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Dual Molecular Signals Mediate the Bacterial Response to Outer-Membrane Stress

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Abstract

In Gram-negative bacteria, outer-membrane integrity is essential for survival and is monitored by the σ^E stress-response system, which initiates damage-repair pathways. One activating signal is unassembled outer-membrane proteins. Using biochemical and genetic experiments in *Escherichia coli*, we found that off-pathway intermediates in lipopolysaccharide transport and assembly provided an additional required signal. These distinct signals, arising from disruptions in the transport and assembly of the major outer-membrane components, jointly determined the rate of proteolytic destruction of a negative regulator of the σ^E transcription factor, thereby modulating expression of stress-response genes. This dual-signal system permits a rapid response to dysfunction in outer-membrane biogenesis, while buffering responses to transient fluctuations in individual components, and may represent a broad strategy for bacteria to monitor their interface with the environment.

The outer membrane (OM) is essential for survival of Gram-negative bacteria. In *Escherichia coli*, the σ^E stress-response system recognizes signals indicative of OM dysfunction and triggers an adaptive response by activating expression of gene products involved in the biogenesis, transport, and/or assembly of the lipopolysaccharides (LPS), phospholipids, and outer-membrane proteins (OMPs) that comprise the OM, and the proteases and chaperones that maintain or repair OM integrity (1,2). In this system, the RseA and RseB regulatory proteins and the DegS and RseP inner-membrane (IM) proteases transmit the signal that activates the σ^E transcription factor (fig. S1). RseA, a single-pass IM protein, has a cytoplasmic domain that binds and inhibits σ^E and a periplasmic domain ($RseA^P$) that binds RseB (3–5). Following stress, OMPs accumulate in the periplasm and their C-terminal residues bind to DegS and activate cleavage of $RseA^P$ (6), triggering a proteolytic cascade that frees σ^E to activate gene expression (1). However, a signal that inhibits RseB is also required, because RseB binding to $RseA^P$ prevents activated DegS

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from cleaving RseA (4,5,7). The RseB-inhibition signal has not been characterized but may be related to LPS, because alterations in LPS structure activate the σ^E response (8,9). Here we test and provide evidence for a model in which intermediates in LPS transport and assembly are the second signal that activates σ^E.

LPS antagonizes RseA•RseB binding in vitro

We developed an assay for ^{35}S-RseB dissociation from RseA^P-agarose and established that ^{35}S-RseB was efficiently eluted by both unlabeled RseB and RseA^P but not by unrelated proteins or an abundant bacterial phospholipid (Fig. 1A). LPS purified from an *E. coli* K-12 strain also had RseB-elution activity (Fig. 1B), as did LPS variants purified from various mutant strains (fig. S2, S3). LptA, a protein that is part of the periplasmic bridge that shuttles LPS from the IM to the OM (10), inhibited LPS elution of RseB (Fig. 1C), indicating that RseB and LptA compete for LPS. RseB competed more efficiently at 45 °C, a temperature that activates the cellular σ^E response, than at lower temperatures (Fig. 1C). Thus, altered competitive binding could contribute to σ^E activation in vivo.

Minimal LPS fragments bind RseB and facilitate cleavage of RseA

We sought to test whether fragments of LPS containing lipid A or di-[keto-deoxyoctulosonate]-lipid A (Kdo_2-lipid-A; Fig. 1D) also bound RseB, but both molecules had very low solubilities, precluding interpretation of biochemical studies. However, NaOH hydrolysis of lipid A or Kdo_2-lipid A, a treatment that partially removes acyl chains, improved solubility and allowed purification of active fragments (called L-IIA and Kdo_2-L-IIA) with masses expected for retention of two N-linked acyl chains but loss of all four O-linked acyl chains (Fig. 2A; fig. S4). The activities of both fragments were similar (Fig. 2B), indicating that the Kdo sugars in Kdo_2-L-IIA are not essential for RseB elution. Derivatives of lipid A devoid of acyl chains had no RseB-elution activity. LptA also inhibited RseB elution by Kdo_2-L-IIA, with inhibition being less efficient at higher temperature (Fig. 2C). Thus, any LPS derivative that contains the phosphorylated GlcNAC disaccharide and N-linked acyl chains of the lipid-A moiety (colored dark gray in Fig. 1D) appears to bind RseB and displace RseA^P.

