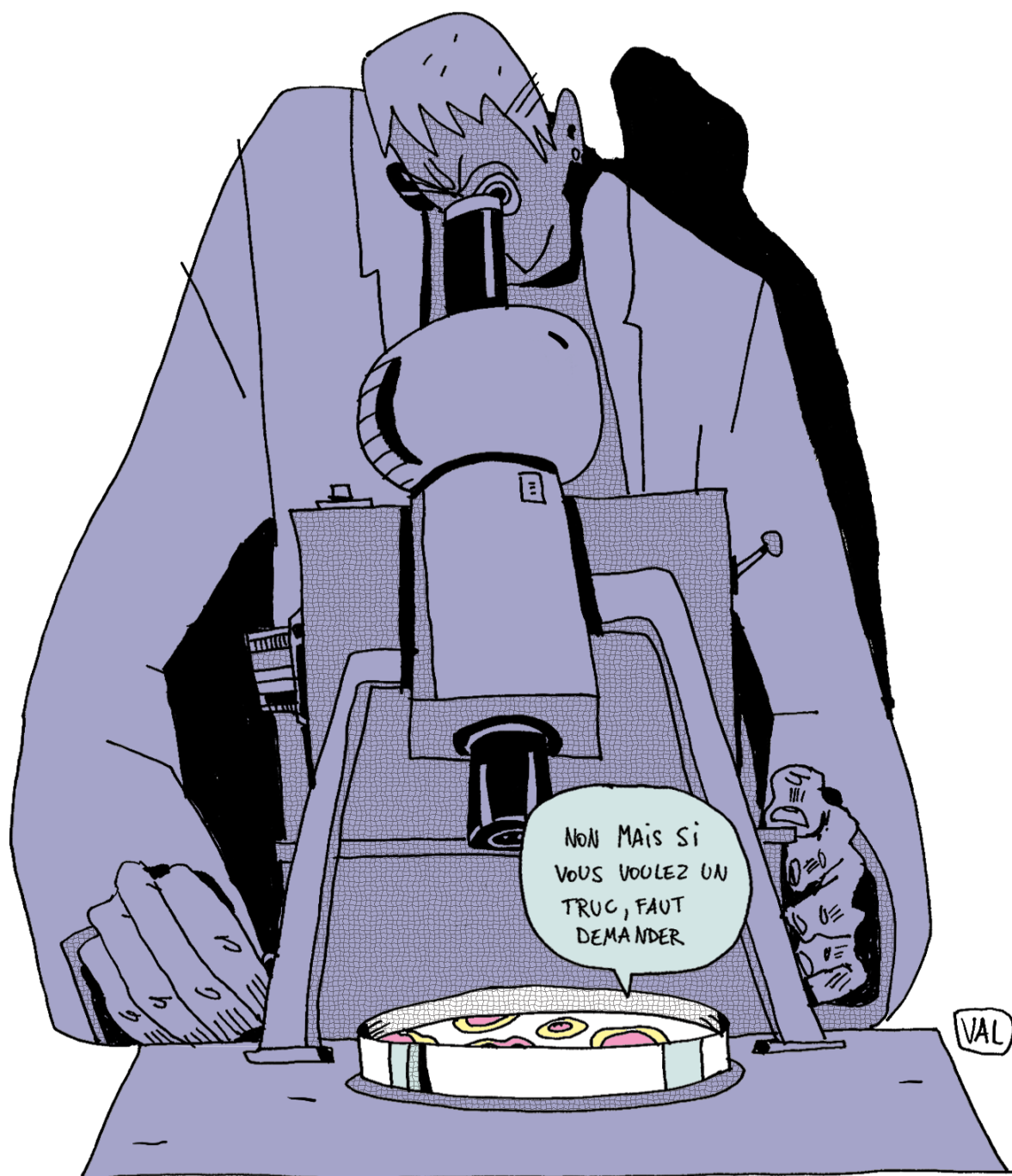


It's a trap: New findings into the
role of outer membrane proteins in
the Rcs stress response system of
Escherichia coli.

Kilian Dekoninck



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Université de Rouen Normandie, Rouen, France.

If we knew what is was we were doing,

It would not be called research,

Would it ?

Presumably by Albert Einstein

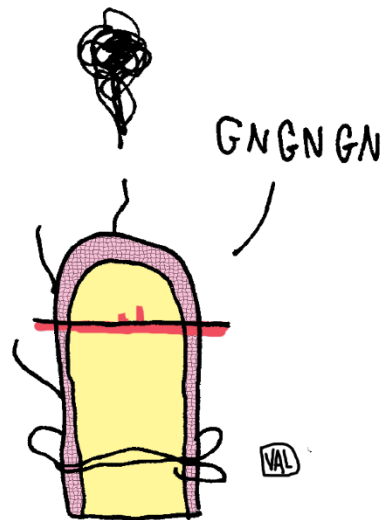
No! Try not.

Do. Or do not.

There is no try.

Yoda

SALMONELLE,
ELLE A RÉUSSI À
SE FAIRE UN NOM,
ELLE.



Acknowledgments

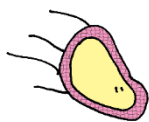
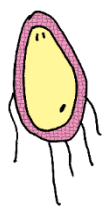
Nous y voici !

Une thèse c'est la synthèse de plusieurs années de travail qui termine en quelques pages, qui termine en un petit livre. Petit livre qui terminera sans doute sur une étagère, à prendre la poussière. Et pourtant, en ces quelques pages se résume l'essence de ma passion, de mon obsession, de mon projet de thèse. Il est certain que cela n'aurait pas été possible sans un nombre incalculable de personnes. Alors à toi cher lecteur, je te souhaite la bienvenue dans la section des remerciements !



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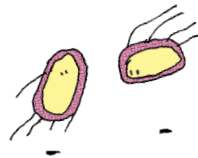
Je remercie aussi les membres de mon comité de thèse. Bien sûr pour m'avoir accompagné et m'avoir stimulé scientifiquement (notamment à la défense de thèse) mais aussi pour des raisons bien plus personnelles: Mon président de jury **Jean-Noël Octave** pour avoir à toutes les étapes (et particulièrement ces derniers mois) fais tout pour faciliter mon parcours en tant que doctorant ; **Didier Vertommen**, pour m'avoir offert un aperçu de la puissance et de la beauté de la Mass Spec, merci de m'avoir permis de passer des journées avec toi et ton labo pour en apprendre plus sur cette méthode extraordinaire ; **Patrice Soumilion** que je connais depuis un sacré bout de temps maintenant. Patrice, merci pour avoir cru,, en premier, que participer à iGEM était possible, c'est grâce à cette expérience dans ton laboratoire que j'ai acquis pour la première fois la certitude que je voulais faire une thèse en Microbiologie et Biochimie. Sans iGEM, sans toi, je n'aurai probablement jamais écrit cette thèse. Un immense merci à **Benjamin Elias**, mon professeur de Chimie organique en BAC3. Tu m'a mis le pied à l'étrier pour énormément de choses, tu m'as aidé et conseillé tout au long de mes études (et de ma thèse) que ce soit sur mon choix de master, mon mémoire, ma thèse et la suite. Je me souviendrai toujours de ce jour où je suis venu te demander pour faire un mémoire dans ton laboratoire et que très honnêtement tu m'as répondu : « Soyons honnête Kilian, la chimie organique, ce n'est pas pour toi. » Jamais une phrase aussi censée (et blessante ??) n'aura eu autant d'impact dans ma vie. Merci Benjamin !



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together. You were the best lab partner I could dream of to work together on the Rcs system. See you very soon, sweetie.

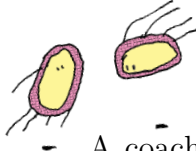
A **Ophélie**, tu occupes une place toute particulière dans mon cœur, jouer sur les planches avec toi était absurde mais tellement drôle. Notre amitié est cette comédie romantique qu'on ne peut que regarder en boucle. Tu es l'incarnation de la bonté et de la gentillesse, tu comptes énormément pour moi (et tu le sais), Merci.

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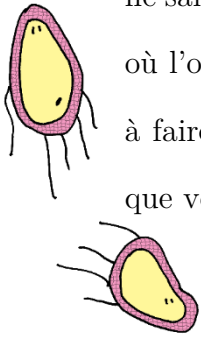


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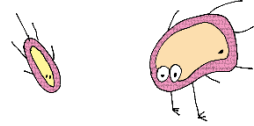
A **Antoine**, mon partenaire d'aventure, ma moitié, mon mari. Je t'aime. C'est à toi que je dédie cette thèse. Tu as été le témoin de chacune des lignes écrites dans ce manuscrit. Tu connais tous les hauts, tous les bas, toutes les frustrations, toutes les victoires, tous les échecs, toutes les réussites qui ont forgés ce travail scientifique. Notre force c'est de construire et de s'épanouir à deux. Cette thèse c'est la mienne, mais c'est la tienne aussi. Je t'aime.

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List of abbreviations

ABC Transporter = ATP-Binding Cassette Transporter

Asp= Aspartate

ATP= Adenosine TriPhosphate

Bae= Bacterial adaptative response

BAM= b-Barrel Assembly Machinery

BLI= Biolayer interferometry

BS3= bis(sulfosuccinimidyl)suberate

CL= Cardiolipin

Cpx= Conjugative pilus expression

D/L-ala= D/L-alanine

DDM= n-Dodecyl-b-D-Maltoside

DegP= Degradation of periplasmic proteins

DiZPK= *N*⁶-((3-(3-methyl-3*H*-diazirin-3-yl)propyl)carbamoyl)-*L*-lysine

DNA= Deoxyribonucleic acid

Dps= DNA protection during starvation

DTSSP= 3,3'-dithio-bis[sulfosuccinimidylpropionate]

ECP= *Escherichia coli* common pilus

ESR = Envelope stress response

GlcNac=N- acetylglucosamine

His= Histidine

IM= Inner membrane

IPTG= Isopropyl β -D-1-thiogalactopyranoside

K_D= Constant of dissociation

kDa= Kilodalton(s)

LB= Luria Broth

LC-MS/MS= Liquid chromatography tandem mass spectrometry

LDT= L,D-transpeptidase

Lgt= Lipoprotein diacyl glyceryl transferase

Lnt= Apolipoprotein N-acyltransferase

Lol= Localization of lipoproteins

LP= Lipoprotein

LPS= Lipopolysaccharide

Lpt= Lipopolysaccharide transport

LspA= Signal peptidase II

mDAP= Meso-diaminopimelic acid

Mla= Maintenance of outer membrane lipid asymmetry
 MOPS= 3-(N-morpholino)propanesulfonic acid
 MurNac= N-acetylmuramic acid
 NMR= Nuclear Magnetic Resonance
 OM= Outer membrane
 OMP= Outer membrane protein
 Pal= Peptidoglycan associated lipoprotein
 PBP= Penicillin Binding Protein
*p*Bpa= *p*-benzoyl-L-phenylalanine
 PDB= Protein Data Bank
 PE= Phosphatidylethanolamine
 PG= Peptidoglycan
 PGI= Phosphatidylglycerol
 PLs= Phospholipids
 POTRA= POlypeptide TRanslocation Associated
 PS= Phosphatidylserine
 Psp= Phage shock protein
 Rcs= Regulator of capsule synthesis
 RMSD= Root mean square deviation
 SDS-PAGE= Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis
 Sec= General secretory machinery
 Skp= Seventeen kilodalton protein
 SLAM= Surface lipoprotein assembly modulator
 SLP= Surface exposed lipoprotein
 Spy= Spheroplast protein Y
 SS= Signal sequence
 SurA= Survival protein a
 T2SS= Type II secretion system
 T5SS= Type V secretion system
 TAM= Translocation assembly module
 Tat= Twin-arginine transport
 TBST= Tris-Buffered Saline Tween®20
 TCA= Trichloroacetic acid
 TCS= Two-component system
 TPR= Tetratricopeptide repeat
 UDP-GlcNAc= UDP-N-acetylglucosamine
 WT= Wild-type

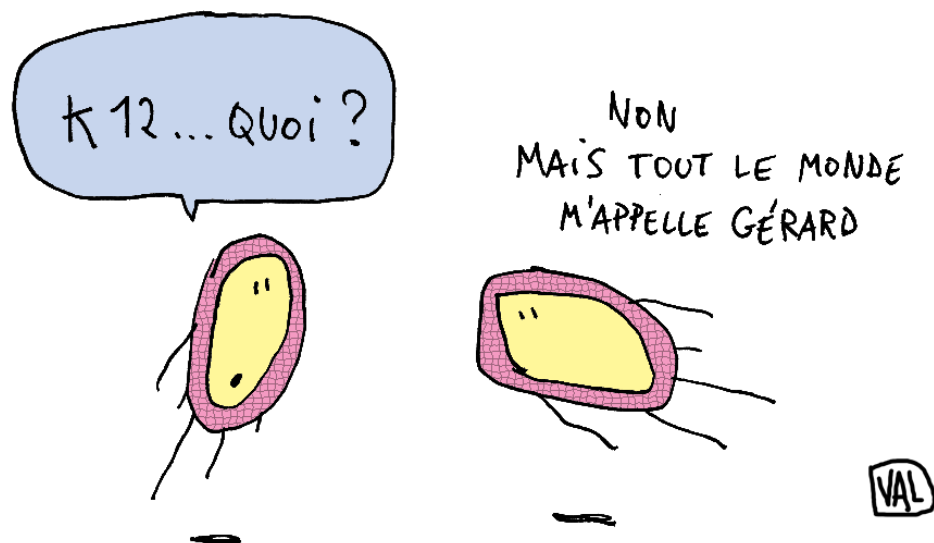
Summary

The cell envelope of Gram-negative bacteria is essential for their survival. It serves as their initial line of defense as well as a physical barrier between the cell and its surrounding environment. Furthermore, the cell envelope protects bacteria by allowing them to respond to changes in their environment. This complex structure comprises an outer membrane (OM) and an inner membrane (IM) separated by a viscous compartment, the periplasmic space. The cell wall, a sacculus with a mesh-like structure comprised of peptidoglycan, is found in the periplasm (PG).

