Detecting Envelope Stress by Monitoring β-Barrel Assembly

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INTRODUCTION

The cell envelope protects bacteria from their surroundings. Defects in its integrity or assembly are sensed by signal transduction systems, allowing cells to rapidly adjust. The Rcs phosphorelay responds to outer membrane (OM)- and peptidoglycan-related stress in enterobacteria. We elucidated how the OM lipoprotein RcsF, the upstream Rcs component, senses envelope stress and activates the signaling cascade. RcsF interacts with BamA, the major component of the β-barrel assembly machinery. In growing cells, BamA continuously funnels RcsF through the β-barrel OmpA, displaying RcsF on the cell surface. This process spatially separates RcsF from the downstream Rcs component, which we show is the inner membrane protein IgaA. The Rcs system is activated when BamA fails to bind RcsF and funnel it to OmpA. Newly synthesized RcsF then remains periplasmic, interacting with IgaA to activate the cascade. Thus RcsF senses envelope damage by monitoring the activity of the Bam machinery.

SUMMARY

The cell envelope protects bacteria from their surroundings. Defects in its integrity or assembly are sensed by signal transduction systems, allowing cells to rapidly adjust. The Rcs phosphorelay responds to outer membrane (OM)- and peptidoglycan-related stress in enterobacteria. We elucidated how the OM lipoprotein RcsF, the upstream Rcs component, senses envelope stress and activates the signaling cascade. RcsF interacts with BamA, the major component of the β-barrel assembly machinery. In growing cells, BamA continuously funnels RcsF through the β-barrel OmpA, displaying RcsF on the cell surface. This process spatially separates RcsF from the downstream Rcs component, which we show is the inner membrane protein IgaA. The Rcs system is activated when BamA fails to bind RcsF and funnel it to OmpA. Newly synthesized RcsF then remains periplasmic, interacting with IgaA to activate the cascade. Thus RcsF senses envelope damage by monitoring the activity of the Bam machinery.
RcsF is an OM lipoprotein that is absolutely required for sensing envelope damage caused either by chemicals targeting the LPS or the PG (Farris et al., 2010; Laubacher and Ades, 2008) or by mutations in genes involved in envelope assembly processes (Evans et al., 2013; Majdalani and Gottesman, 2005).

The Rcs pathway is the only signal transduction system which has an OM component that is necessary for sensing nearly all inducing cues. Yet, how RcsF senses envelope defects and conveys the signal to the downstream Rcs components has remained unknown. We found that RcsF is displayed on the cell surface by forming a complex with the abundant β-barrel protein OmpA. BamA, the central component of the β-barrel protein assembly machinery, plays a key role in this process by interacting with RcsF and funneling it to OmpA. When the Bam machinery cannot assemble the OmpA-RcsF complex, newly synthesized RcsF remains exposed in the periplasm. There, RcsF can interact with the less abundant protein IgaA, which we show is the downstream component of the signaling cascade, and activate the Rcs response.

RESULTS

RcsF Activates the Rcs System via IgaA

We first examined whether the two upstream components of the Rcs system, RcsF and IgaA, use a common pathway to transduce information to downstream components. In S. Typhimurium IgaA is an essential IM protein that inhibits the Rcs system (Cano et al., 2002; Domínguez-Bernal et al., 2004). We verified...
that igaA is also essential in E. coli and using an established pipeline for large-scale testing of genetic interactions (Typas et al., 2008), we showed that, among a collection of knockout mutants for all E. coli nonessential genes (Baba et al., 2006), an igaA deletion was viable only when combined with deletions of rcsB, rcsC, and rcsD (Figure S1A available online). Thus, E. coli and S. Typhimurium igaA play similar roles. Importantly, deletion of rcsF did not suppress igaA lethality, implying that igaA lies downstream of RcsF in the signaling cascade. In agreement with this configuration, depletion of igaA activated the Rcs system independently of RcsF (Figures 1B and S1B).

We next tested whether IgaA and RcsF are physically linked by expressing a tagged version of the only periplasmic domain of IgaA (IgaAper: ~32 kDa) in the periplasm and pulling down its interaction partners after crosslinking with DTSSP (3,3′-dithiobis(sulfosuccinimidylpropionate)), which cannot cross the IM. RcsF was identified by both mass spectrometry (MS) and western blot (Figure S1C and Table S1). Likewise, a purified tag-less version of IgaAper, and a soluble His-tagged version of RcsF interacted directly, forming a complex with a 1:1 stoichiometry (Figures 1C and S1D). These results support a model in which RcsF activates the Rcs system by interacting with IgaA, likely alleviating its inhibitory effect on the signaling cascade.

**By Forming a Complex with the β-Barrel Proteins OmpA and BamA, RcsF Is Occluded from IgaA**

Wild-type RcsF turns on the signaling cascade only upon envelope stress, suggesting that RcsF is physically occluded from IM IgaA under steady-state growth. This physical occlusion is tightly interconnected with the OM location of RcsF, as rerouting RcsF to the IM (RcsFIM) or expressing it as a soluble periplasmic protein (RcsFpen) constitutively activates the Rcs system (Farris et al., 2010; Tao et al., 2012). We therefore looked for the underlying occlusion mechanism.