Although metabolically irrelevant, L-IIA and Kdo_2-L-IIA were useful LPS surrogates because they did not scatter light, permitting the use of fluorescence anisotropy to monitor RseB binding (4). L-IIA dissociated a complex of fluorescent RseA^P fl-RseA^P) and RseB in a concentration-dependent manner but did not alter anisotropy of fl-RseA^P alone (Fig. 2D), confirming competition between L-IIA and fl-RseA^P for RseB binding (also see fig. S5). Dissociation of the RseB•fl-RseA^P complex was complete within 30 s of addition of L-IIA (Fig. 2E), a time rapid enough to account for the kinetics of the cellular σ^E response following a stress treatment (11).

Purified RseB is a mixture of dimers and tetramers, with only the dimer binding RseA (4,12). When we added L-IIA to freshly purified RseB dimers and re-chromatographed the mixture, most protein co-eluted with L-IIA at an RseB-tetramer position (Fig. 3A). L-IIA alone eluted near the column salt volume (fig. S6). Thus, L-IIA binds directly to RseB, L-IIA complexes with RseB largely persist during the ~1 h required for chromatography, and L-IIA binding stabilizes RseB in a tetrameric state that does not bind RseA.

We tested the effects of RseB, L-IIA, and OMP peptide on DegS cleavage of RseA^P in vitro (Fig. 3B). As expected (4–6), DegS alone did not cleave RseA^P, addition of OMP peptide activated cleavage, and further addition of RseB inhibited this cleavage. However, addition of L-IIA to OMP peptide restored robust DegS cleavage of RseA^P in the presence of RseB.

Other combinations did not restore cleavage. L-IIA alone did not activate DegS, confirming that it acts to inhibit RseB.

LPS plays an evolutionarily conserved role

Most γ and β proteobacteria have RseA, RseB, and DegS orthologs, with MucA, MucB, and AlgW being their functional equivalents in *Pseudomonas aeruginosa*, a distant relative of *E. coli* (12). We tested the generality of our findings by examining the interaction of *E. coli* LPS fragments with the *P. aeruginosa* proteins. L-IIA largely converted MucB dimers to tetramers (Fig. 3C) and eluted ^{35}S-MucB from a MucA^{P}-affinity column (Fig. 3D), supporting conservation of an LPS-mediated displacement mechanism. L-IIA also allowed OMP-activated AlgW to cleave MucA in the presence of MucB (Fig. 3E). Thus, in both the *E. coli* and *P. aeruginosa* systems, an LPS molecule and OMP peptide mimic the signals that result in RseA/MucA cleavage in vivo by inactivating RseB/MucB and activating DegS/AlgW, respectively.

σ^{E} activation by wild-type and mutant LPS

Our results predict that increased periplasmic LPS should activate the cellular σ^{E} response. Following synthesis on the IM, LPS is shuttled over an Lpt protein bridge and inserted into the OM by the LptD/E translocon at the distal end of the bridge (13–15) (see Fig. 5). The LptD$^{\Delta 330–352}$ variant has fewer functional bridges because of the loss of key disulfide bonds and should cause LPS accumulation in the periplasm (15–17). We found that the *lptD*$^{\Delta 330–352}$ allele increased expression of a β-galactosidase reporter under σ^{E}-transcriptional control in a strain requiring inhibition of RseB but not requiring OMP signal (*degS*$^{\Delta PDZ}$) and in a strain requiring both inhibition of RseB and an OMP signal (*degS*$^{+}$) (Fig. 4A; fig. S7). Although RseA cleavage neither requires nor is activated by OMPs in *degS*$^{\Delta PDZ}$ cells because the autoinhibitory PDZ domain of DegS is missing, RseB normally keeps σ^{E} activity low (4,7,18). Thus, increased σ^{E} activity in the *lptD*$^{\Delta 330–352}$ *degS*$^{\Delta PDZ}$ strain supports a model in which accumulation of periplasmic LPS relieves RseB inhibition. The activation of σ^{E} in *lptD*$^{\Delta 330–352}$ *degS*$^{+}$ cells suggests that OMP intermediates also accumulate (6,7), probably as a consequence of defects in LPS assembly, as we discuss below. We also decreased the level of properly disulfide-bonded LptD by deleting the DsbA disulfide oxidoreductase (16,17). Although the *ΔdsbA* mutation affects many proteins in addition to LptD, it increased σ^{E} activity in *degS*$^{\Delta PDZ}$ and *degS*$^{+}$ strains (fig. S8).