The Rcs (Regulator of capsule synthesis) system responds to OM and PG damage in *Enterobacteriaceae*. The OM lipoprotein RcsF senses stress and activates the signaling cascade. The β -Barrel Assembly Machinery (BAM) inserts OM β -barrel proteins (OMPs) in the OM of Gram-negative bacteria. BAM also most likely exports RcsF to the cell surface, probably by forming RcsF-OMP complexes. The main objective of my thesis project was to investigate the relationship between RcsF and its OMP partners.

Hereby by dissecting the protein-protein interaction interface (PPI) between OmpA, an abundant *E. coli* OMP, and RcsF, I identified an unexpected role of OmpA in the Rcs stress response system. In this complex, OmpA, folded as a two-domain protein, interacts *via* its periplasmic domain with the globular domain of RcsF. This mode of interaction between OmpA and RcsF does not allow OmpA to be the vehicle for the surface exposure of RcsF. Instead, my results suggest that OmpA plays the role of a periplasmic buffer for the Rcs system: The periplasmic

domain of OmpA, when interacting with RcsF, prevents the interaction of RcsF with IgaA, the Rcs repressor. To identify the PPI of the OmpA-RcsF complex, I used a site-specific photo-crosslinking method based on non-natural photoactivable amino acids. By doing so, my work identified a flaw in this considered foolproof method, which I investigated to raise the attention of scientists working on protein-protein interaction or protein complexes on the probable existence of artifacts.



Organization of the thesis

This thesis is organized in three main sections: Introduction, Results, and General discussion, conclusion and perspectives. An annex can be found at the end of the manuscript.

The introduction itself is divided into two chapters. The first chapter includes an overview of the Gram-negative bacteria's cell envelope and describes the major envelope biogenesis routes. A particular attention was given to the biogenesis of lipoproteins and β -barrel proteins. The last part consists in a brief introduction of the envelope stress response systems that monitor and respond to cell envelope perturbations in *E. coli*. The second chapter of the introduction is dedicated to Rcs, an envelope stress response system, which was at the core of my thesis research.

The results section is divided into two chapters. In the first chapter (Chapter III), I describe the investigation of the interaction between OmpA, an abundant outer membrane protein of *E. coli*, and RcsF, the stress sensor lipoprotein of the Rcs system. The results of this work was the fruit of numerous methods and collaborations that allowed us to gain insights into the function of OmpA in the Rcs stress response system. This work was published in *eLife* in September 2020. The second chapter (Chapter IV) dissects a potential flaw existing in a widely used method used to study protein-protein interaction interfaces. We submitted this work to *Protein Science* in April 2022.

The general discussion, conclusions and perspectives section has one chapter, chapter V, which discusses the main findings presented in the results section and contains a short conclusion.

Finally, the annex contains the full text of the published version of the *eLife* paper. Each chapter in this thesis has its own figure numbering and references.

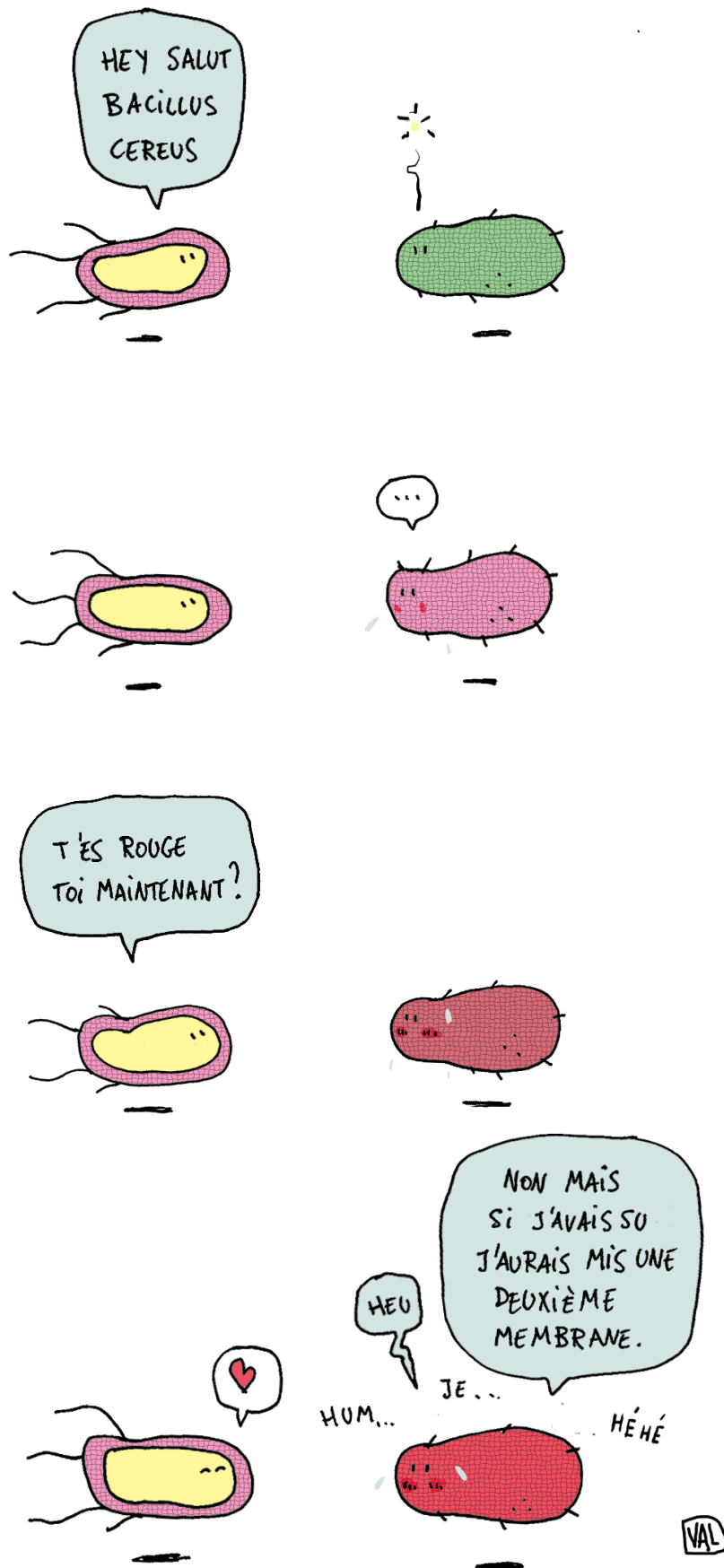
Research objectives

Gram-negative bacteria employ envelope stress response systems to monitor the extracytoplasmic space and guarantee that the cell can efficiently respond to stress by regulating their genes expressions. The Rcs stress response system is critical for detecting damage to the outer membrane (OM) and the peptidoglycan (PG). RcsF is an OM lipoprotein that serves as the Rcs stress sensor. In 2014, our group discovered that RcsF is likely exposed to the cell surface by forming complexes with β -barrels OMP partners. While displaying lipoproteins on the cell surface is a well-known characteristic in certain bacterial phyla, such as in *Borrelia spp.*, it was unsuspected in *E. coli*.

The main objective of my thesis was to gain insight into the protein-protein interaction interface (PPI) existing between RcsF and OmpA, an abundant *E. coli* OMP. I found that RcsF does not use OmpA as a vehicle for surface exposure. Instead, RcsF and OmpA interact with each other in the periplasm to buffer the activity of the Rcs system. The results are presented in Chapter III.

The study of protein-protein interaction interface of protein complex is a common approach in understanding biological pathways at a molecular level. Among other methods, site-specific photo-crosslinking is a gold standard method often considered foolproof. However, the second objective of my thesis focused on the identification and characterization of a flaw in this method. We found that OmpA displays a hydrophobic pocket with a strong affinity for a commonly used non-natural amino acid, precluding the reliable identification of interaction interfaces. The results are presented in Chapter IV.

Section I: Introduction



Chapter I: The cell envelope of Gram-negative bacteria: Biogenesis and Maintenance

I.1 Introduction

The cell envelope, which is the morphological hallmark of Gram-negative bacteria, acts as the cell's outermost defense line, insuring cell survival. Because bacteria express several unique membrane protein machineries to maintain cell envelope function and structure, the cell envelope allows them to adapt to a constantly changing environment. Briefly, the cell envelope consists of two concentric membranes: the inner membrane (IM) surrounding the cytoplasm (viscosity of $9 \pm 2.1 \mu\text{m}^2\text{s}^{-1}$) and the outer membrane (OM) in direct contact with the extracellular medium (Mullineaux, Nenninger et al. 2006). The IM and OM are separated by an aqueous compartment, the periplasm (diffusion rate of $2.6 \pm 1.2 \mu\text{m}^2\text{s}^{-1}$), which contains a thin cell wall composed of peptidoglycan (PG)(Mullineaux, Nenninger et al. 2006, Silhavy, Kahne et al. 2010) (**Figure 1**). Beyond its defensive role, the cell envelope is involved in many other functions such as cell shape determination, motility, division, and growth. It is also the site of many essential processes such as selective nutrient uptake, respiration, or secretion of macromolecules in the extracellular environment (Saha, Lach et al. 2021).

Given the key function of the cell envelope in the lifecycle of bacteria, these organisms have developed tools to monitor envelope integrity and homeostasis. In particular, they rely on pathways called envelope stress responses (ESRs). When envelope integrity or biogenesis is compromised, ESRs are activated and transmit information to cytoplasmic transcriptional machineries to induce an adaptive cell response (Saha, Lach et al. 2021).

As with great powers comes great responsibility, the cell envelope is a perfect target for biological warfare. Indeed, many clinically used antibiotics, e.g., β -lactams, specifically target envelope biogenesis pathways. However, by mediating ESRs activity, Gram-negative bacteria quickly adapt their cell envelope to acquire resistance to antibiotics (Saha, Lach et al. 2021). As a result, nowadays, antimicrobial resistance is becoming a frightening reality.

Throughout this chapter, I will describe the *E. coli* bacterial cell envelope and its main characteristics, as well as a brief description of the different ESRs. By doing so, I will highlight the intricate relationship between ESRs and cell envelope biogenesis.

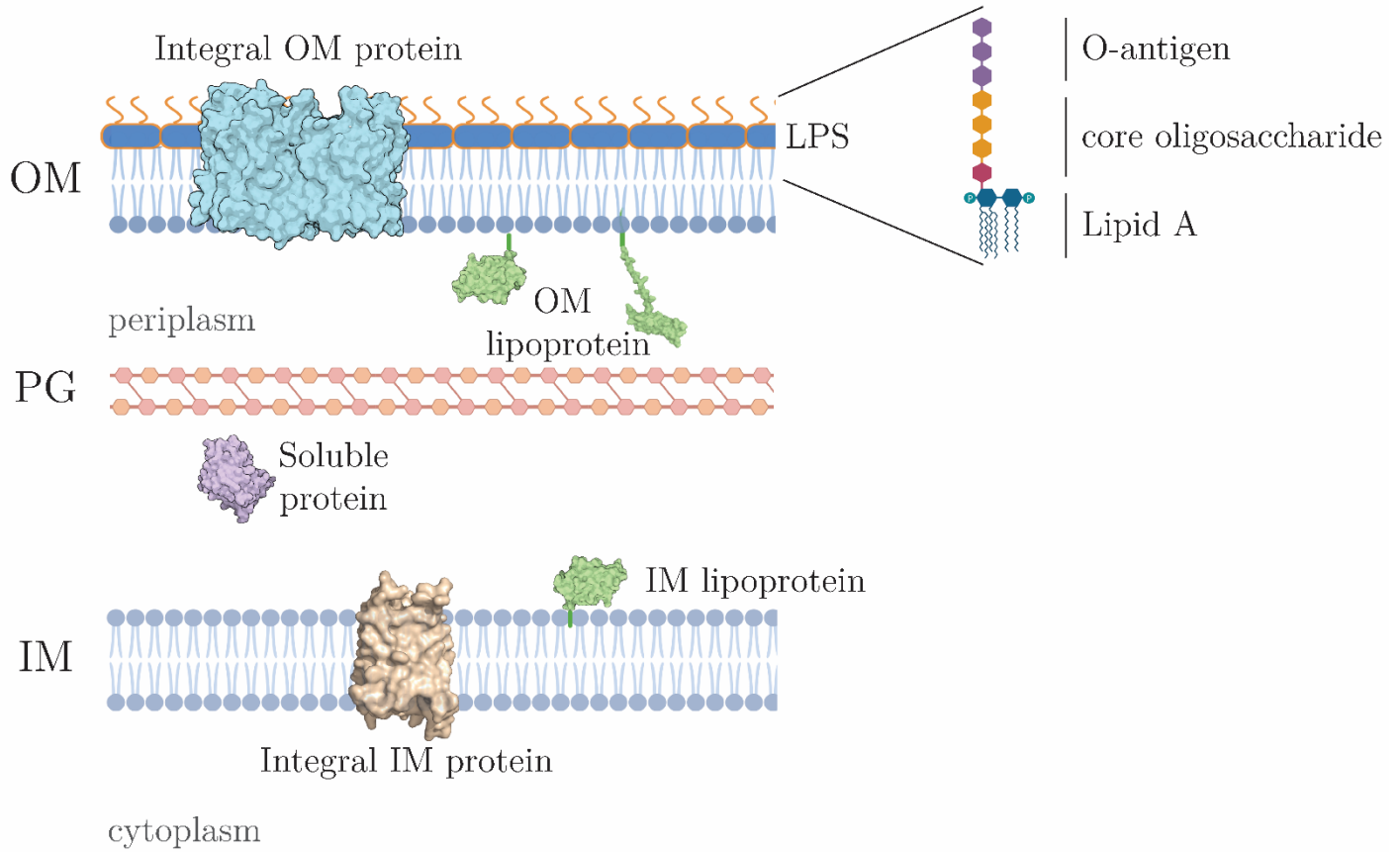
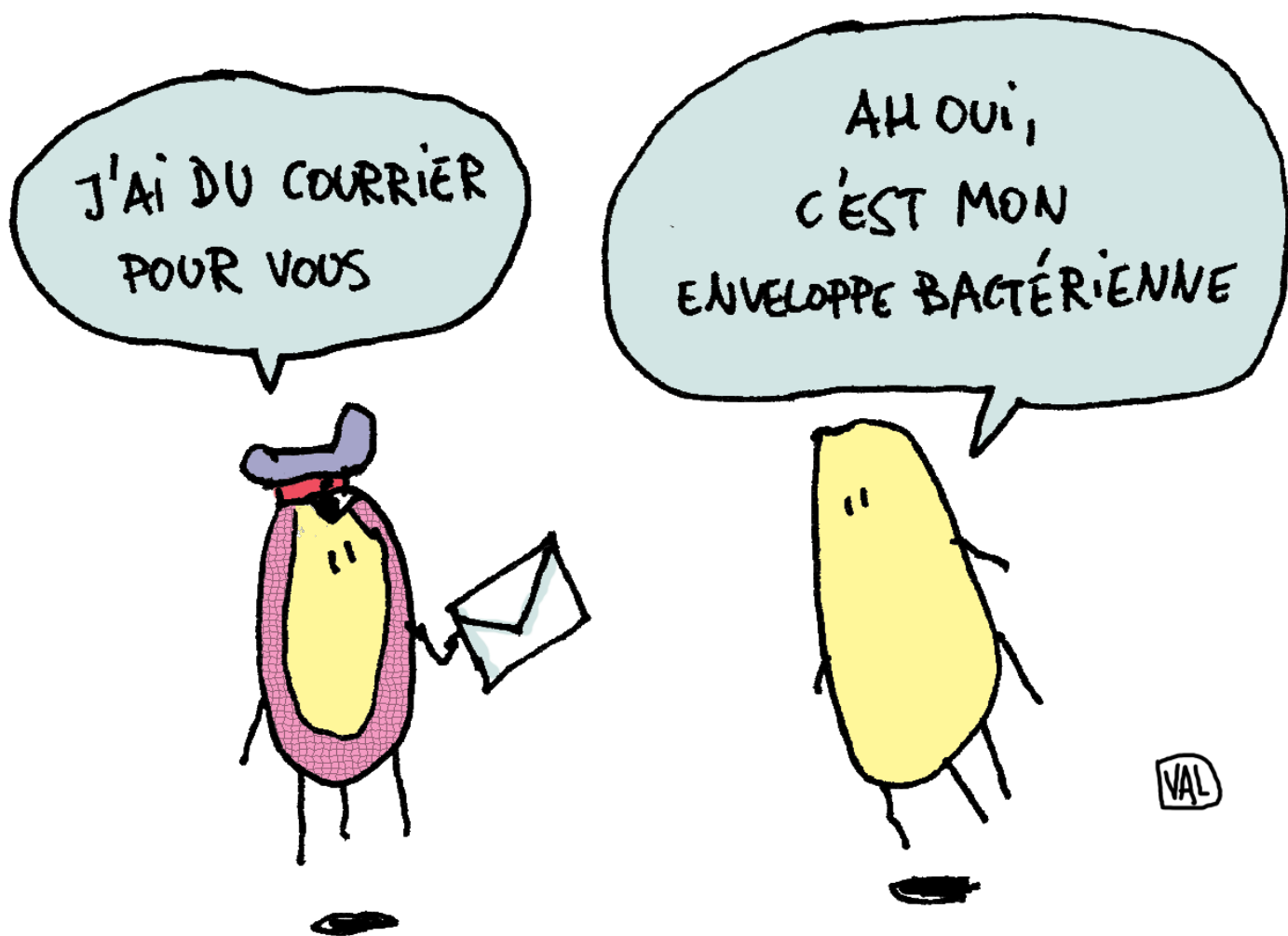