RcsF is composed of a 31-residue intrinsically disordered N-terminal linker (S17-T47), which connects its globular domain (P48-K134; referred to as “signaling domain”) to the lipidated cysteine residue anchoring the protein to the OM (Figure S2A) (Levernier et al., 2011). We first tested whether this linker is cleaved under stress, releasing RcsF in the periplasm, by fractionating cells exposed to various Rcs-inducing stresses. RcsF was never detected in the soluble fraction (Figure S2B). We then determined whether RcsF was occluded from IgaA by other proteins. To find proteins interacting with RcsF, we performed in vivo DTSSP crosslinking in both ΔrcsF and wild-type cells. Three RcsF-containing protein complexes were detected (Figure 2A; marked as 1, 2, and 3). To identify them, the RcsF-interacting complexes were immunoprecipitated and analyzed by MS after reversing the crosslinks. We identified BamA, the core protein for β-barrel assembly, and the β-barrels OmpA, OmpC and OmpF as potential RcsF interacting partners (Table S2A). We further verified these interactions by analyzing the immuno-precipitated samples by western blot using antibodies specific for the interacting proteins. We confirmed that the ~115 kDa band (complex 1) contained BamA (~100 kDa) (Figure S2C). Only OmpA (38 kDa) could be detected in the ~55 kDa complex (complex 2) (Figure S2C), but not OmpC (40 kDa) and OmpF (39 kDa) (data not shown), suggesting that OmpA was the major interaction partner involved in this complex. Consistently, upon deleting the essential BamA or deleting ompA, the respective complexes disappeared (Figure 2B). Using an lpp deletion mutant, we identified that the protein involved in complex 3 (~25 kDa) was Lpp (8 kDa), the most abundant OM lipoprotein (Figure S2D).

We further verified the specificity of these interactions by site-specific photocrosslinking, inserting the crosslinkable amino acid p-benzoyl-L-phenylalanine (pBpa) at 25 specific positions in RcsF. Thereby we could map the interaction interface of RcsF with its binding partners more precisely, and lower the risk of nonspecific interactions (pBpa can only form covalent bonds with residues at a very close proximity [3 Å], whereas DTSSP has a 12 Å spacer). We selected 21 residues located on the surface of the signaling domain and 4 located in the N-terminal linker (Figure S3A). Following UV-exposure, 6/25 variants formed the previously observed 55 kDa complex with OmpA (Figures 2C and S3B). The identity of OmpA was confirmed by MS after immunoprecipitating RcsF(40pBpa)-OmpA (OmpC and OmpF were not detected in the sample, Table S2B). High complexity levels were observed when pBpa was inserted in the N-terminal linker and at the tip of the signaling domain of RcsF (Figure S3C). Four of these variants could also form the 115 kDa complex corresponding to BamA-RcsF (Figures 2C and S3D), which was confirmed with a BamA antibody (Figure S3D). As none of the 25 pBpa-containing variants was found in complex with Lpp, and as Lpp is the most abundant protein in E. coli, which could lead to nonspecific interactions, we decided not to follow up on this interaction. Altogether, these results indicated that RcsF interacts specifically with BamA and OmpA. Importantly, the levels of the RcsF(40pBpa)-OmpA complex were ~30%–40% of these of free RcsF, indicating that 25%-30% of total RcsF is bound to OmpA (Figures S3E and S3F). Given that photocrosslinking efficiency at optimal conditions can reach 40% (Zhang et al., 2011), we concluded that most RcsF is in complex with OmpA.

**The Bam Machinery Assembles the RcsF-OmpA Complex and Is Key for the Sensing Role of RcsF**

The interactions of RcsF with BamA and OmpA suggested that in nonstress conditions RcsF is occluded from IgaA by interacting with OM proteins, but that these interactions are disturbed upon envelope stress, enabling RcsF to interact with IgaA. If OmpA and BamA occlude RcsF from IgaA under nonstress conditions, then the Rcs system should be activated when bamA or ompA are knocked down/out. We found that an ompA deletion induced the Rcs system by ~3-fold, with induction being dependent on RcsF (Figure 2D). As OmpC and OmpF were also identified as RcsF partners, we tested the effect of deleting ompC or ompF on Rcs activity. Whereas the system was only marginally induced in the ompC mutant, the ompF deletion had no impact (Figure 2D). When ompC and ompF deletions were combined together or with ompA, synergistic effects were observed, but the absence of OmpA was clearly the most important contributor of the three to the activation of Rcs (Figure 2D), consistently with our interaction data (Figures 2 and S3). In contrast to the omp mutants, the Rcs system was fully induced in the bamA knockout (bamA101) mutant (Figure 2D). In this strain, BamA levels decrease ~5-fold without
significantly compromising β-barrel assembly (Aoki et al., 2008). These results are in agreement with the idea that OMPs (mainly OmpA) and BamA occlude RcsF from IgaA and suggest a dominant role for BamA in this process.