Using *degS*$^{+}$ and *degS*$^{\Delta PDZ}$ strains, we tested σ^{E} activity in a panel of 11 LPS-biosynthesis mutants to determine if they relieved RseB inhibition and/or activated DegS. The mutant LPS variants all contained the minimal RseB-interaction motif (fig. S9). Nine mutants elevated σ^{E} activity in *degS*$^{+}$ and *degS*$^{\Delta PDZ}$ strains (Fig. 4B,C), supporting a model in which activation is linked to LPS inhibition of RseB and to the generation of an OMP signal that activates DegS. In a *ΔrseB degS*$^{\Delta PDZ}$ strain in which OMP signal is not required and negative regulation by RseB is abrogated, the most strongly inducing LPS biosynthesis mutations did not further activate σ^{E} (Fig. 4D).

RseB as a periplasmic LPS sensor

Our results support a model in which LPS prevents RseB from blocking RseA cleavage. Intact LPS or LPS fragments containing a portion of the lipid-A scaffold bind RseB and release RseA, allowing its cleavage by DegS. Functionally, detection of part of the lipid-A moiety of LPS ensures that RseB can sense and respond to many mislocalized LPS species, including wild-type LPS, incompletely synthesized LPS molecules that reach the periplasm, and LPS variants lacking O-linked acyl chains or core sugars that support *E. coli* viability but decrease the ability of the OM to protect against cytotoxic agents.

Mutant LPS molecules with the fewest core sugars and phosphates activated σ^E to the highest levels in *rseB*$^+$ cells (Fig. 4B,C; fig. S9). By analogy with the activation observed when LPS transport was disrupted by partially disabling LptD, we propose that these mutations disrupt synchrony between one or more discrete steps in LPS biosynthesis, transport or OM insertion in ways that increase periplasmic LPS (Fig. 5), which could transit to RseB either through free diffusion or handoff. During exponential growth, the flux of LPS through the periplasm is ~70,000 molecules min^{-1}. Changes in the coordination of individual biogenesis and transport steps that diverted ~10% of these molecules would result in a ~40 μM min^{-1} rise in periplasmic LPS (19). Likewise, environmental stress could affect the integrity or function of the Lpt machinery for LPS transport or OM insertion, causing periplasmic accumulation of LPS. Temperature stress might have additional direct effects. LptA, a key component of the transenvelope bridge (10,15), competed less efficiently with RseB for binding to LPS at 45 °C, suggesting that heat shock facilitates transfer of LPS from LptA to RseB, providing a "feedforward" mechanism for σ^E activation.

Dual-signal logic and σ^E control

Two signals are required for DegS cleavage of RseA when RseB is present in vitro. An OMP signal activates proteolysis by DegS, whereas an LPS signal prevents RseB from blocking cleavage of RseA. The importance of both signals is also clear in certain genetic backgrounds in vivo. For example, mutations expected to increase periplasmic LPS activate the σ^E response in *degS*$^{\Delta PDZ}$ cells, which require RseB inhibition but not OMP activation. Similarly, expression of OMP signals alone is sufficient to strongly induce the σ^E response in *ΔrseB* cells (7). This requirement for dual signals is reminiscent of an 'AND' operation in symbolic or digital logic.

σ^E activates transcription of genes for the chaperones and machines that transport and insert both LPS and OMPs into the OM. Crosstalk between these pathways could add an additional layer of regulation (2,13,20,21). If defects in LPS biogenesis created problems with OMP biogenesis and vice versa, then both signals needed to activate σ^E would be produced when either pathway is perturbed. Indeed, *rfaC*, *rfaF*, and *rfaP* LPS mutations activate σ^E in strains requiring only RseB inhibition (*degS*$^{\Delta PDZ}$ *rseB*$^+$; Fig. 4C), only OMP activation (*degS*$^+$ *ΔrseB*; fig. S11), or both (*degS*$^+$ *rseB*$^+$; Fig. 4B). Supporting this idea, the OM of *rfaC*, *rfaF*, and *rfaP* strains have significantly reduced levels of OMPs (22–24), suggesting problems with OMP biogenesis. Conversely, the LptD component of the LPS translocon is transported and inserted into the OM by the same chaperones and machinery as other OMPs (16,25), and thus defects in OMP biogenesis would soon lead to problems in LPS transport and assembly. Additionally, the combination of 'AND' logic and cross signaling permits the σ^E response to reflect the stress level. Low levels of off-pathway OMPs and LPS may be constantly present in the periplasm, as basal cleavage of RseA by DegS is essential for survival (26). Thus, transient increases in either signal would result in an increased but buffered response, whereas a full and metabolically expensive response would be mounted only when RseB and DegS detect high concentrations of off-pathway LPS and OMPs, a condition indicative of extensive dysfunction in OM biogenesis.

