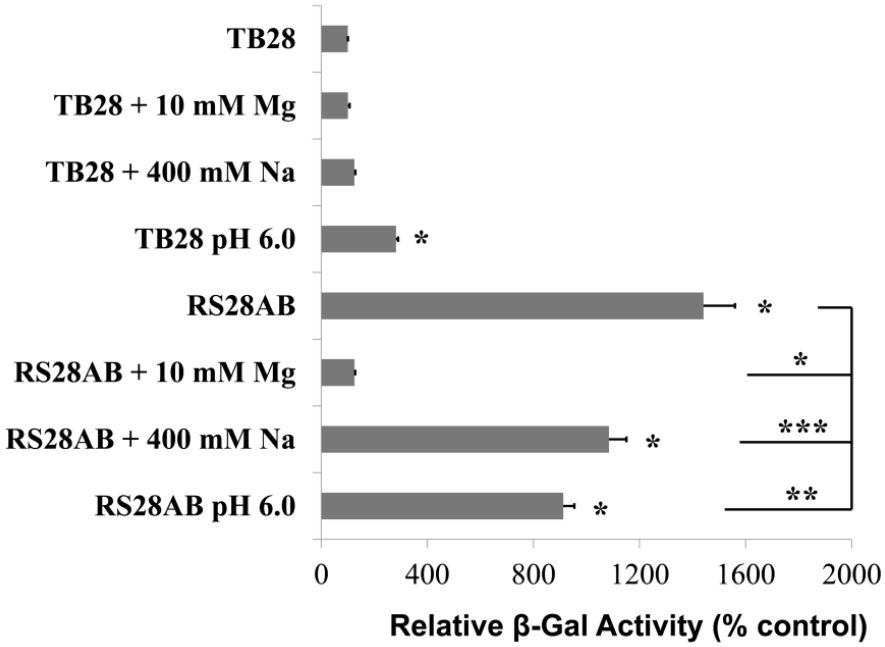

Figure S4: Attenuation of Rcs activation in RS28AB is observed in the presence of 10 mM Mg^{2+} or 400 mM NaCl or lowering of pH in the growth media. Strains TB28 and RS28AB harboring plasmid pRS103 (Table 1) were grown in rich media at 30 °C supplemented with appropriate antibiotics with and without 10 mM $MgCl_2$ or 400 mM NaCl as well as in LB media of pH 6.0 buffered with 100 mM MES. Cells were harvested during exponential growth and assayed for β-galactosidase activity. Addition of 10 mM $MgCl_2$ in the growth media completely attenuates the Rcs activation in RS28AB. On the other hand, 400 mM NaCl in the growth media or growth in LB media of pH 6.0 causes a partial yet significant attenuation of Rcs activation in RS28AB. Standard deviation and statistical significance were determined as in Figure S3.

Figure S5.