To dissect the signaling system further, we examined RcsF-BamA and RcsF-OmpA complex formation during Rcs activation by polymyxin B, A22, or mecillinam. All three chemicals induce the Rcs by targeting different cellular structures but always in an RcsF-dependent manner (Figure S4A) and without significantly affecting the transport of RcsF to the OM (Figure S4B). The cationic antimicrobial peptide polymyxin B damages the OM by perturbing the LPS leaflet, A22 inhibits the actin-like MreB, and the β-lactam antibiotic mecillinam inhibits the essential transpeptidase PBP2. After addition of subinhibitory amounts of each drug, we observed a sharp decrease in the levels of the BamA-RcsF complex within the timeframe that the Rcs system would be activated, while OmpA-RcsF remained largely unaffected (Figures 3A–3C). We also probed a galU

Figure 2. RcsF Forms Complexes with BamA and OmpA In Vivo, Which Prevents It from Activating the Signaling Cascade

(A and B) In vivo chemical crosslinking of RcsF in the periplasm. Wild-type and ΔrcsF cells were harvested at mid-log phase, washed and incubated with or without 1 mM DTSSP for 30 min. The reaction was quenched by addition of glycine (0.1 M), proteins were isolated by TCA precipitation, resuspended in sample buffer (without DTT) and subjected to SDS-PAGE and immunoblot analysis with an anti-RcsF antibody. Three complexes were observed (A), which were identified as RcsF-BamA (1), RcsF-OmpA (2) and RcsF-Lpp (3) (Figures S2C and S2D). Complexes 1 and 2 disappeared when repeating the DTSSP crosslinking in bamA101 and ΔompA cells, respectively (B).

(C) In vivo site-specific photocrosslinking of RcsF. Cells expressing RcsF(K40pBPA)-Flag-His or RcsF(Q79pBPA)-Flag-His from low-copy plasmids were irradiated with UV light (lanes 2 and 3) or not (lane 1), and protein samples were subjected to immunoblot analysis with an anti-RcsF antibody. BamA and OmpA were crosslinked with both RcsF mutants.

(D) ompA deletion and BamA depletion activate the Rcs system. An ompA deletion and a bamA knockout (bamA101) activated the Rcs system only in the presence of RcsF. Overexpression of bamA could restore basal Rcs activity in the ΔompA mutant. Deletions of ompC or ompF had marginal or no effects on Rcs activity. Double omp mutants induced the Rcs system further with OmpA being the most contributing factor. A chromosomal rprA::lacZ fusion was used to monitor Rcs activity, and specific β-galactosidase (β-gal) activity was measured from cells at mid-log phase (OD670 = 0.2–1). Error bars depict standard deviations (n > 4). See also Figures S2 and S3.
mutant that cannot produce UDP-D-glucose, a precursor for LPS and other surface-exposed sugars, and in which the Rcs system is constitutively turned on in an RcsF-dependent manner (Figure S4C) (Girgis et al., 2007). Similarly, the impact on the BamA-RcsF complex was stronger (Figure S4D). Thus, the BamA-RcsF complex was more responsive than the OmpA-RcsF complex, regardless of the stress applied. RcsF seems to be in a "locked" conformation with OmpA, which is not disrupted upon stress.

BamA is required for assembly of β-barrel proteins in the OM, including OmpA (Hagan et al., 2011). We postulated that BamA is also required for assembling the OmpA-RcsF complex. In this model, the BamA-RcsF complex would be an intermediate in the RcsF-OmpA formation during the assembly of the latter in the OM. Consistently decreasing BamA levels (bamA101) led to lower OmpA-RcsF levels (Figure 2B). Moreover, overexpressing BamA alone, without the other components of the Bam machinery (BamA alone cannot assemble OMPs [Hagan et al., 2010]), resulted in significantly higher levels of the BamA-RcsF complex, while the OmpA-RcsF complex almost disappeared (Figure 3D). BamA overexpression also restored basal Rcs activity in the ΔompA mutant (Figure 2D). These results indicated that: (1) overexpressed, nonfunctional BamA can act as a sink for RcsF, preventing Rcs activation, and (2) a functional Bam machinery is required to assemble the OmpA-RcsF complex, with BamA funneling RcsF to OmpA.

**Newly Synthesized RcsF Monitors the Activity of the Bam Machinery**

We established that the BamA-RcsF interaction is key in the activity of RcsF to activate the Rcs system and in the assembly of the OmpA-RcsF complex. However, it remained unclear if the two events are connected, i.e., does formation of the OmpA-RcsF complex play a role in the ability of RcsF to sense stress? We reasoned that as only active BamA can form the OmpA-RcsF

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*Figure 3. RcsF-BamA Is More Sensitive than RcsF-OmpA to Envelope Stress*