Most unicellular organisms are enclosed by cell walls or envelopes that provide both a barrier to and an interface with the environment. These external structures are built from components that are synthesized in the cytosol but are assembled outside of the cytoplasmic membrane. Monitoring the flux of these molecules in the vicinity of the membrane coupled with dual-signal logic to buffer spurious responses may be a common regulatory strategy enabling precise homeostatic control of these critical barrier structures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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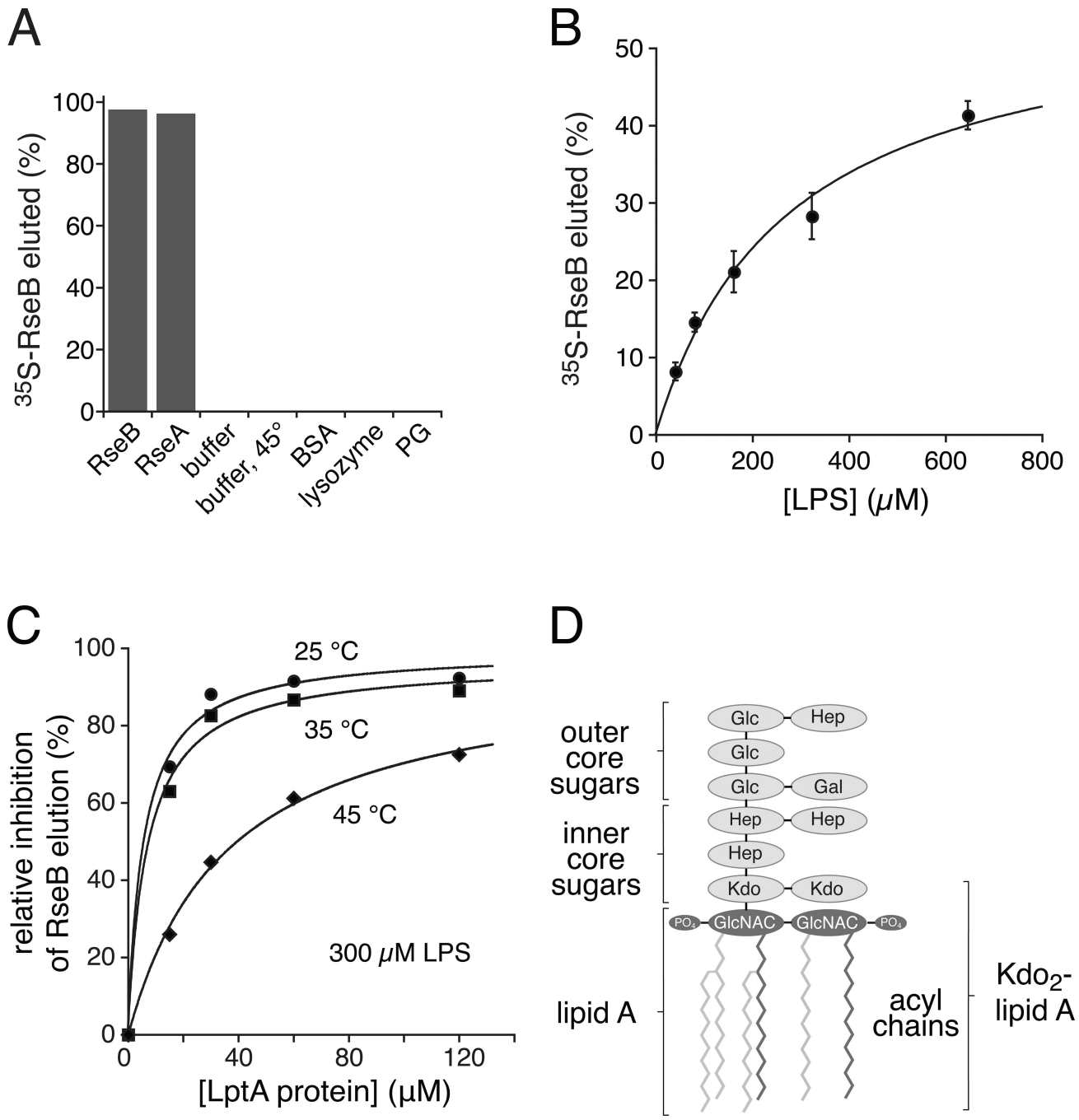

Fig. 1.