Figure 1. The cell envelope of Gram-negative bacteria. The cell envelope is composed of two concentric membranes: the inner membrane (IM) surrounding the cytoplasm and the outer membrane (OM) in direct contact with the extracellular environment. A viscous compartment separates the two membranes, the periplasm, in which lies a thin cell wall, a sacculus with a mesh-like structure made of peptidoglycan (PG). The IM is a classical phospholipid (PLs) bilayer. The OM is an asymmetric bilayer: its inner leaflet is composed of PLs, while the outer leaflet is made of lipopolysaccharide (LPS). LPS is an amphipathic molecule which consists of three different regions: the hydrophobic lipidA, the core oligosaccharide, and the O-antigen. Note that the *E. coli* K12 strain used in this thesis does not contain the O-antigen. Various proteins can be found in the envelope, IM or OM integral protein, soluble proteins, and lipoproteins anchored either in the IM or the OM.



I.2 The architecture of the cell envelope

I.2.1 The structure of *E. coli* cell envelope

A. The inner membrane

The IM or cytoplasmic membrane consists of a phospholipid bilayer (~6 nm thickness) separating the cytoplasm from the periplasm and ultimately from the extracellular environment (**Figure 1**) (De Geyter, Smets et al. 2019). The IM is composed of phospholipids: ~75% of phosphatidylethanolamine (PE), ~20% of phosphatidylglycerol (PGI), ~5% cardiolipin (CL) and less than 1% phosphatidylserine (PS) (**Figure 2**) (Langley, Hawrot et al. 1982, Silhavy, Kahne et al. 2010).

IM integral proteins (made of α -helical transmembrane domains spanning the membrane) and lipoproteins that face the periplasm are the two types of proteins that are present in the IM. (Luirink, Yu et al. 2012). They are involved in processes such as the respiratory chain, cell division, and the import or export of ions or sugars polymers (De Geyter, Smets et al. 2019).

Approximately 35% of *E. coli* proteins are localized outside the cytoplasm. Therefore, bacteria evolved specific pathways to allow proteins to cross the IM (Orfanoudaki and Economou 2014, De Geyter, Tsirigotaki et al. 2016, Sueki, Stein et al. 2020). The two dedicated systems in charge of protein secretion are the twin-arginine translocation pathway (Tat) and the general secretion pathway (Sec) (see

further) (Cline 2015, De Geyter, Tsirigotaki et al. 2016, Tsirigotaki, De Geyter et al. 2017).

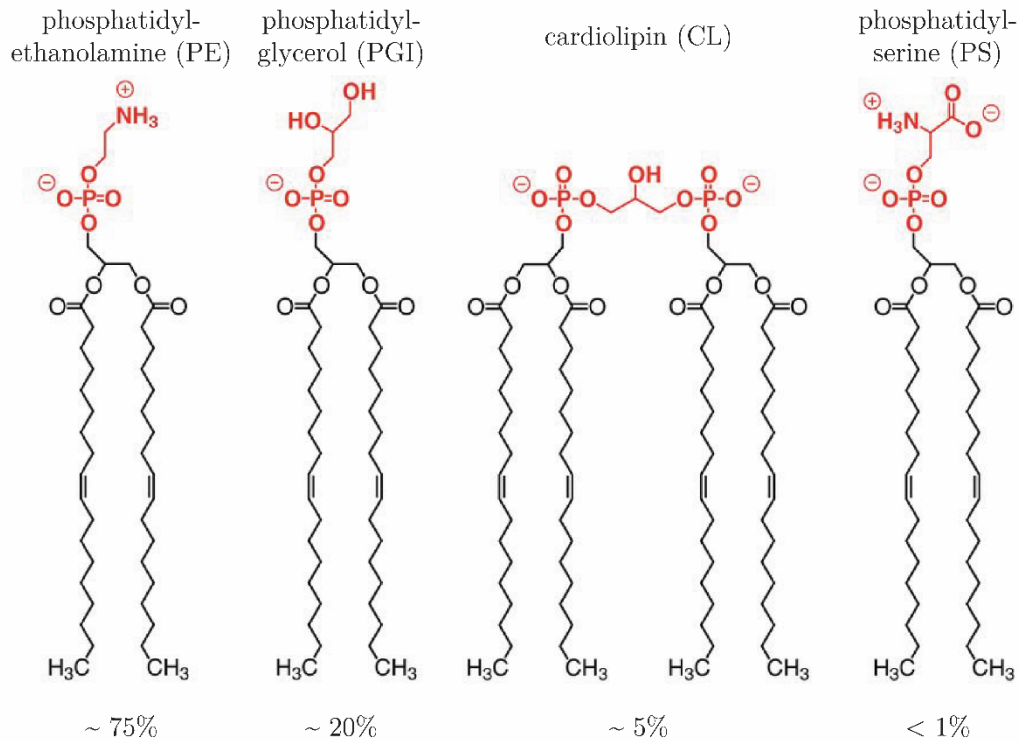


Figure 2. Chemical structure of the major phospholipids in *E. coli*. For simplicity, all phospholipids (colored in red) are represented with an 18-carbon tail. Figure adapted from (Oliver, Crooks et al. 2014).

B. The periplasm

General features of the periplasm

The periplasm is the cell compartment comprised between the IM and the OM of Gram-negative bacteria. Although the periplasm is small in size (~25 nm thickness, 19 nm for the periplasmic space in itself) compared to the global cell

dimension (1 μm of diameter, 2 μm of length for *E. coli*) and ATP-deprived, it fulfills many purposes (Silhavy, Kahne et al. 2010, Asmar, Ferreira et al. 2017), including essential steps involved in cell division, protein transport, protein folding, or signaling function (Miller and Salama 2018).

The cell wall is made up of a single-layered biopolymer called peptidoglycan (PG), which is composed of repeated units of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc). MurNAc units are cross-linked together via short peptides attached to MurNAc via an amide bond and contain both L- and D-amino acids (**Figure 3**) (Typas, Banzhaf et al. 2012). In *E. coli*, the MurNAc oligopeptide is a pentapeptide whose amino sequence is: L-ala – D-glu – mDap – D-ala – D-ala. The peptidoglycan sacculus forms a continuous mesh-like structure with glycan strands of about 20 to 50 glycan units (Harz, Burgdorf et al. 1990). Note that a glycan units is composed of one unit of GlcNAc followed by one unit of MurNAc. The peptidoglycan synthesis is a dynamic process that requires both synthetases to produce and insert new glycan strands into the polymer and hydrolases to cleave the sacculus and allow the insertion of new glycan units (Holtje 1998). The PG contains pores of $\sim 2\text{nm}$ diameter that can theoretically allow the diffusion of globular protein up to 25 kDa molecular weight (Demchick and Koch 1996). Under certain conditions, such as hyperosmotic shocks, the PG pores can stretch and accommodate the diffusion of proteins with up to 100 kDa sizes (Demchick and Koch 1996, Pazos and Peters 2019).

The main features of PG biogenesis

PG synthesis requires the polymerization of new glycan strands by glycosyltransferases and the crosslinking of their oligopeptide side chains by transpeptidases (Typas, Banzhaf et al. 2012). There are two sorts of peptide cross-links: first, D, D-transpeptidase reactions mediated by Penicillin Binding Proteins (PBPs) that form mDap-alanine cross-links (also named 3-4 cross-link) between adjacent peptides stems. Second, L-D-transpeptidase reactions catalyzed by LDTs forming mDap-mDap cross-links (3-3 cross-link) (Magnet, Bellais et al. 2007, Typas, Banzhaf et al. 2012, Egan, Errington et al. 2020). Newly synthesized glycan units are constantly produced and inserted into the cell wall. To avoid an increase in the thickness and to allow the newly synthesized glycan unit to be incorporated into the peptidoglycan, old PG material is removed by hydrolysis (Kraft, Prabhu et al. 1999). N-acetylglucosaminidases, N-acetylmuramidases, amidases, carboxypeptidases, and endopeptidases are among the most common types of PG hydrolases (Egan, Errington et al. 2020).

The PG is attached to the OM via dedicated connectors

The three layers of the Gram-negative cell envelope are not just stacked upon each other. Many proteins make junctions between the PG and the membranes. In *E. coli*, the OM lipoprotein Lpp is covalently attached via its C-terminal lysine to mDap residues found in pentapeptides by three LDTs. In addition, the OM lipoprotein Pal, the β -barrel protein OmpA, and the BAM complex (β -barrel assembly machinery, see further) also interact, but in a non-covalent manner, with the PG via hydrogen bonds and hydrophobic contacts (Koebnik 1995, Cascales, Bernadac et al. 2002, Parsons, Lin et al. 2006, Consoli, Collet et al. 2021). Very recently, it was reported that in other Gram-negative

bacteria (such as *Coxiella burnetii*, *Agrobacterium tumefaciens*, *Legionella pneumophila*, and *Brucella abortus*), the OM is covalently attached to the PG via β -barrel outer membrane proteins (Godessart, Lannoy et al. 2021, Sandoz, Moore et al. 2021).

The PG is a target for numerous antibiotics

The synthesis and remodeling of the PG is a dynamic and essential process for bacterial growth and survival. As a result, PG assembly is the target of several antibiotics, such as β -lactams and glycopeptides. β -lactams antibiotics covalently bind to PBPs, inhibiting PG synthesis, resulting in inhibition of cell growth which leads to cell death (Bajaj, Singh et al. 2016). However, the effectiveness of β -lactams is challenged by the expression of β -lactamases, enzymes able to bind and hydrolyze β -lactams. Also, reducing β -lactam access to the periplasmic space due to regulation in porin expression and mutations is another widely used mechanism to resist β -lactam. Glycopeptides (such as vancomycin) target PG precursors by binding the terminal D-ala-D-ala of MurNAc pentapeptides. By doing so, glycopeptides inhibit the transpeptidase activity involved in PG cross-linking (Stokes, French et al. 2016). Note that Gram-negative bacteria are naturally resistant to glycopeptides as this class of antibiotics has a molecular size that does not allow its diffusion through porins. Glycopeptides can only cross the OM when LPS or OM defects are observed (Ruiz, Falcone et al. 2005, Stokes, French et al. 2016).