(A–C) RcsF-BamA and RcsF-OmpA complex formation upon treatment with different cues sensed by RcsF (see also Figure S4A). (A) Cells were treated with 0.5 μg/ml polymyxin B when they reached an OD_{600} of 0.4, and samples were collected 10 min later, crosslinked with DTSSP and immunoblotted with an anti-RcsF antibody. (B and C) Cells were treated with mecillinam (0.3 μg/ml) or A22 (5 μg/ml) when they reached an OD_{600} of 0.2, samples were collected at indicated time points after stress induction, and were subjected to DTSSP crosslinking and immunoblot. In all three stresses the RcsF-BamA complex disappeared when the Rcs system was activated (Figure S4A), whereas the RcsF-OmpA complex remained largely unaffected. (D) BamA overexpression shifts all RcsF to BamA. In vivo DTSSP crosslinking of wild-type cells harboring an empty vector (pET3a) or a vector expressing BamA (pBamA). In all panels, DTSSP crosslinking and immunoblot were done as in Figure 2A, and a representative experiment is shown (n = 3–4). See also Figure S4.
complex, if RcsF sensed the ability of BamA to funnel it to OmpA, then RcsF would actually monitor the activity of BamA. To test this hypothesis, we carefully dissected the interplay between RcsF, BamA, and OmpA. We first probed whether dissociation of preformed BamA-RcsF or inability of newly synthesized RcsF to bind to BamA upon stress triggers the Rcs system. To test this, we examined whether continuing RcsF synthesis was necessary for induction. We expressed RcsF to slightly higher steady-state levels than wild-type (130%), using an IPTG inducible promoter. We then stressed cells with A22, and simultaneously either shut down RcsF expression or kept it at the same steady-state levels. Only cells with continued RcsF synthesis rapidly induced the Rcs system. Cells with only “old” RcsF, albeit to wild-type levels, could not induce the Rcs system (Figure 4A). Similar results were obtained when we overexpressed RcsF and then completely shut down its expression, letting cells dilute RcsF to nearly wild-type levels before the A22 stress. A chromosomal rprA::lacZ reporter was used to measure Rcs activity. Empty dots: OD; filled dots: specific β-gal activity. RcsF levels were monitored in parallel (bottom). A representative experiment of four replicates is shown. (B) Preformed RcsF-BamA does not dissociate upon A22 stress. Cells were treated with chloramphenicol (300 μg/ml) when they reached an OD600 of 0.17, to block new protein synthesis, and subjected to A22 (5 μg/ml) 10 min later (OD was then ~0.2). Samples were collected 20 min after stress induction and subjected to DTSSP cross-linking and immunoblot. A control strain was grown without drug for the same time. The levels of the BamA-RcsF complex remained constant. (C) The Rcs system is more sensitive to RcsF levels in cells lacking OmpA. ΔrcsF and ΔrcsF ΔompA cells carrying rcsF under the same controllable expression system described in (A) were grown without inducer till an OD578 of 0.3. At this point, IPTG (20 μM) was added and Rcs activity (chromosomal rprA::lacZ) and RcsF levels (bottom) were monitored as a function of time. In ΔompA cells the Rcs system was activated almost instantaneously, before RcsF reached the steady state levels observed in wild-type (with chromosomal rcsF). In contrast, in cells carrying OmpA, the Rcs system was activated only when RcsF reached a >3-fold excess over the wild-type steady state levels, indicating that the presence of OmpA increases the capacity of the cell to “store” RcsF. As BamA is required for funneling RcsF to OmpA, the cell senses this way the BamA activity—i.e., the ability to form the OmpA-RcsF complex. Error bars depict standard deviations (n = 4). See also Figure S5.
period of time (Figure S4A). In contrast, mecillinam-mediated activation was slow, while polymyxin B-mediated activation was short-lived (Figure S4A) (Farris et al., 2010). The BamA-RcsF complex levels remained unchanged when protein synthesis was stopped before A22 addition (Figure 4B), whereas they decreased when protein synthesis was ongoing (Figure 3B). This suggests that the newly arriving RcsF cannot bind to BamA when cells are stressed, leading to the activation of the system. Consistently the BamA-RcsF complex disappeared faster than OmpA-RcsF after adding A22 or polymyxin B under ongoing protein synthesis (Figures 3A–3B). This is because BamA could presumably keep funneling RcsF to OmpA to a certain degree, preventing OmpA-RcsF from disappearing with dilution-like kinetics. We also stopped new protein synthesis by chilling the cells, then added polymyxin B and probed RcsF-BamA and RcsF-OmpA complex formation after 10 min. Both complexes remained intact (Figure S5B), which is in agreement with the inability of preformed RcsF-BamA to respond to stress.

These results indicated that constant synthesis of RcsF is required for RcsF to act as a sensor, and supported a model in which activation results from newly synthesized RcsF being unable to bind BamA. However, it remained unclear whether irreversible sequestration of RcsF by BamA was sufficient to keep the Rcs system off or whether continuous funneling of RcsF to OmpA was also required. To discriminate between these two possibilities, we compared the levels of RcsF that were required to activate the system in ΔompA and wild-type strains. While both strains have similar BamA levels (Figure S5C), BamA can funnel RcsF to OmpA only in the wild-type, thereby theoretically increasing its capacity for RcsF. We found that in the strain lacking ompA, the Rcs system was induced at a fraction (~80%) of wild-type RcsF levels, whereas wild-type cells could tolerate a ~3-fold increase in RcsF levels before inducing the Rcs system (Figure 4C). Thus, by funneling RcsF to OmpA, BamA increases its capacity for RcsF and maintains the Rcs system in an off state. This means that RcsF can monitor the capacity of BamA to assemble the OmpA-RcsF complex, which is presumably affected during stress. Since active BamA is required for OmpA-RcsF assembly (Figure 3D), RcsF senses this way the activity of the Bam machinery.

**RcsF, a Sensitive but Robust-to-Noise Sensor of BamA Activity**

Newly synthesized RcsF represents a small fraction of the RcsF pool, but is able to rapidly activate the system under stress. This led us to probe for the minimal RcsF protein levels required for activation. Taking advantage of the fact that periplasmic or IM-located RcsF constitutively activate the Rcs system (Farris et al., 2010), we established that RcsF_{perm} or RcsF_{IM} activated the Rcs system when they reached ~10% of the wild-type RcsF levels (Figures 5A and S6). Interestingly, protein abundance estimates based on ribosomal profiling indicate that RcsF (~3,100 copies/cell) is in ~10-fold excess over IgaA (~220 copies/cell) and in similar amounts to BamA (~3,900 copies/cell) (Li et al., 2014). As RcsF activates the Rcs system via IgaA, these numbers are consistent with the levels of RcsF required to remain exposed to the periplasm for activation. Thus, RcsF is a sensitive sensor, able to trigger the Rcs response as soon as a small fraction escapes the BamA-OmpA pathway due to envelope perturbations.