LPS displaces RseA from RseB. (**A**) RseB (35 μM) and $RseA^P$ (100 μM) eluted ^{35}S-RseB (~1 μM) from $RseA^P$-agarose, whereas buffer controls, albumin (BSA; 125 μM), lysozyme (125 μM), or phosphatidylglycerol (PG; 13 mM) did not. (**B**) LPS from *E. coli* K12 eluted ^{35}S-RseB from $RseA^P$-agarose (25 °C). Data (mean ± SD; *N*=3) were fit to a hyperbolic function (K_{app} = 270 μM). (**C**) LptA inhibition of RseB elution by LPS (300 μM) was more efficient at lower temperatures. (**D**) Structure of *E. coli* K12 LPS (GlcNAC, N-acetylglucosamine; Kdo, keto-deoxyoctulosonate; Hep, heptose; Gal, galactose; Glc, glucose). We show that the dark gray elements mediate RseB binding.

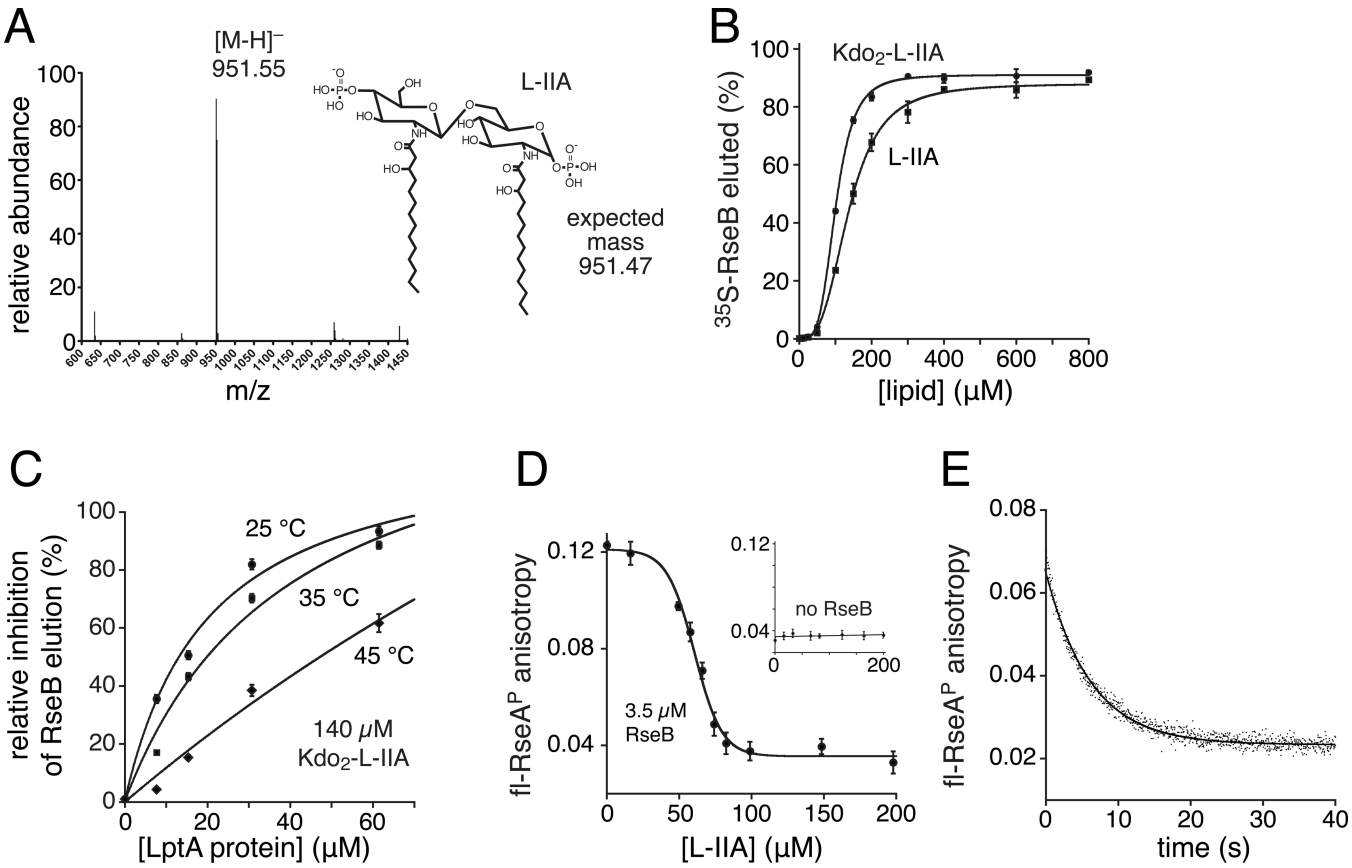

Fig. 2.