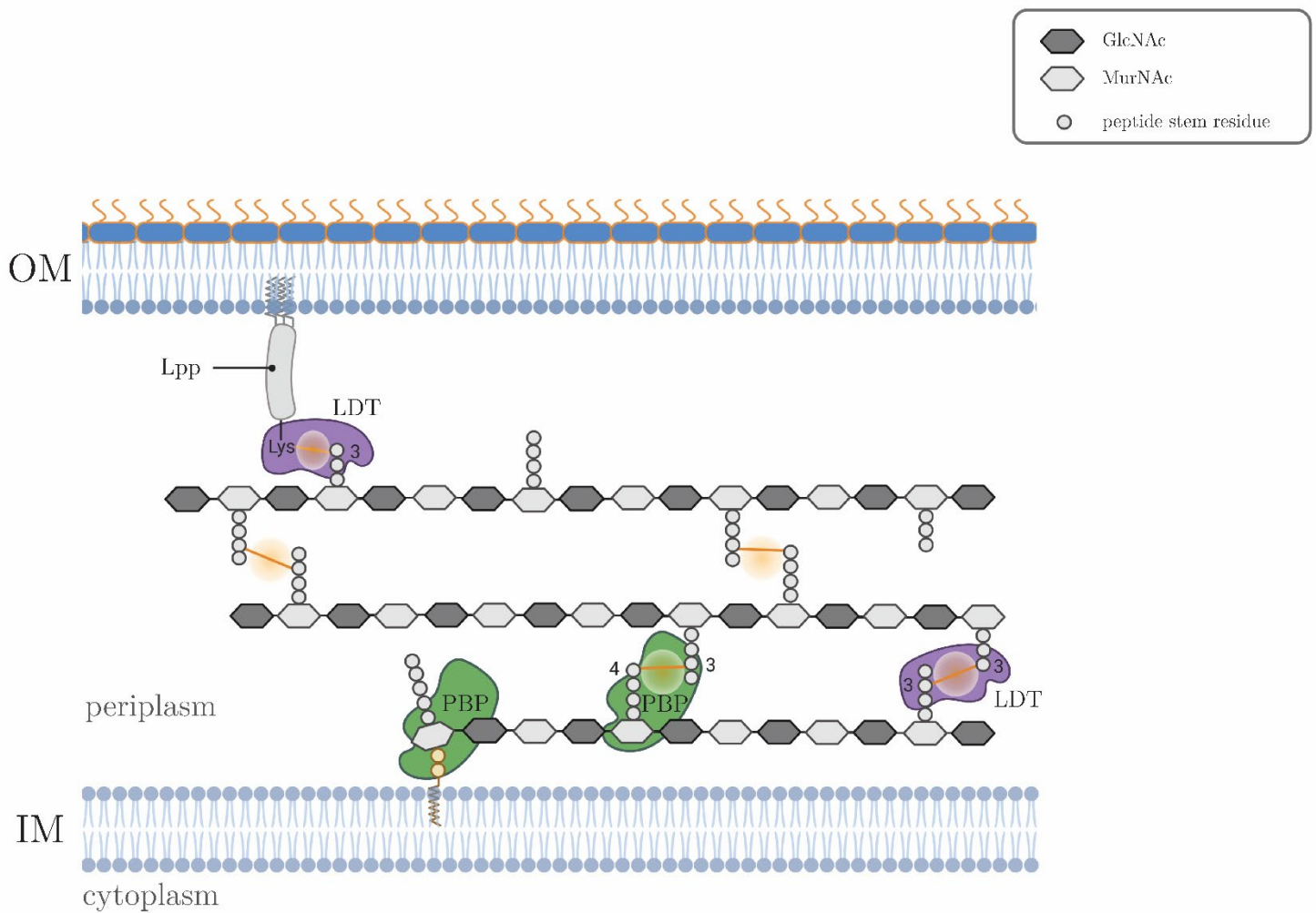


Figure 3. Peptidoglycan synthesis. The peptidoglycan (PG) is a polymer forming a mesh-like structure composed of glycan units: MurNAc linked to GlcNAc. In the periplasm, Penicillin Binding Proteins (PBP) and L-D transpeptidases (LDT) cooperate in PG assembly and crosslinking. In *E. coli*, Lpp, the most abundant protein in the periplasm, is crosslinked to the PG, creating a direct link between the OM and the PG.

C. The outer membrane

General features of the outer membrane

The OM differs from the IM in terms of structure and chemical composition. The OM is a one-of-a-kind asymmetric lipid bilayer that consists of a phospholipid inner leaflet and lipopolysaccharide (LPS)-based outer leaflet. The inner leaflet of the OM is made up of phospholipids, but it contains more PE and is enriched in saturated fatty acids compared to the IM. (Lugtenberg and Peters 1976, Ishinaga, Kanamoto et al. 1979). The LPS present in the outer leaflet is a glycolipid facing the extracellular environment (Doerrler 2006). Similar to the IM, there are two major groups of proteins present in the OM, integral outer membrane proteins (OMPs, see details below) and lipoproteins, which are anchored to the OM by a lipid moiety facing either the periplasm or the extracellular environment (**Figure 1**) (Konovalova, Kahne et al. 2017, El Rayes, Rodriguez-Alonso et al. 2021).

Structure and biosynthesis of the lipopolysaccharide

LPS molecules have a tripartite structure: a lipidic part called “Lipid A”, a core oligosaccharide divided into a conserved inner and a variable outer core, and a variable O-antigen polysaccharide (**Figure 1**) (Heinrichs, Yethon et al. 1998, Bertani and Ruiz 2018). Divalent cations (Mg^{2+} and Ca^{2+}) are associated with the LPS and prevent the repulsion of the LPS molecules caused by their negatively-charged phosphate group. LPS lateral interactions are crucial as they participate in the hydrophilic behavior of the outer leaflet of the OM, which protects bacteria against compounds like antibiotics (glycopeptides, such as Vancomycin), bile salts, or detergents.

The biosynthesis of LPS starts at the cytoplasmic leaflet of the IM where LPS molecules are synthesized in two different parts independently: the core-LipidA and the O-antigen. Then, both components are flipped to the periplasmic side of the IM where they are fused to each other. Finally, the LPS is transported to the OM and flipped from the inner leaflet of the OM to the outer leaflet using a dedicated pathway (see below)(Polissi and Sperandio 2014).

Briefly, synthesis of the core-LipidA moiety is achieved by two enzymatic pathways, the Lpx for the LipidA synthesis and the Waa pathway for the core oligosaccharide addition to the LipidA (Raetz and Whitfield 2002, Mamat, Skurnik et al. 2011, Okuda, Sherman et al. 2016). The LipidA consists of two glucosamine residues carrying a phosphate group on each saccharide which are linked to two fatty acids chains (Raetz, Guan et al. 2009). The starting building block of LipidA is UDP-N-acetylglucosamine (UDP-GlcNAc) which will be processed by the Lpx pathway and generate the LipidA. Later the LipidA will be processed by the Waa enzymatic pathway that will add sugars to it. After synthesis, the core-LipidA is flipped across the IM via the homodimeric and energy-driven MsbA flippase, an ATP Binding Cassette transporter (ABC transporter) (Voss and Trent 2018).

The third component of the LPS is the O-antigen, a highly variable polysaccharide formed by repeated oligosaccharide units themselves composed of four to six sugar residues (Samuel and Reeves 2003). The enzymatic Wec pathway synthesizes the O-antigen at the cytoplasmic side of the IM. At this stage, the O-antigen is linked to an undecaprenyl diphosphate lipid IM anchor, allowing it to reside in the IM (Rai and Mitchell 2020). The flippase Wzx flips the O-antigen-linked lipid from the cytoplasmic to the periplasmic side of the IM (Whitfield, Williams et al. 2020). The final step for LPS assembly occurs at the periplasmic

side of the IM with the linkage of the O-antigen to the Lipid A – core oligosaccharide via the action of the WaaL ligase (Ruan, Monjarás Feria et al. 2018).

LPS are shuttled through the periplasm to reach the outer leaflet of the OM

The transport of LPS molecules across the periplasm is mediated by seven essential proteins named LptA-F. LptA-F forms a protein complex (the Lpt system) that spans from the IM to the OM. The Lpt system comprises two subunits: the LptB₂FGC complex in the IM and the LptDE complex in the OM. The soluble periplasmic protein LptA connects these two subunits (**Figure 4**)(Sperandeo, Martorana et al. 2019).

LptB₂FG forms an ABC transporter which is associated with an extra IM protein: LptC. The LptB₂FG-LptC complex extracts LPS from the IM (Dong, Tang et al. 2017). The OM LptDE complex is formed by the 24-stranded β -barrel LptD plugged by the OM lipoprotein LptE (Freinkman, Chng et al. 2011). LptA creates a bridge between the two Lpt subunits. The Lpt complex functions as an elevator: an LPS molecule is extracted from the IM and pushes the previously extracted molecule across the LptA bridge. LPS molecules finally reach the LptDE complex, where they are flipped into the outer leaflet of the OM (**Figure 4**)(Okuda, Sherman et al. 2016). A recent studies suggest that LPS are releases from LptD *via* a LptD lateral gate (Botte, Ni et al. 2022).

The OM offers protection and enhances cell survival

The OM acts as a selective permeability barrier, protecting the cell from harmful chemicals like detergents and antibiotics while allowing nutrients to pass through diffusion channels in some OMPs (porins). The lipid and protein content of the OM has a significant impact on bacteria's antibiotic sensitivity as well as the development of drug resistance.

To be effective against Gram-negative bacteria, antibiotics must overcome the dual challenge posed by the OM's asymmetrical chemical composition. Antimicrobial compounds are either hydrophilic and small enough (e.g., β -lactams) to diffuse through bacterial porins (like OmpC and OmpF) or hydrophobic (e.g., kanamycin) and have to use a lipid friendly way to enter the cell. However, when the LPS layer is disrupted, cells show increased sensitivity to hydrophobic antibiotics or detergents like bile salts, and to large hydrophilic antibiotics (e.g., Vancomycin). Characterizing LPS biogenesis and transport can therefore lead to the development of new promising therapeutical molecules (Guest, Rutherford et al. 2021).

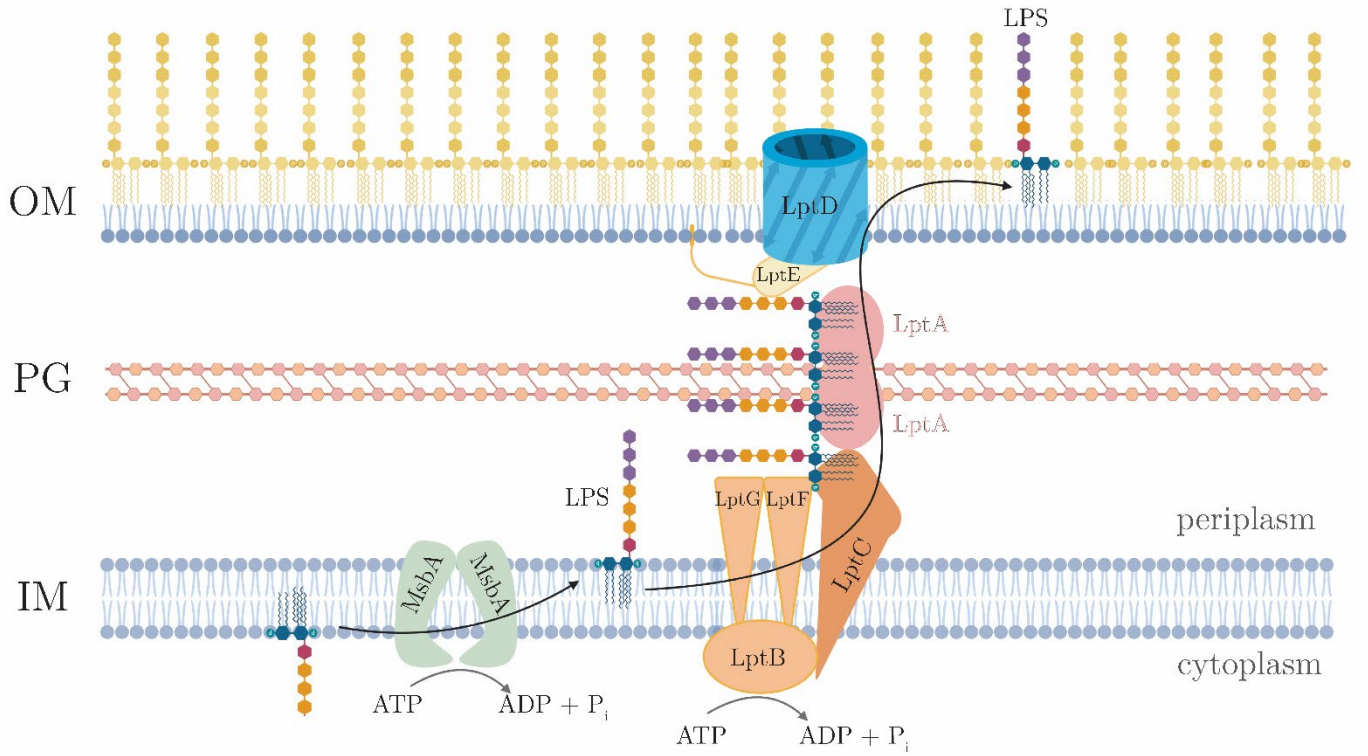
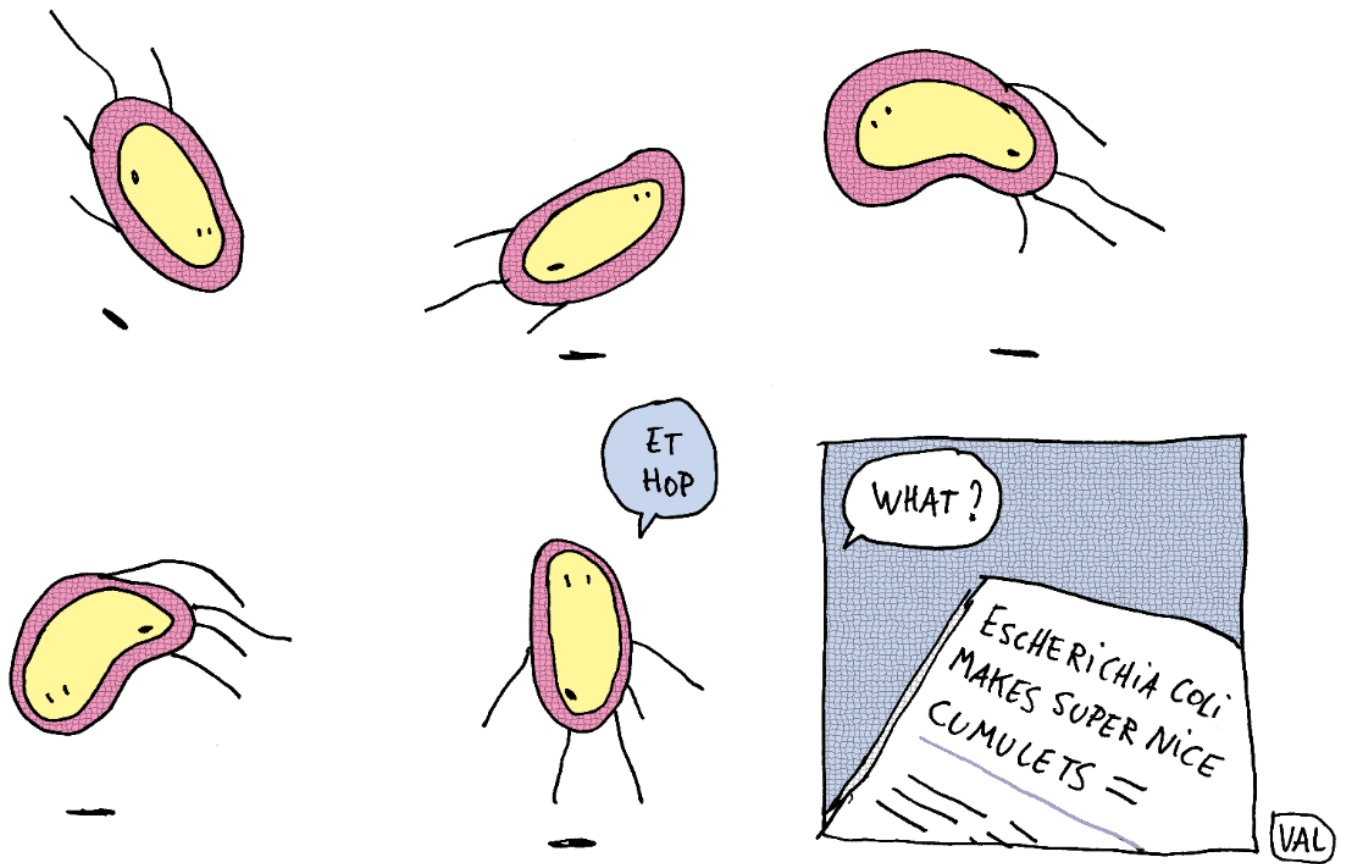