This setup raised the possibility that small fluctuations of RcsF levels could trigger the Rcs phosphorelay, resulting into a leaky signaling system. This was not the case, as wild-type cells could tolerate significantly higher RcsF levels without triggering the Rcs response (Figure 4C) by funneling more RcsF to OmpA (Figure 5B). This suggests that during steady-state, BamA is not forming RcsF–OmpA at maximal capacity, thereby insulating the Rcs response from small fluctuations of RcsF levels. In comparison to RcsF and BamA, each cell contains ~210,000 copies of OmpA (Li et al., 2014), suggesting that OmpA levels are not the limiting factor in this process.

**Portions of RcsF Are Displayed on the Cell Surface via OMPs**

Although *E. coli* OM lipoproteins are considered to be all facing the periplasm, some were recently proposed to be surface-exposed (Zückert, 2014). Because the OmpA-RcsF complex was stable and unresponsive to stress, we hypothesized that the interaction between these two proteins may lead to partial exposure of RcsF on the surface. In this scenario, OmpA would be the vehicle for lipoprotein surface exposure, and BamA the means. To test this hypothesis, we performed immunofluorescence (IF) microscopy on intact and OM-permeabilized cells using antibodies specific for the signaling domain of RcsF, LamB3xFlag, an abundant OM porin fused to a triple Flag tag at its periplasmic C-terminus, was used as negative control (Figure S7A). RcsF was clearly labeled in intact cells, while LamB3xFlag was only marginally labeled in the same cells (Figure 6A). Similar results were obtained with immunodot blotting on intact cells without fixation (Figure S7B). Therefore, we propose that the signaling domain of RcsF is at least partially exposed on the cell surface.

We next tested whether surface exposure of RcsF required OmpA. We were unable to address this question using a ΔompA mutant, as deleting ompA rendered the OM permeable to antibodies, making it impossible to obtain reliable results. Instead, we used BamA overexpressing cells, in which the OmpA-RcsF complex was almost absent (Figure 3D). In this case, the surface-exposed RcsF decreased significantly (Figure 6B) without an overall change in RcsF levels (Figure S7C), suggesting that RcsF reaches the surface at least partially via OmpA. Conversely, overexpression of RcsF that increased OmpA-RcsF levels (Figure 5B) resulted in more RcsF being detected on the cell surface (Figure 6C). Altogether these results suggest that RcsF reaches the cell surface mainly via OmpA, but possibly also through other OMPs (see Discussion).

**DISCUSSION**

**RcsF Senses the Bam Machinery Activity**

After IM translocation, β-barrels are ushered by chaperones through the periplasm to the Bam machinery, which folds and inserts them into the OM (Goemans et al., 2014). To monitor malfunctioning at different levels of this multistep process, the cell would need multiple signal transduction systems. We already
know that accumulation of unassembled OMPs in the periplasm is the primary signal for the Rcs stress response (Walsh et al., 2003). We now report that the activity of the Bam machinery is monitored by the Rcs system through RcsF. BamA interacts with RcsF and, when active, funnels it to OmpA. When bound to BamA or OmpA, RcsF is occluded from IgaA and cannot activate the Rcs system. Yet, BamA cannot sequester all RcsF molecules and funneling of newly synthesized RcsF to OmpA is necessary for maintaining the Rcs system off. This is especially important because preformed BamA-RcsF does not disassociate upon stress, and only newly arriving RcsF can sense stress. Thus, this constant flow of RcsF from BamA to OmpA is what defines the availability of BamA and what RcsF is sensing. Stress conditions impair BamA availability for newly arriving RcsF, which ends up facing the periplasm, free to activate the Rcs cascade (Figure 7).

In addition to its well-known activity in OMPs assembly, we report that BamA funnels RcsF to OmpA and other OMPs. Since functional Bam machinery is required for both events, we suggest that they are coupled (Figure 7), which implies that RcsF senses by default both activities. Further structure-function analysis will be required for deciphering if and how the two events are connected and how RcsF intervenes.

An interesting feature of the Rcs system is that RcsF is in ~10-fold excess over its downstream partner IgaA (Li et al., 2014), despite the two forming a 1:1 complex (Figure 1C). This results into only a fraction of RcsF being required for fully activating the Rcs system (Figure 5A). The cell presumably maintains RcsF in excess over IgaA to efficiently monitor the Bam machinery, which is present at a similar copy/cell ratio as RcsF (Li et al., 2014). At the same time, the steady-state RcsF levels are kept low enough to prevent activation of the Rcs system by small fluctuations. Indeed, a ~3-fold increase in RcsF levels was required for the Rcs system to be activated without stress (Figure 4C). An interesting hypothesis that we are currently pursuing is that RcsF levels are optimized for high sensitivity and low noise.