Lipid-A fragments disrupt $RseA^P$•RseB complexes. (**A**) Mass spectrometry and structure of the L-IIA fragment. (**B**) L-IIA and Kdo_2-L-IIA eluted ^{35}S-RseB from $RseA^P$-agarose (25 °C). Data (mean ± SEM; *N*=2) were fit to a Hill equation; L-IIA (K_{app} ~140 μM); Kdo_2-L-IIA (K_{app} ~100 μM). (**C**) Temperature dependence of LptA competition for Kdo_2-L-IIA (140 μM) elution of ^{35}S-RseB from $RseA^P$-agarose. Data are means ± SEM (*N*=2). (**D**) L-IIA competed for RseB (3.5 μM) binding to fl-$RseA^P$ (60 nM). Data (mean ± SD; *N*=5) were fit to a competition equation (EC^{50} ~65 μM). Inset: L-IIA did not change fl-$RseA^P$ (60 nM) anisotropy. (**E**) After premixing RseB (5 μM) and fl-$RseA^P$ (60 nM), dissociation was initiated by adding an equal volume of L-IIA (5 mM). The line is a single exponential fit (k = 0.16 s^{-1}).

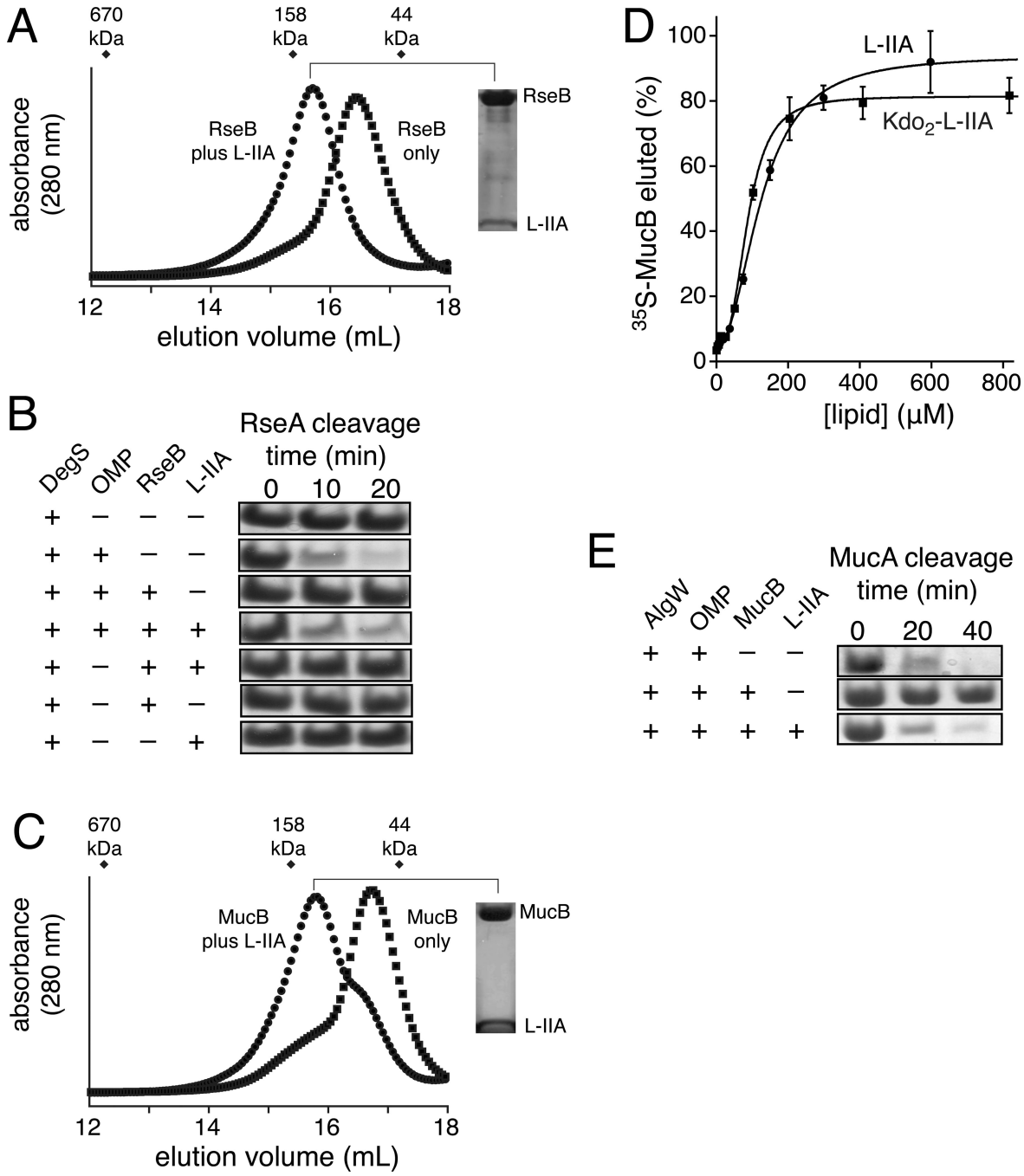

Fig. 3.