Figure 4. The lipopolysaccharide transport machinery. After synthesis, the core-LipidA flips across the IM via the ABC transporter MsbA. The LPS is then matured and extracted from the IM by LptBFG at the expense of ATP hydrolysis. Following extraction, LPS is transferred to LptC before transiting through the LptA bridge formed across the periplasm. In the OM, LptD in complex with LptE inserts LPS at the outer leaflet of the OM.

LES BACTÉRIES SONT FLEXIBLES SUR LEUR GÉNOME:



I.2.2 The biogenesis of outer membrane proteins

A. Structure, biogenesis, and functions of outer membrane proteins

The general structure, biogenesis, and functions of *E. coli* OM proteins will be discussed in this section. Three OM proteins of *E. coli*, OmpA, OmpC, and OmpF, will receive special attention. Indeed, these proteins are at the center of the work presented in this thesis.

General features of β -barrels

Outer membrane proteins (OMPs) are proteins embedded in the OM thanks to amphipathic, antiparallel β -strands. OMPs acquire a β -barrel structure via hydrogen bonds formation between their first and last β -strand. Polar amino acids face the interior of the barrel while hydrophobic residues face the exterior, interacting with the lipidic environment of the OM (**Figure 5**)(Tamm, Hong et al. 2004). The variety of shapes and sizes of OMPs (as pictured in **Figure 6**) allows them to carry out diverse cellular functions such as envelope biogenesis, signal transduction, cell adhesion, protein secretion, or nutrient uptake.

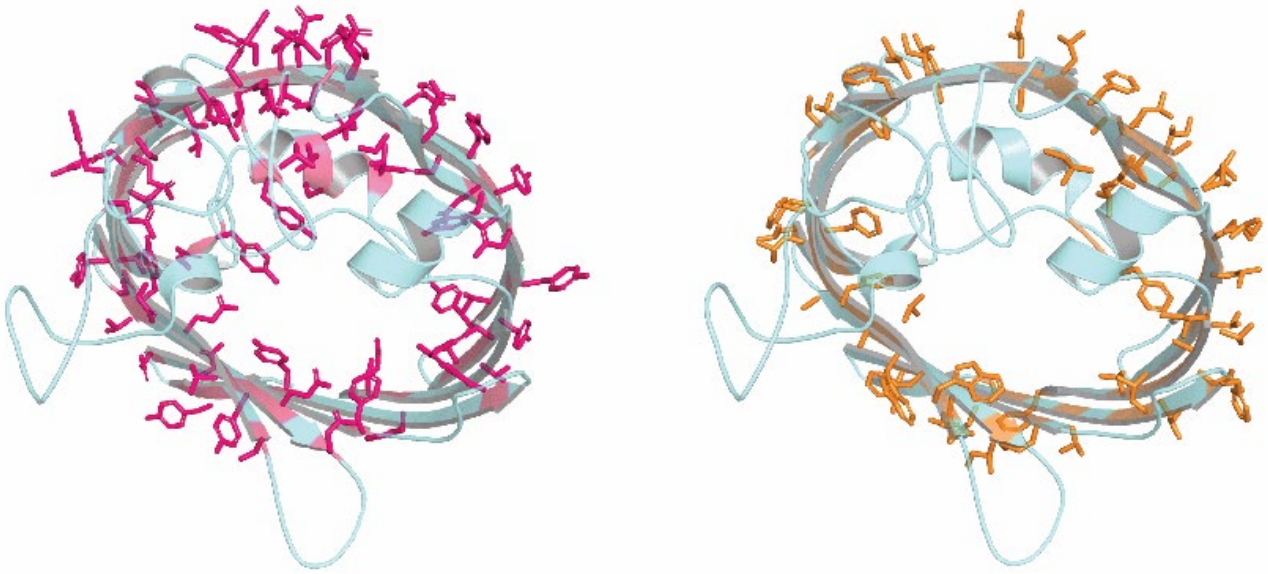


Figure 5. The general β -barrel fold. OmpC is an OMP exhibiting typical features of β -barrels proteins. The structure shown is a top view of a monomer of OmpC (PDB: 2J1N) forming a 16-stranded β -barrel with short periplasmic turns and large extracellular loops. On the left, hydrophilic residues (Ser, Thr, Cys, Asn, Gln, and Tyr) are shown in purple and point mainly inward. On the right, hydrophobic residues (Gly, Ala, Val, Leu, Ile, Pro, Phe, Met, and Trp) are shown in orange and point mainly outward.

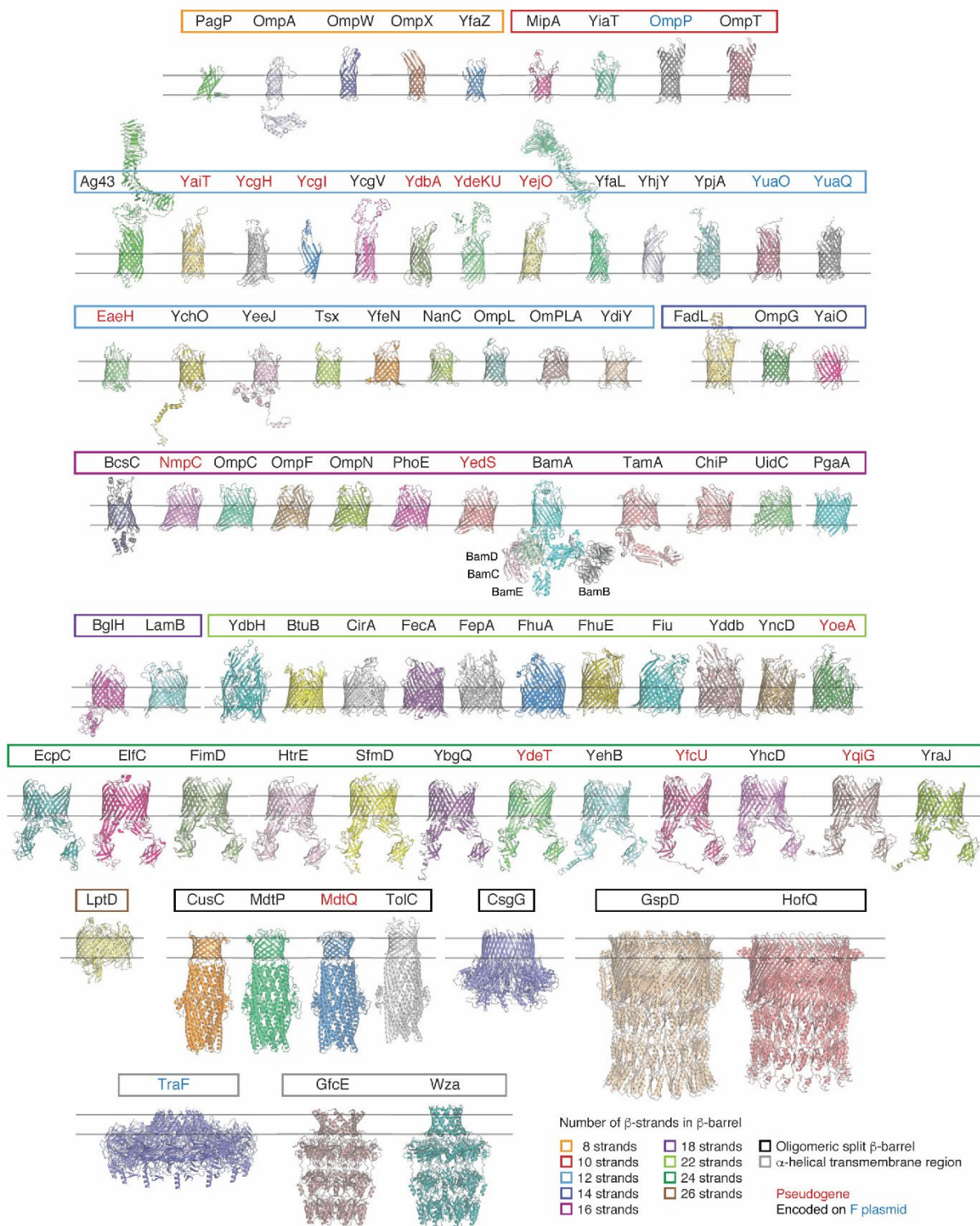


Figure 6. Structures of OMPs found in *E. coli* (K12, MG1655). PDB extracted structures of all known and predicted OMPs of the *E. coli* OM. OMPS are grouped by the number of β -strands, as determined by their color box. Note that the BAM complex is

represented with its non-OMPs proteins. This figure was extracted from (Horne, Brockwell et al. 2020).

A well-known group of OMPs are the pore-forming proteins (also known as porins), primarily studied for their ability to create channels in the OM. Porins can be separated into two subgroups: selective and non-selective channels. For instance on one hand, LamB is a selective channel that forms an 18-stranded β -barrel protein (Schirmer, Keller et al. 1995) which is involved in the specific transport of maltose and maltodextrins across the OM (Dutzler, Wang et al. 1996). On the other hand, OmpC and OmpF are non-selective channels of high importance in maintaining the osmotic homeostasis in the periplasm of *E. coli*. OmpC and OmpF, two highly abundant and structurally similar porins ($\sim 150,000$ copies/cell)(Li, Burkhardt et al. 2014), form trimers in the OM thanks to hydrophobic interactions between β -barrel surfaces. Each monomer consists of a 16-stranded β -barrel containing an aqueous and continuous pore with a diameter of ~ 6 Å and a measured conductance of ~ 2.2 nS (**Figure 7**)(Biro, Pezeshki et al. 2010, Bremer and Kramer 2019). OmpC and OmpF β -strands have short connections at the periplasmic side and long, irregular loops at the extracellular surface; the loop L3 folds into the β -barrel and forms a constriction zone; as a result, L3 deletion results in a much larger pore (~ 20 Å diameter) and a loss of OM permeability (Cowan, Schirmer et al. 1992, Lou, Saint et al. 1996). Due to the constriction of their pore, OmpC/F adopt an hourglass shape, as depicted in **Figure 7B**. The pore displayed at the center of OmpC and OmpF allows small hydrophilic (e.g., sugars, ions, and amino acids) compounds of less than 600 Da to diffuse (O'Shea and Moser 2008). Note that the 600 Da threshold varies depending on the charge and hydrophobicity of the compound to be diffused (Nikaido 2003). Besides their role as non-selective channels, OmpC and OmpF have been shown to directly interact with MlaA, a component of the Mla

pathway that maintains lipid asymmetry in the OM (Chong, Woo et al. 2015, Abellon-Ruiz, Kaptan et al. 2017).