How can PG and OM stress affect Bam activity? Although PG perturbations could affect the journey of RcsF bound to the lipoprotein-specific chaperone LolA through the porous PG layer, we did not see RcsF accumulating in the periplasm upon mecillinam or A22 treatment. On the other hand, transport of the bulkier BamA may be more impaired, creating a bottleneck in BamA availability/activity. Alternatively the POTRA domains of BamA that extend deep into the periplasm could be affected by changes in PG integrity, with direct consequences

Figure 5. RcsF Is a Sensitive but Robust-to-Noise Sensor of BamA Activity

(A) Only a fraction of RcsF is required to be in the periplasm for Rcs activation. ΔrcsF cells carrying RcsFIM or RcsFperi under an IPTG-inducible promoter on a low-copy vector (pSC202) and a higher-copy plasmid encoding lacF (pREP4) were grown for three generations in LB and before adding inducer (100 μM IPTG). RcsF protein levels (bottom) and Rcs activity (top left; chromosomal rprA::lacZ fusion) were closely monitored onward. Note that in this setup, no matter how much IPTG was added, or when added, rcsF expression remained undetectable until cells reached an OD578 of ~0.6. Quantification of RcsFIM or RcsFperi protein levels at the time point of Rcs activation is shown at the top right. Error bars depict standard deviation (n = 3). The time point of activation was considered as the point at which a linear curve fitted on specific β-gal activity versus time crossed the basal activity, minus 3 min required for β-gal synthesis and folding. For quantifying RcsF levels, we always ensured that the signal detected from cells expressing RcsFIM or RcsFperi (40 μg) was within linear range by loading a titration of total protein extracts from wild-type cells (2.5–20 μg). An example western blot is shown (bottom). Empty dots: OD; filled dots: specific β-gal activity. The full gel can be seen in Figure S6.

(B) The capacity of BamA to form the OmpA-RcsF complex is not maxed out in wild-type cells. Increasing RcsF expression resulted into more OmpA-RcsF complex being formed, but the levels of the BamA-RcsF complex remained largely unchanged. Thus, in nonstressed cells, BamA has the ability to funnel more RcsF to OmpA. In lane 1 the wild-type levels of the OmpA-RcsF and BamA-RcsF complexes are shown. In lane 2, RcsF was expressed in ΔrcsF cells carrying rcsF under an IPTG-inducible promoter on a low-copy vector, pSC202. In the absence of lacF, the RcsF steady-state levels were ~3- to 4-fold higher than in the wild-type. DTSSP crosslinking and immunoblot were performed as described in Figure 2A, and a representative experiment is shown (n = 3).
on the Bam activity. Defects in LPS composition and assembly could also affect the RcsF journey in many ways, as they vastly reorganize the OM and periplasm (Sperandeo et al., 2008). Further work is required to mechanistically dissect how particular envelope stresses impair the availability and activity of BamA.

**Figure 6. Portions of RcsF Are Surface-Exposed**

(A) Wild-type and ΔrcsF cells were probed for RcsF and LamB3xFlag localization by IF microscopy using anti-RcsF/-Flag antibodies, with/without cell permeabilization. LamB3xFlag expressed from a plasmid, serves as a permeabilization marker—the 3xFlag tag is fused to the periplasmic C terminus (Figure S7 A). The ΔrcsF strain is used as specificity control for the anti-RcsF antibody. Phase contrast, fluorescence signals and overlay images (green: RcsF, red: LamB3xFlag) are shown for representative cells. Scale bar, 4 μm. P, permeabilized; NP, nonpermeabilized. Unlike LamB, RcsF is detected even on nonpermeabilized wild-type cells.

(B) BamA overexpression reduces RcsF surface exposure. RcsF was visualized by IF as described above in nonpermeabilized cells carrying an empty vector (WT) or a vector overexpressing BamA (pBamA). Phase contrast, fluorescence signal and overlay images are shown for representative cells. Scale bar: 4 μm. Right: a distribution of the total fluorescence intensity per cell, normalized by cell area, is shown for populations of WT (blue) and pBamA (red) cells. AU, arbitrary units; n, number of cells. Significantly less RcsF is detected on the surface when BamA is overexpressed.

(C) RcsF overexpression increases its surface exposure. RcsF and LamB3xFlag were visualized by IF as described above in wild-type cells containing an empty vector, pBAD33 (WT), and ΔrcsF cells carrying pSC216 (pBAD33–RcsF). Phase contrast, fluorescence signal and overlay images are shown for representative cells. Scale bar, 4 μm. Note that the RcsF-associated signal is not visible in nonpermeabilized WT cells because of scaling applied—to avoid saturation of the RcsF signal in pRcsF cells. Middle, distributions of the total fluorescence intensity (associated with RcsF, left, or LamB3xFlag, right) per cell, normalized by cell area, are shown for populations of wild-type and pRcsF cells imaged on the left. Inset: the ratio of the mean value of normalized fluorescence associated with RcsF or LamB3xFlag in nonpermeabilized (NP) and permeabilized (P) wild-type and pRcsF cells is depicted. Abbreviations are like above. A significantly higher fraction of RcsF could be labeled from outside, compared to LamB. Although more RcsF was detected on the surface of cells overexpressing RcsF than in WT cells, the fraction of surface-exposed RcsF (NP/P ratio) remained similarly high. See also Figure S7.

**The Bam Machinery Exports RcsF to the Cell Surface**

Although the general view has been that E. coli OM lipoproteins face the periplasm (Okuda and Tokuda, 2011), a handful were recently reported to be surface exposed (Zückert, 2014). Yet no machinery has been identified that would allow such translocation through the OM bilayer. Here, we report that BamA allows...
RcsF to reach the surface by funneling it to the β-barrel OmpA. It is plausible that the BamA-mediated formation of lipoprotein-β-barrel complexes is a more general mechanism of lipoprotein export to the surface, but further experimentation is needed to establish this. As the Bam machine is highly conserved among Gram-negative bacteria, this would explain why surface-exposed OM lipoproteins are exported to the outside when expressed in heterologous systems (Arnold et al., 2014; Pride et al., 2013).