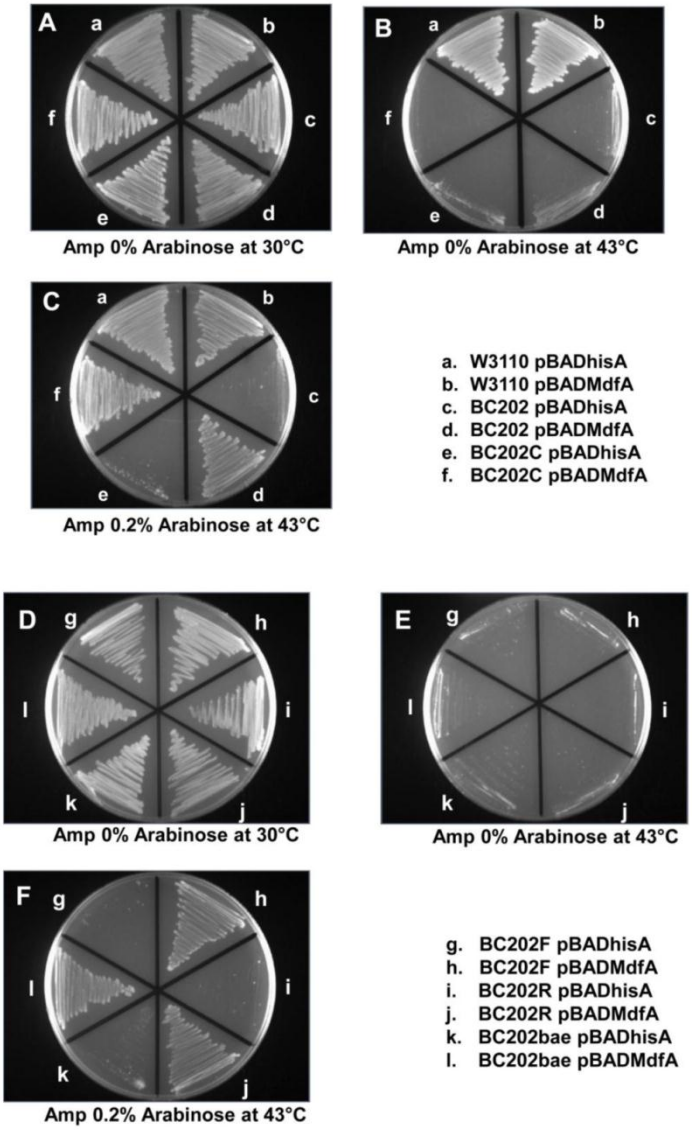

Figure S5: MdfA overexpression restores growth of strains BC202C, BC202R, BC202F and BC202bae at 43 °C. Strains W3110 (a, b), BC202 (c, d), BC202C (e, f), BC202F (g, h), BC202R (i, j), and BC202bae (k, l), transformed with either empty vector pBADhisA (a, c, e, g, i, k) or with plasmid pBADMdfA (b, d, f, h, j, l), were streaked on LB-Amp-agar plates with (C, F) or without (A, B, D, E) 0.2% arabinose and incubated overnight at 30 °C (A, E) or 43 °C (B, C, E, F). Overexpression of MdfA from plasmid pBADMdfA restored growth to BC202 (Bd versus Cd), BC202C (Bf versus Cf), BC202F (Eh versus Fh), BC202R (Ej versus Fj) and BC202bae (El versus Fl).

Figure S6.

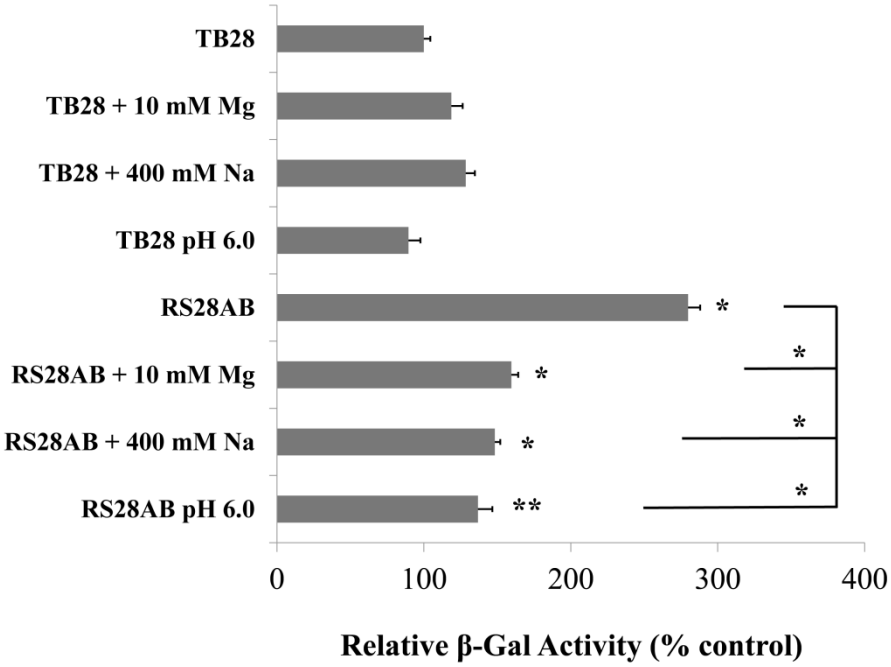

Figure S6: Partial attenuation of Bae activation in RS28AB is observed in the presence of 10 mM Mg^{2+}, 400 mM NaCl or by lowering the pH in the growth media. Strains TB28 and RS28AB harboring plasmid pRS104 (Table 1) were grown in rich media at 30 °C supplemented with appropriate antibiotics with and without 10 mM $MgCl_2$ or 400 mM NaCl as well as in LB media of pH 6.0 buffered with 100 mM MES. Cells were harvested during exponential growth and assayed for β-galactosidase activity. Addition of 10 mM $MgCl_2$ and 400 mM NaCl or growing the cells at pH 6.0 attenuates the Bae activation in RS28AB by about 2.0 - 2.5 fold but the Bae stress response pathway still remains significantly activated when compared to the wild type RS28AB. Standard deviation and statistical significance were determined as in Figure S3.