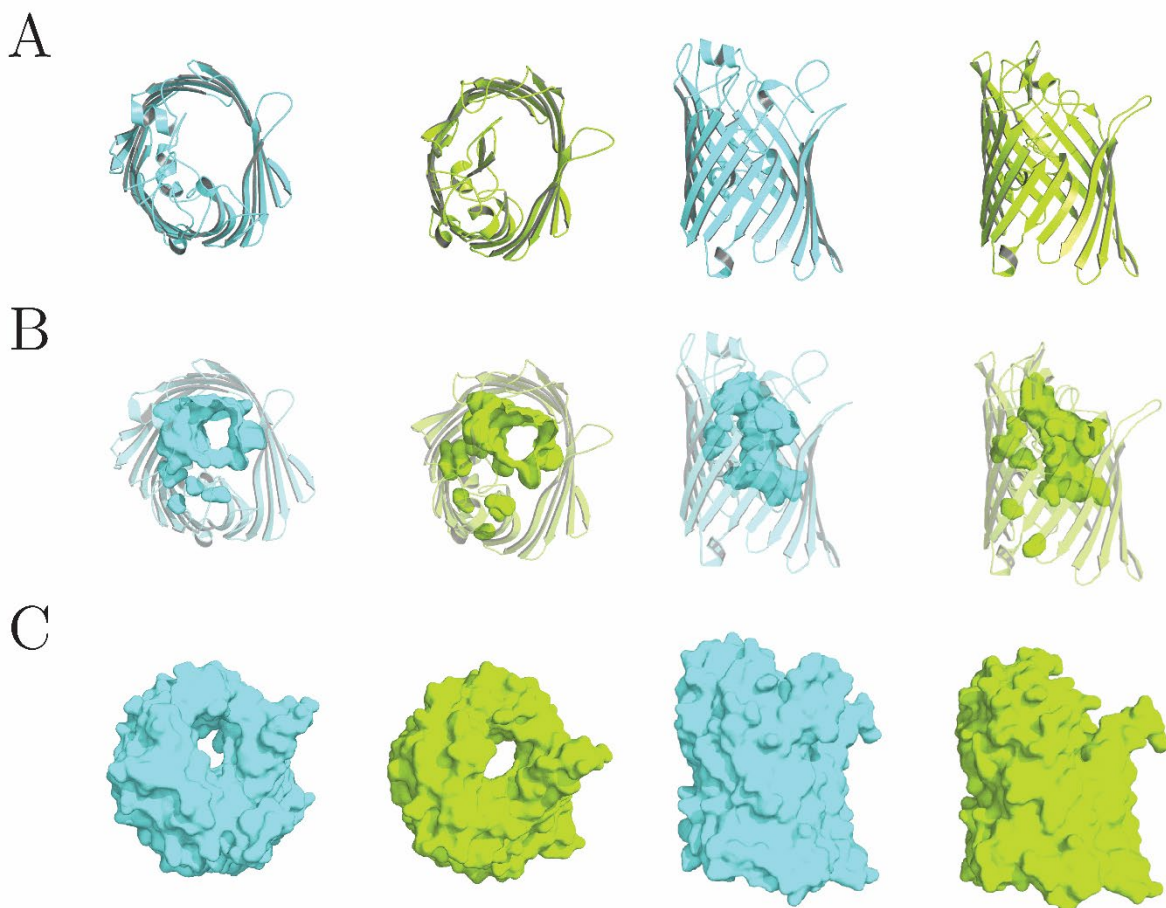


Figure 7. Structural views of OmpC and OmpF, two abundant and non-selective channels of *E. coli*. Top and side views of monomeric OmpC colored in blue (PDB: 2J1N), and monomeric OmpF colored in green (PDB: 2ZFG). **A)** Ribbon diagram of OmpC and OmpF forming a 16-stranded β -barrel. **B)** Ribbon diagram of OmpC and OmpF, the pore is highlighted via a surface view. It shows the hourglass shape that OmpC and OmpF adopt as non-selective channels. **C)** Surface representation of OmpC and OmpF. It shows the pore size difference between the two porins, OmpF has a larger pore than OmpC.

However, all OMPs are not functioning as channels, and some are involved in other processes. For instance, BamA and LptD, two essential proteins, are involved in the biogenesis of the OM (Silhavy, Kahne et al. 2010). I want to raise the reader's attention on another OMP, OmpA, which is not involved in envelope biogenesis and does not function as a channel but is important for adhesion, toxicity, invasiveness, and biofilm formation. OmpA has therefore been proposed to be a valid target for new therapeutic approaches against bacteria (Guan, Bhowmick et al. 2021). Supporting this, OmpA β -barrel is required for bacterial conjugation and also as a bacteriophages receptor and a colicines target (Foulds and Barrett 1973, Schweizer and Henning 1977, Morona, Klose et al. 1984, Morona, Tommassen et al. 1985, Ried and Henning 1987). OmpA is highly abundant in *E. coli* (~200 000 copies/cell)(Li, Burkhardt et al. 2014). Although OmpA has been studied for many years, how it folds remains controversial. The most accepted view is that OmpA folds into a two domain-protein with an 8-stranded β -barrel and a soluble globular periplasmic C-terminal domain (De Mot and Vanderleyden 1994, Koebnik 1995, Pautsch and Schulz 1998, Pautsch and Schulz 2000, Park, Lee et al. 2012, Ishida, Garcia-Herrero et al. 2014). However, an alternative conformation has been proposed in which OmpA folds into a large, 16-stranded β -barrel (Stathopoulos 1996, Zakharian and Reusch 2005, Negoda, Negoda et al. 2010).

In the two domains' conformation, the periplasmic region of OmpA harbors a structure commonly found in proteins that are associated in a non-covalently manner with the peptidoglycan (Parsons, Lin et al. 2006). In the reported structure of the periplasmic domain of OmpA (PDB: 2MQE), a PG precursor was identified, and two OmpA residues (D241 and R256) were identified as interacting with it, supporting the role of OmpA in mediating cell wall attachment (Ishida, Garcia-Herrero et al. 2014, Samsudin, Ortiz-Suarez et al. 2016, Samsudin, Boags et al.

2017, Boags, Samsudin et al. 2019). On the other hand, the N-terminal domain of OmpA forms a 8-stranded β -barrel which in the reported structure (PDB: 1QJP) display large extracellular loops and short turns at the periplasmic side (Pautsch and Schulz 2000). Note that in the two domain's conformation no pore is observed in the N-terminal β -barrel domain of OmpA (Pautsch and Schulz 1998, Pautsch and Schulz 2000). Consistent with the two domains conformation, OmpA forms dimers in the OM of bacteria, primarily *via* formation of salt bridges in the periplasmic domain between residues K192, E310 and E312 and secondly via the OmpA N-terminal domain in which polar and non-polar residues of loop L1,L2 and L4 are involved in weak interactions (Stenberg, Chovanec et al. 2005, Ortiz-Suarez, Samsudin et al. 2016).

In the alternative conformation, based on prediction, OmpA folds as a one 16-stranded β -barrel protein in which, compared to the two-domain structure, the periplasmic domain would be folded in the OM forming an OmpC/F-like β -barrel protein (Stathopoulos 1996). Note that so far no structure has been solved supporting the one domain conformation of OmpA. Moreover, in the work of *Stathopoulos, 1996*, they explain that a large OmpA would exhibit peculiar features at its C-terminal region compare to other OMPs: (1) long periplasmic loops and short extracellular ones, (2) a lack of hydrophobic residues in the carboxy region that would shortens the β -strands and (3) an enrichment in proline residues compared to other OMPs (Stathopoulos 1996). However studies on the folding of OmpA have shown that the conductance of OmpA varies according to the temperature. For instance, at room temperature OmpA forms low conductance pores (0.06 ± 0.02 nS) which at 37°C the conductance was increased up to 0.4 ± 0.06 nS (Zakharian and Reusch 2005, Negoda, Negoda et al. 2010, Reusch 2012). Such a variation in the conductance would suggest a shift from an narrow (at low

temperature) to a large (at higher temperature) OmpA pore. In this model, OmpA forms first a two-domain structure that represents an intermediate state (kinetically stable at low temperature) as it later folds into a large 16-stranded β -barrel (Zakharian and Reusch 2005). However, I want to raise the reader's attention, to mention that even a 16-stranded β -barrel conformation for OmpA as suggested based on the shift in conductance is unlikely to result in an OmpC/F-like structure. Indeed, the conductance at 37°C of various 16-stranded β -barrel pore forming proteins such as OmpC, OmpF, PhoE or LamB has been measured as ranging from ~2 to 4 nS conductance (Benz, Darveau et al. 1984, Benz, Schmid et al. 1986, Biro, Pezeshki et al. 2010). On the other hand, a non-pore forming 16-stranded β -barrel such as BamA have a conductance of ~0.5 nS, which is in better agreement to the conductance observed for OmpA at 37°C (~0.4 nS) (Robert, Volokhina et al. 2006). To conclude, the exact topology of OmpA remains controversial and more works are required to unambiguously assess its conformation.

Biogenesis of OMPs: from the cytoplasm to the outer membrane

OMPs must be transported and inserted into the OM in order to perform their function. To do so, bacteria have evolved dedicated pathways. In this section, I will briefly describe the journey of a nascent OMPs from the cytoplasm to the OM.

When unfolded OMPs emerge from the ribosome, they present an N-terminal signal sequence (SS) of approximately 22 amino acids (von Heijne 1985, Hegde and Bernstein 2006). The OMPs SS has a positively charged N-terminal part (residues 1-3) followed by a hydrophobic part (9-15 residues) and a polar C-terminal region containing a consensus cleavage site for a signal peptidase (von Heijne 1985). Upon release from the ribosome, hydrophobic and aromatic regions of fully

synthesized proteins are recognized and protected by the SecB chaperone, which maintains the pre-OMP in a translocation competent state (mainly in an unfolded state)(Bechtluft, Kedrov et al. 2010, De Geyter, Portaliou et al. 2020). SecB, in complex with the polypeptide, then interacts at the membrane with the ATPase SecA, which is associated with the SecYEG translocon, a heterotrimeric transmembrane complex embedded in the IM (Komar, Alvira et al. 2016). The interaction between SecB and SecA induces the dissociation of the complex between SecB and the pre-OMP, which is then transferred to SecA. The SS of the pre-OMP forms an α -helix and is transferred inside the SecY pore in an ATP-dependent manner (Hartl, Lecker et al. 1990, Randall, Crane et al. 2004). Upon several rounds of ATP hydrolysis, the pre-OMP is translocated through SecY (White and Von Heijne 2008). After complete translocation, the SS is cleaved at the periplasmic side of the IM by the signal peptidase SPaseI (Paetzel, Karla et al. 2002). Several IM and periplasmic factors facilitate the protein release from the translocon. For instance, SecD-F and YajC, which associate with the SecYEG translocon, pull out the protein at the periplasmic side of SecY (Tsukazaki, Mori et al. 2011, Kuhn and Kiefer 2017).

Once in the periplasm, pre-OMPs interact with periplasmic chaperones that help them to travel through the periplasm and reach their final destination. Unfolded OMPs have indeed a high tendency to aggregate and therefore need to be protected. This is the reason why *E. coli* possesses periplasmic chaperones, such as Survival protein A (SurA), Seventeen-kilodalton protein (Skp), and the serine endoprotease DegP, that shuttle OMPs to the OM (Sklar, Wu et al. 2007). It has been shown that SurA, Skp, and DegP are involved in two partially redundant pathways. The first pathway involves SurA (Mas, Thoma et al. 2019), while the second involves Skp and DegP. Note that DegP acts either as a chaperone or as a

protease depending on the temperature (<28°C it acts as a chaperone)(Spiess, Beil et al. 1999) (Sklar, Wu et al. 2007). Supporting evidence for these two pathways come from genetic experiments showing that while the *skp degP* mutant or the *surA* mutant are viable, *surA skp* or *surA degP* mutants are not (Denoncin, Schwalm et al. 2012). Strains lacking SurA display severe OM defects, which indicates that SurA plays a major role in OMPs assembly while the role of Skp and DegP appears to be minor unless SurA is absent (Sklar, Wu et al. 2007). Skp and DegP act more as a quality control mechanism monitoring proper OMP incorporation in the OM. Indeed, results show that β -barrels failing to be incorporated in the OM are removed from the BAM complex by Skp, which acts as a sacrificial adaptor protein: the Skp-unfolded OMP complex is indeed degraded by DegP (Chen and Henning 1996, Jarchow, Luck et al. 2008, Burmann, Wang et al. 2013, Thoma, Burmann et al. 2015, Combs and Silhavy 2022). Overall the exact mechanism by which SurA-OMPs are delivered remains largely unknown, yet recent work shows that SurA interacts with POTRA 2 and 3 of BamA which suggests that the BAM complex would form a supercomplex with the Sec translocon across the periplasm, helping to direct the delivery of nascent OMPs to BAM (Wang, Wang et al. 2016).

In addition to these three major chaperones, *E. coli* expresses other proteins that assist the OMPs in their journey throughout the periplasmic space. For instance, BepA, a metallopeptidase seems to function both as a chaperone and a protease; it specifically acts in the quality control of LptD and interacts with the BAM complex (Daimon, Iwama-Masui et al. 2017, Shahrizal, Daimon et al. 2019, Daimon, Narita et al. 2020). FkpA is a chaperone that, together with Skp, helps in the folding of LptD. It has been suggested to be functionally redundant to SurA under heat shock conditions, as a $\Delta surA \Delta skp$ lethal mutant is viable at heat shock temperature thanks to FkpA activity (Ge, Lyu et al. 2014).

OMPs easily aggregate in aqueous environment, to protect and assist them through the journey across the periplasm, periplasmic chaperones are present in *E. coli*. Once at the OM, the chaperones deliver the OMPs to multiprotein machinery, which catalyzes their insertion in the OM. In *E. coli*, the primary OM receiver for OMPs is the BAM complex (see below for details); another receiver is the translocation and assembly module (TAM) that inserts subgroups of OMPs: the autotransporters (Stubenrauch and Lithgow 2019). In vitro experiments shows that OMPs can spontaneously fold and be OM inserted (Gessmann, Chung et al. 2014, Thoma, Burmann et al. 2015). So it remains unclear why exactly OMPs require assembly machinery to be inserted into the OM (Schiffrin, Brockwell et al. 2017, Hussain and Bernstein 2018, Horne, Brockwell et al. 2020, Iadanza, Schiffrin et al. 2020). The OMPs biogenesis pathway is summarized at **Figure 8**.