OmpA is the major β-barrel acting as terminal acceptor of RcsF. Not only most RcsF binds OmpA (Figures 2B and S3E and S3F), but also the steady-state Rcs activity is higher in ΔompA cells than in cells deleted for other β-barrels (Figure 2D). Moreover, BamA has lower capacity for RcsF in ΔompA cells (Figure 4C). Yet our MS and Rcs-activity data (Table S2A and Figure 2D) indicated that RcsF is also funneled to other abundant β-barrels, such as OmpC and OmpF (~165,000 and 90,000 copies/cell, respectively [Li et al., 2014]). The cellular levels of the 3 OMPs cannot explain alone the preference of BamA for funneling RcsF to OmpA, suggesting that a more selective process, which remains to be discovered, is at play. The redundancy of OMPs as terminal acceptors of RcsF could explain why ΔompA cells or cells missing 2 OMPs have the Rcs system still only partially activated, in comparison to cells where BamA is depleted. Interestingly, if many OMPs are used as terminal RcsF acceptors, this could mean that BamA, on its own, has very limited capacity to sequester RcsF, and has to always funnel new RcsF to OMPs for keeping the Rcs system off. As BamA levels are slightly higher than that of RcsF (Li et al., 2014), there may be more (lipoprotein) substrates competing with RcsF for BamA.

RcsF interacts with OmpA via its N-terminal linker and the tip of the signaling domain (Figures 2C and S3). A simple model to explain our results is that the N-terminal linker of RcsF traverses the OmpA pore to allow (portions of) the globular domain to locate outside the OM (Figure 7). This would not be unprecedented as OM lipoproteins with their entire globular domain present on the surface have been reported, such as the Vibrio cholerae lysophospholipase VolA (Pride et al., 2013). As OmpA-RcsF is a dead-end complex for the signaling role of RcsF, the physiological role of RcsF when bound to OmpA is
enigmatic. Additional work will be required to clarify how OmpA and RcsF interact and the role of RcsF in this complex.

How can RcsF use a β-barrel such as OmpA to access the surface? OM porins act as gates for peptides coming from outside (Housden et al., 2013) and for periplasmic proteins secreted by the cell, such as YeF (Prehna et al., 2012). The lipoprotein LpE was also recently shown to reside inside the β-barrel LpD, presumably acting as a controllable plug for the LPS assembly machinery (Freinkman et al., 2011). Thus, it is not uncommon that a β-barrel pore can accommodate a polypeptide. OmpA, in one of its two known conformations, forms a 16-stranded β-barrel structure with a large pore (Reusch, 2012). This conformation could accommodate a disordered segment such as the RcsF linker. In its second conformation, which has been proposed to be an intermediate state, OmpA assumes a 2-domain structure, with a smaller N-terminal β-barrel and a C-terminal periplasmic domain interacting with the PG (Reusch, 2012). In this conformation, the β-barrel diameter is too small for a polypeptide, but an OmpA-RcsF interaction at this stage could be an important intermediate for the funneling of RcsF from BamA.

Finally, we detected an RcsF–Lpp complex (Figure S2D). As this interaction was not recapitulated with any of the 25 pBpa-containing RcsF variants, we deduced that it might be indirect. This would be consistent with the very high abundance of Lpp and its shared localization with RcsF at both OM leaflets (Cowles et al., 2011). In addition, the absence of Lpp did not affect the RcsF–BamA and RcsF–OmpA interactions (Figure S2D). It remains to be tested if Lpp has any direct effect on Rcs signaling.

Integrating Envelope Stresses: RcsF Monitors the Journey of Lipoproteins through the Envelope

There are ~100 lipoproteins in E. coli. The vast majority is localized in the OM. Although the function of most is unknown, some are components of essential OM assembly machineries (Silhavy et al., 2010) and others regulate core envelope processes (Paradis-Bleau et al., 2010; Typas et al., 2010; Uehara et al., 2010). Thus lipoprotein targeting is vital for the cell.

OM lipoproteins are escorted across the periplasm by the essential chaperone LtpA (Okuda and Tokuda, 2011). RcsF senses defects in: (1) phosphatidylglycerol biosynthesis (Shiba et al., 2004), which is required for lipoprotein maturation; and (2) in the LtpA-mediated transport of lipoproteins to the OM, presumably because it gets stuck in the IM when LtpA’s function is impaired (Tao et al., 2012), gaining access to IgaA. RcsF activation resulting from RcsF accumulation in the IM leads to higher IgaA expression, creating a feedback loop to fix the damage (Tao et al., 2012).

For RcsF, and at least a few other lipoproteins, the journey does not end at the inner leaflet of the OM, as they are finally translocated to the cell surface. As we have shown here, it is the Bam machine that mediates the export of RcsF to the surface by inserting it into β-barrels such as OmpA. Malfunctioning of this process results into newly translocated RcsF remaining exposed in the periplasm, where it can reach IgaA and trigger the signaling cascade (Figure 7). Therefore, RcsF also monitors the ability of BamA to insert OM lipoproteins to OMPs. Altogether this means that Rcs can sense the entire lipoprotein journey across the envelope, from maturation to OM exposure, adjusting the envelope composition in response to failures at any step.