Lipid-A fragments bind RseB/MucB and coactivate cleavage of $RseA^P$/$MucA^P$. (**A**) After adding L-IIA (2 mM) to RseB (180 μM), the protein eluted from a gel-filtration column as expected for an RseB tetramer (~143 kDa). SDS-PAGE and silver staining of the "tetramer" peak fraction showed co-elution of RseB and L-IIA. See fig. S6 for gels across the entire included volume. (**B**) SDS-PAGE assay of $RseA^P$ (40 μM) degradation by DegS (4 μM trimer) in the presence or absence of RseB (40 μM monomer), OMP peptide (200 μM), and L-IIA (2 mM). (**C**) After adding L-IIA (2 mM), MucB (180 μM) largely eluted as a tetramer during gel filtration, although a dimeric shoulder was still evident. (**D**) L-IIA and Kdo_2-L-

IIA eluted ^{35}S-MucB from MucA^{P}-agarose. Data (mean ± SD; N=3) were fit to a Hill equation; L-IIA (K_{app} ~120 μM); Kdo$_2$-L-IIA (K_{app} ~83 μM). (**E**) SDS-PAGE assay of MucA^{P} (25 μM) degradation by AlgW (2 μM trimer) in the presence or absence of MucB (40 μM monomer), OMP peptide (75 μM), and L-IIA (2 mM).

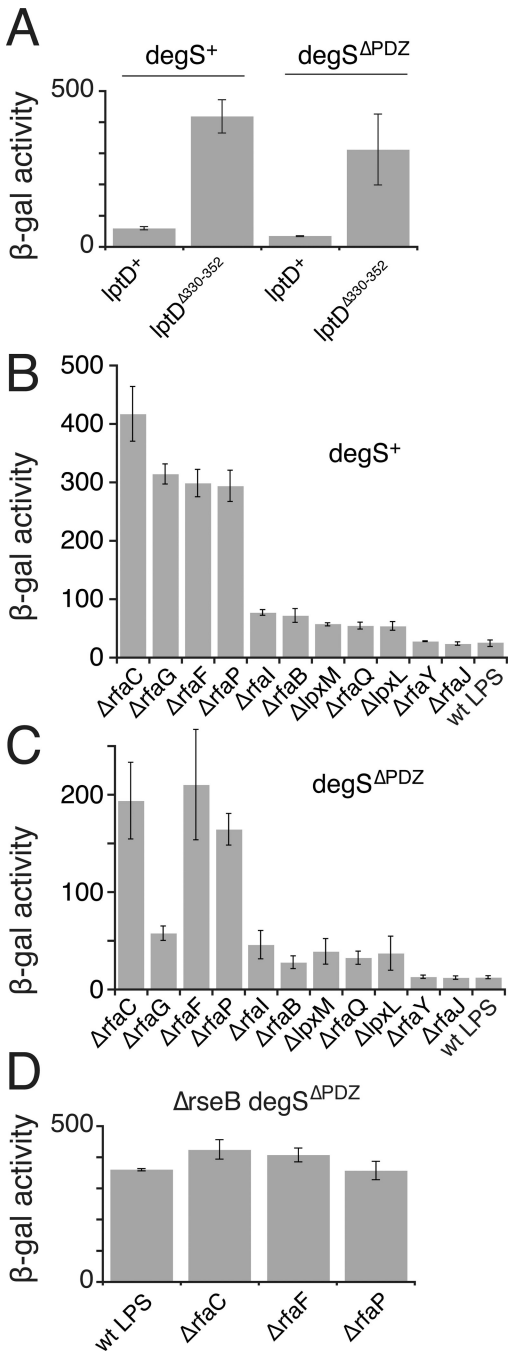

Fig. 4.