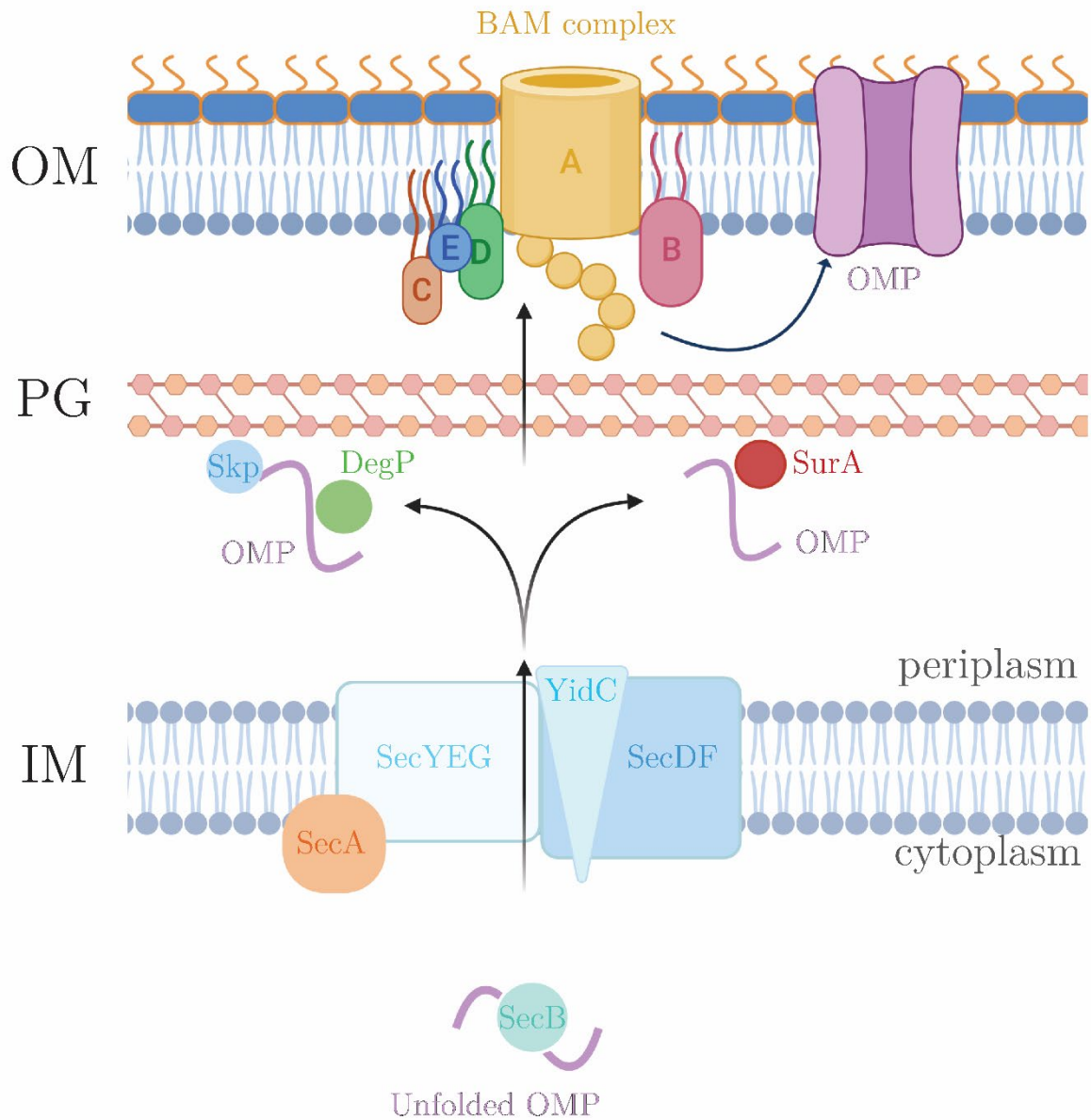


Figure 8. Overview of the β -barrel outer membrane proteins biogenesis pathway. The cytoplasmic chaperone SecB directs unfolded OMPs to the SecYEG translocon. SecB interacts with SecA, the ATPase associated with SecYEG, at the membrane. The proteins are then transferred from SecB to SecA. Proteins are translocated through the SECYEG translocon when ATP is hydrolyzed. SecDF and YidC are inner membrane (IM) proteins associated with SecYEG that help unfolded OMPs to be released in the periplasm. Upon release in the periplasm, chaperones bind to the OMP and assist their transport to the BAM complex at the outer membrane (OM). The three main

periplasmic chaperones known to interact with β -barrel OMPs are SurA, DegP, and Skp. These chaperones function in parallel pathways, one involving SurA and one involving DegP and Skp. SurA is believed to be the primary chaperone in the periplasm, while DegP and Skp rescue OMPs that fail to be folded by the BAM complex. The BAM complex will mediate the folding and insertion of the OMP into the OM.

B. The β -barrel assembly machinery inserts OMPs into the OM

In this section, I will briefly describe the components of the BAM complex, which is responsible for OMP folding and insertion in the *E. coli* OM. The BAM complex is composed of five proteins: BamA, a β -barrel, and four accessory lipoproteins BamB/C/D/E (Wu, McCandlish et al. 2006, Sklar, Wu et al. 2007). The four lipoproteins are anchored in the OM via a lipidic moiety and are associated with BamA at the periplasmic side of the OM. Although BamD and BamA are the only essential component of this complex, the absence of any other BAM lipoproteins causes defects in OMPs assembly (Malinverni, Werner et al. 2006).

BamA is a 16-stranded β -barrel harboring at its N-terminal part a periplasmic extension of five polypeptide translocation associated domains named POTRA domains. While the five POTRA domains have little primary sequence identity, they share a similar structural organization and are important for initiating the folding of OMPs into the BamA β -barrel (Noinaj, Gumbart et al. 2017). BamB adopts an 8-bladed β -propeller fold with a central pore. BamB binds to the POTRA domains and is believed to stabilize BAM by stiffening it (Noinaj, Gumbart et al. 2017, Wu, Bakelar et al. 2021). BamC has an unstructured N-terminal domain followed by two α -helix grips. Results suggest that one of the α -helix grip of BamC is surface-exposed, but the function of such surface exposure

remains mysterious (Webb and Lithgow 2015). BamD, the only essential lipoprotein of the BAM complex, is believed to serve as an OMPs substrate receptor by recognizing the β -signal motif present in OMPs. BamD is composed of multiple tetratricopeptide repeat domains (TPR) packed together into a superhelical structure (Noinaj, Gumbart et al. 2017). BamE interacts with the POTRA domains as well as with BamD, bridging BamA to BamD (Noinaj, Gumbart et al. 2017).

In 2016, four independent research teams obtained BAM complex structures using either X-Ray crystallography or cryo-EM (Bakelar, Buchanan et al. 2016, Gu, Li et al. 2016, Han, Zheng et al. 2016, Iadanza, Higgins et al. 2016). Briefly, these structures reveal that BamB/D/E interact with the BamA POTRA domains, while BamC interacts with BamD and BamE (Noinaj, Gumbart et al. 2017). In addition, the four structures reveal that BamA adopts two conformations. First is the “inward-open” conformation in which BamA displays an open pore at its periplasmic side. The POTRA domains surround the β -barrel, forming a ring that allows nascent OMPs to be delivered to the lumen of the BamA periplasmic pore (Gu, Li et al. 2016, Han, Zheng et al. 2016). Second, in the “outward-open” conformation, the BamA β -barrel opens between $\beta 1$ and $\beta 16$, creating a lateral gate to the membrane. The five POTRA domains are rearranged and occlude the periplasmic mouth of the β -barrel. In this conformation, the nascent OMPs cannot be delivered to BAM (Bakelar, Buchanan et al. 2016, Iadanza, Higgins et al. 2016). Although the exact mechanism remains unclear, the transition between the closed and open states of the BamA-barrel, called the scissor-like movement, seems important for the OMPs insertion into the OM. The BAM complex is thought to wait for the OMPs substrate to arrive in the "inward-open" state, while in the "outward-open" state, the BAM complex releases OMPs in the OM. (Tomasek and Kahne 2021).

C. Current view on β -barrel OMP assembly in the OM

The OMPs are client proteins of the BAM complex and are specifically recognized as such. This section will first go over how the BAM complex recognizes OMPs as substrates, and then go over the main models of OMPs insertion into the OM.

OMPs are specifically recognized as BAM substrates

OMPs possess at the C-terminal part of their amino acid sequence a conserved region located in the 5th-to-last β -strands named the β -signal, as well as a highly conserved phenylalanine at the C-terminal position. While the exact mechanism is not known, evidence suggests that BamA and BamD recognize the β -signal and the terminal phenylalanine, which allow the OMPs delivery to the BAM complex. (Struyve, Moons et al. 1991, de Cock, Struyve et al. 1997, Robert, Volokhina et al. 2006, Kutik, Stojanovski et al. 2008, Germany, Ding et al. 2021).

Models of insertion of OMPs mediated by the BAM complex

Up to date, three mechanisms for OMPs assembly by the BAM complex have been proposed (Gessmann, Chung et al. 2014, Noinaj, Gumbart et al. 2017, Schiffrin, Brockwell et al. 2017, Wang and Bernstein 2022).

The first model, called the **BamA-assisted model**, suggests that OMPs folding spontaneously occurs in the periplasm or near the BAM complex. Membrane incorporation occurs near the lateral gate of BamA, which would locally disrupt the lipidic bilayer, leading to a thinner membrane and promoting OMPs insertion

(see **Figure 9**) (Jain and Goldberg 2007, Ieva and Bernstein 2009, Fleming, Patel et al. 2016, Gu, Li et al. 2016, Lee, Xue et al. 2016).

In the second model, known as **BamA-hybrid barrel model**, the nascent OMP zips its last C-terminal β -strand with BamA $_{\beta 1}$. Then, by progressive intercalation of more N-terminal β -strands near BamA $_{\beta 16}$, a BamA-OMPs hybrid barrel is created. At the final stage, the OMP is released from BamA into the OM (see **Figure 9**) (Sapra, Damaghi et al. 2009, Thoma, Bosshart et al. 2012).

In the third model, named as **BamA-triggered barrel-elongation**, the nascent OMP zips its last C-terminal β -strand with BamA $_{\beta 1}$. But in this model, the BamA $_{\beta 1}$ -OMP $_{\text{last-}\beta}$ is served as a template for the rest of the OMPs folding, occurring via β -augmentation. This process continues until an elongated β -sheet is formed and eventually leads to the sealing of the OMPs on themselves. The scissor-like movement of BamA is probably the driving force of the β -augmentation as it pulls out the nascent β -sheet into the OM prior to its release in the OM (see **Figure 9**) (Ranava, Caumont-Sarcos et al. 2018, Doyle and Bernstein 2019, Tomasek,

Rawson et al. 2020). This model was recently supported by structural work from Doyle and co-workers (Doyle, Jimah et al. 2022).

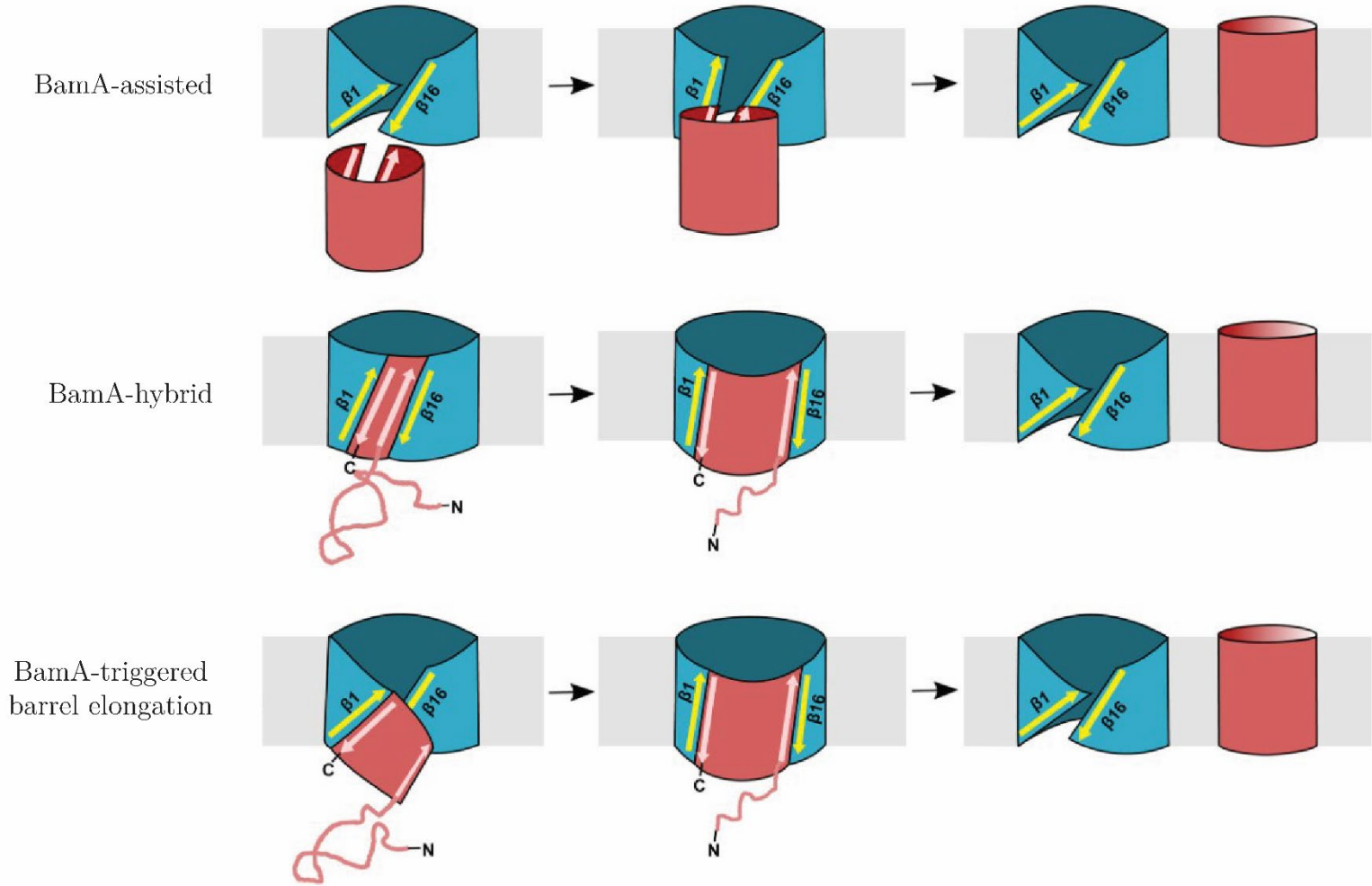


Figure 9. Possible mechanism for the assembly of β -barrel proteins. Three models of OMP (colored in red) assembly by BamA (colored in blue) are depicted. The BamA-assisted model suggests the OMPs are prefolded in the periplasm and are integrated at the BamA lateral gate, where a membrane thinning occurs. In the BamA-hybrid model, the C-terminal β -strand of the nascent OMPs is zipped to BamA $_{\beta 1}$, then BamA intercalates progressively the N-terminal β -strand of OMPs near BamA $_{\beta 16}$. In the BamA-triggered barrel elongation, the C-terminal β -strand of the nascent OMPs is zipped to BamA $_{\beta 1}$. Then,

a nascent β -sheet elongates via β -augmentation across the OM, likely driven by the scissor-like movement of BamA. At a final stage, the BamA-OMPs is released in the OM. For clarity, the POTRA domains and the accessories lipoproteins of the BAM complex are omitted. This figure was adapted from (Ranava, Caumont-Sarcos et al. 2018).