Rcs, a Complex Signal Transduction System

Rcs is one of the most complex signaling systems known in bacteria with key steps remaining unresolved. We have shown that RcsF interacts with the large periplasmic domain of IgaA, which likely triggers the signaling cascade. As the two membranes are separated by ~200 Å, it remains to be determined how this interaction occurs. RcsF has an intrinsically disordered 31 amino acid-long N-terminal linker. It is likely that, when extended, this region allows RcsF to reach the large periplasmic domain of IgaA. The OM lipoprotein LpoB uses a similar configuration to access its IM counterpart, PBP1B (Egan et al., 2014).

It also remains to be proven whether the RcsF–IgaA interaction is sufficient for conveying the signal downstream and activating the Rcs cascade; our genetic data that put IgaA downstream of RcsF strongly suggest so. How IgaA itself mechanistically controls the Rcs phosphorelay, whether it directly interacts with the other IM components RcsC and RcsD, and whether it plays additional roles in the cell remain unknown and will all be fields of future research. Moreover, further work will be required to elucidate how the few genetic perturbations that activate the Rcs system independently of RcsF (Majdalani and Gottesman, 2005, 2007) are sensed by the system.

CONCLUSIONS

We elucidated how the OM lipoprotein RcsF senses stress and talks to the downstream signaling cascade. RcsF monitors the activity of the machinery for OM β-barrel assembly, Bam, triggering the signaling cascade when Bam is malfunctioning. Moreover, we identified the formation of complexes between RcsF and the β-barrel OmpA as a novel mechanism for lipoprotein translocation through the bacterial OM. We propose that this may be a conserved system for lipoprotein export. Although many of the molecular details of both processes described here remain to be fully elucidated, these findings generate a number of intriguing hypotheses on the mechanisms that the cell uses to sense the activity of the protein machineries that build its envelope.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Media, and Plasmids

Cells were grown in LB at 37°C and, when necessary, growth media were supplemented with spectinomycin (50–100 μg/ml), ampicillin (100–200 μg/ml), chloramphenicol (20–25 μg/ml), or kanamycin (50 μg/ml). The bacterial strains and plasmids used in this study are listed in Tables S3 and S4, respectively, and information on their construction is provided in Extended Experimental Procedures.

In Vitro RcsF–IgaA Binding

RcsF with a C-terminal 6-Histidine tag (RcsF–His) and an untagged version of the periplasmic IgaA domain were purified as described in Extended Experimental Procedures. RcsF–His (0.15 nmol) was coupled to 20 μl Talon beads and washed with PD buffer (25 mM Tris [pH 7.5], 200 mM NaCl, 10% glycerol) to remove residual RcsF–His. IgaA was then added to the RcsF (2.5 μM) containing Talon beads in a concentration range: 0.375–10 μM (assay volume = 60 μl; 0.625–10 μM range is shown in Figure 1C). The RcsF–IgaA suspension
was incubated for 15 min at room temperature and pelleted by brief centrifugation. Half of the supernatant was aspirated to quantify unbound IgA by SDS-PAGE. The pellet was washed with 500 μl PD buffer and half was also analyzed by SDS-PAGE to quantify the pulled-down fraction of IgA.

**In Vivo DTSSP Crosslinking**

In vivo chemical crosslinking experiments were performed as described by Thanabal et al. (1998) with some modifications. The detailed procedures are described in Extended Experimental Procedures.

**In Vivo Site-Specific Photocrosslinking**

Site-specific photocrosslinking was performed essentially as described by Okuda et al. (2012) with some modifications. The detailed procedures are described in Extended Experimental Procedures.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Extended Experimental Procedures, seven figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2014.11.045.

**AUTHOR CONTRIBUTIONS**

S.H.C., J.S., C.P., A.T., and J.F.C. conceived this study; S.H.C., J.S., C.P., M.Z., M.B., A.T., and J.F.C. designed research; S.H.C., J.S., C.P., M.Z., P.R., A.A., G.L., M.B., A.-K.H., P.L., C.V.d.H., D.V., and A.T. performed all experiments (S.H.C., J.S. performed all DTSSP experiments, except for the pull down-MS for IgA-peri which were performed by C.P. and M.Z.; S.H.C. performed all photocrosslinking experiments; C.P., M.Z., and A.K. performed all li-gal assays; C.P. performed the quantitative western blotting; M.B. and A.K. performed the in vitro binding assays; P.R., A.A., and G.L. performed the IF microscopy; J.S. performed the dot blotting; A.T. performed the IgA suppressor screen); S.H.C., J.S., C.P., M.Z., P.R., A.A., G.L., M.B., D.V., A.T., and J.F.C. interpreted the data; A.T. and J.F.C. wrote the manuscript; S.H.C., J.S., C.P., A.A., G.L. prepared the figures; S.H.C., J.S., and C.P. edited the manuscript; A.T. and J.F.C. supervised all aspects of the project.

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Note Added in Proof
While this paper was under revision, Konovalova et al. reported the surface exposure of portions of RcsF via OM ß-barrels.


Note Added in Proof
While this paper was under revision, Thanabal et al. and Sperandeo et al. reported the surface exposure of portions of RcsF via OM ß-barrels.