Activation of a σ^E-dependent rpoHp3-LacZ reporter under non-stress conditions by LPS-biogenesis mutations. (**A**) σ^E activity was enhanced by *lptD*$^{\Delta 330–352}$ in *degS*$^+$ and *degS*$^{\Delta PDZ}$ backgrounds. (**B**) σ^E activity was enhanced in a *degS*$^+$ strain by numerous LPS-biosynthesis mutations (see fig. s9 for structures). (**C**) Same as panel B but *degS*$^{\Delta PDZ}$. DegS$^{\Delta PDZ}$ has lower RseA-cleavage activity than OMP-activated DegS (18), probably accounting for the lower σ^E activities in this panel compared to panel B (also, see fig. S10). (**D**) σ^E activities were similar in *ΔrseB degS*$^{\Delta PDZ}$ strains producing wild-type LPS and some of the strongest LPS-biosynthesis mutations from panel C. In all panels, data are means ± 1 SD ($N \geq 4$).

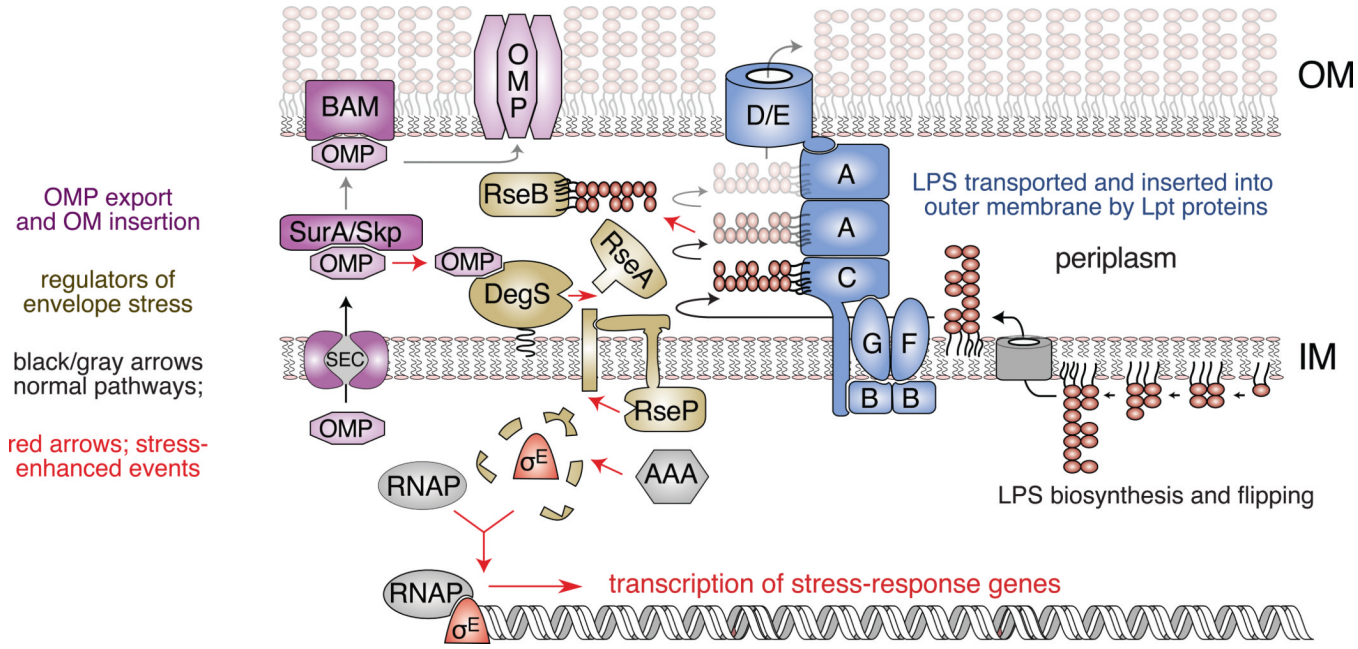

Fig. 5.

Dual-signal model for σ^E activation. LPS and OMPs accumulate in the periplasm when stress interferes with the normal pathways of transport and/or OM insertion. Periplasmic LPS binds RseB, freeing RseA to be cleaved by OMP-activated DegS, which initiates the σ^E stress response.