Given its central role in OMPs biogenesis, the BAM complex has been identified as an attractive target for developing antimicrobial compounds. As proof of this, darobactin, a natural antibiotic produced in nematodes' gut, has been shown to inhibit BAM function by mimicking a β -strand that binds to the lateral gate of BamA with higher affinity than OMP substrates. Doing so, darobactin inhibits BAM function, which leads to OM defects and ultimately to cell death (Kaur, Jakob et al. 2021).

I.2.3 The biogenesis of lipoproteins

A. Maturation and processing of lipoproteins in the cell envelope

The genome of *E. coli* encodes ~100 lipoproteins (Juncker, Willenbrock et al. 2003). These globular proteins, while being anchored either in the IM or in the OM (~90 % of *E. coli* lipoproteins), are present in the periplasm (Horler, Butcher et al. 2009, Narita and Tokuda 2010, Zuckert 2014). The general view states that *E. coli* lipoproteins face the periplasm.

Biogenesis of lipoproteins: from the cytoplasm to the periplasm

The lipoproteins maturation and biogenesis pathway have been recently reviewed (El Rayes, Rodriguez-Alonso et al. 2021). Lipoproteins are synthesized as precursors in the cytosol and, like other envelope proteins, present a signal peptide which is ~25 amino acids long (Hayashi and Wu 1990, Babu, Priya et al. 2006). In the case of lipoproteins, the signal sequence is followed by a particular motif: L/V/I – A/S/T/V/I – G/A/S – C, known as the lipobox. Note that only the last cysteine of the motif is strictly conserved; it will later become the first residue of the mature lipoprotein. (Inouye, Wang et al. 1977, Babu, Priya et al. 2006). This signal sequence directs the lipoproteins to the translocation machineries.

Most lipoproteins will cross the IM using the Sec pathway as described previously for OMPs. Note that a few lipoproteins will cross the cytoplasmic membrane in a partially folded state, using the twin-arginine translocation (TAT) machinery (not discussed here) (Palmer and Berks 2012, Crane and Randall 2017). Once translocated across the IM, lipoproteins, which are anchored to the IM via

the α -helix formed by their signal sequence, undergo a three-step maturation process: (1) a diacyl glyceryl moiety is added to the N-terminal cysteine lipobox by the lipoprotein diacyl glyceryl transferase (Lgt) (Sankaran and Wu 1994, Mao, Zhao et al. 2016). (2) The signal sequence is cleaved by the signal peptidase II (LspA) (Vogeley, El Arnaout et al. 2016) and (3) the N-terminal cysteine residue is aminoacylated by the acyltransferase Lnt (Wiktor, Weichert et al. 2017). Note that Lgt, LspA, and Lnt are essential in most Gram-negative organisms (Okuda and Tokuda 2011, Buddelmeijer 2015, El Arnaout and Soulimane 2019, Legood, Boneca et al. 2021). The lipoprotein maturation process is summarized in **Figure 10**.

The sorting of lipoproteins between the IM and the OM

In *E. coli*, the final localization of lipoproteins is dependent on a lipoprotein localization machinery, the Lol system, which is composed of five essential proteins (LolA/B/C/D/E). Lipoproteins destined for the OM are extracted from the IM through the action of LolCDE, an IM ABC transporter (Narita and Tokuda 2010). Recent work suggests that upon ATP hydrolysis, the LolCDE complex undergoes a conformational change that lifts the lipoprotein lipid moiety out of the IM membrane (Sharma, Zhou et al. 2021, Tang, Chang et al. 2021) and allows the binding of LolA to the periplasmic domain of LolC (Kaplan, Greene et al. 2018, Sharma, Zhou et al. 2021). When fully extracted from the membrane, the lipoprotein is delivered to LolA (Tang, Chang et al. 2021). LolA is an incomplete β -barrel with a hydrophobic cavity large enough to contain the acyl chains of the lipoproteins, protecting them from the hydrophilic periplasmic compartment (Takeda, Miyatake et al. 2003, Boags, Samsudin et al. 2019). The LolA-substrate complex crosses the periplasm to reach the OM. At the OM, LolA delivers its lipoprotein substrate to LolB, a structural homolog of LolA, by a mouth-to-mouth interaction (Okuda and Tokuda 2009, Narita and Tokuda 2010, Grabowicz 2019).

Subsequently, LolB incorporates the client lipoprotein in the OM by a poorly understood mechanism (see **Figure 10**).

Mature lipoproteins reside in the IM if they have a signal at their N-terminus that prevents them from interacting with LolA and hence being transported. This Lol avoidance signal is an aspartate located after the N-terminal cysteine (Yamaguchi, Yu et al. 1988). Note that the nature of the residue at position +3 (relative to the cysteine) also plays a role (e.g., an aspartate, glutamate, or glutamine strengthens IM retention) (Gennity and Inouye 1991, Terada, Kuroda et al. 2001). Furthermore, recent work regarding the specificity of the Lol system for its client protein showed that ~50% of the *E. coli* OM lipoproteins display an intrinsically disordered N-terminal linker which is required for optimal shuttling by the Lol system (El Rayes, Szewczyk et al. 2021).

Lipoproteins perform a variety of roles in cell physiology therefore sorting them correctly between the IM, and the OM is critical for bacteria. The following section will discuss significant roles fulfilled by lipoproteins in *E. coli*.

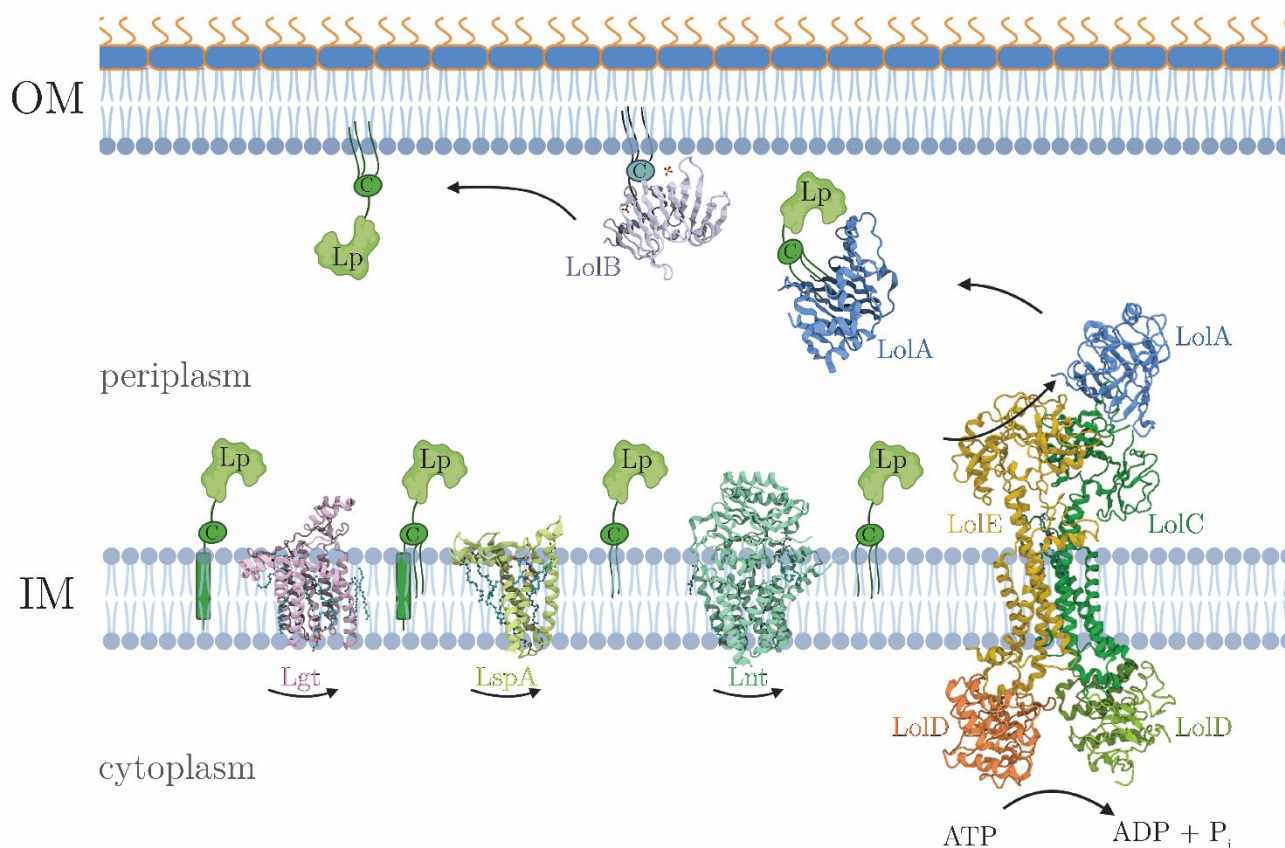


Figure 10. The lipoprotein transport maturation and transport system in the cell envelope. Following translocation of the lipoprotein across the inner membrane, Lgt (PDB: 5AZC) catalyzes the addition of diacyl glyceryl to the cysteine (C) residue of the lipobox. The signal peptide is then cleaved by the peptidase LspA (PDB: 5DIR). As a last step of maturation, Lnt (PDB: 6NWR) catalyzes the acylation of the N-terminal cysteine. Lipoproteins destined for the OM are extracted from the IM by an ABC transporter, LolCDE. LolA (PDB: 2ZPD) in complex with the periplasmic domain of LolC receives the transferred lipoproteins and shuttles them through the periplasm (PDB: 7ARM). The lipoproteins are delivered to LolB (PDB: 3WJV) by the mouth-to-mouth interface between LolA and LolB, which then inserts them into the OM via an unknown mechanism.

B. Role of lipoproteins in the cell envelope of Gram-negative bacteria

Lipoproteins are involved in a wide range of functions. Whereas some, like RcsF, monitor envelope integrity, others play a role in virulence or in envelope biogenesis (Konovalova, Kahne et al. 2017, Laloux and Collet 2017). For instance, Lpp is one of the lipoproteins involved in the OM connection to the PG. Lpp is by far the most abundant protein in *E. coli* (~1,000,000 copies/cell), structured as an α -helical tether covalently linked to the PG. It acts as a distance keeper for the cell envelope (Asmar, Ferreira et al. 2017, Cohen EJ 2017). A detachment of Lpp from the PG leads the OM to be pulled away from the IM, forming blebs (Hoekstra, van der Laan et al. 1976, Asmar, Ferreira et al. 2017).

Another OM lipoprotein critical for OM integrity is DolP. DolP is found in the mid-cell during the late stages of cell division, where it regulates septal peptidoglycan splitting through an unclear mechanism (Tsang, Yakhnina et al. 2017). Moreover, a recent study shows that under stress, DolP supports the proper folding and function of the BAM complex by interacting with BamA (Ranava, Yang et al. 2021).

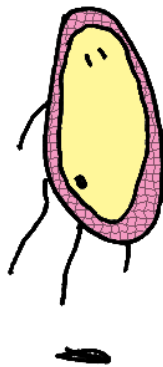
Finally, a lipoprotein extensively studied during my thesis is RcsF, the stress sensor of the Rcs pathway. RcsF will be discussed in detail in the Chapter II of this thesis. RcsF is a remarkable lipoprotein as, besides its stress sensor role, it is the only lipoprotein of *E. coli* that has been demonstrated by different groups as surface-exposed (Cho, Szewczyk et al. 2014, Konovalova, Perlman et al. 2014).

C. Surface exposed lipoproteins

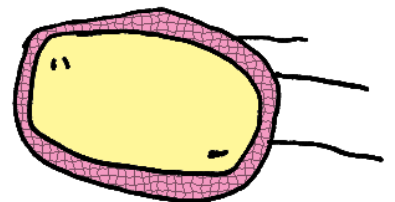
After reaching the OM, some lipoproteins use poorly understood methods to cross the asymmetric lipid bilayer and reach the cell surface. Surface-exposed lipoproteins (SLPs), which are attractive candidates for vaccine development (Vernikos and Medini 2014, Basta and Christensen 2016) are common in certain bacterial species such as *B. burgdorferi*, where two-thirds of the lipoproteins are SLPs (Wilson and Bernstein 2016, Dowdell, Murphy et al. 2017). Yet, in species such as *E. coli*, SLPs are less common, and so far, only RcsF, the Rcs stress sensor, has been confirmed to be surface exposed by different groups (Cho, Szewczyk et al. 2014, Konovalova, Perlman et al. 2014).

Different strategies exist for delivering lipoproteins to the cell surface. For instance, the type II secretion system (T2SS), a large complex spanning both the IM and the OM of many Gram-negative bacteria, allows a wide variety of folded cytoplasmic and periplasmic proteins to be secreted to the extracellular environment (East, Mechaly et al. 2016). Lipoproteins can also use the type V secretion system (T5SS), also called autotransporters, to be surface-exposed (Roussel-Jazede, Grijpstra et al. 2013, Meuskens, Saragliadis et al. 2019). Furthermore, in *Neisseria spp.*, the Surface Lipoprotein Assembly Modulator (SLAM, also known as the T11SS) has been identified as a specific mechanism for lipoproteins transport. SLAM is a 14-stranded β -barrel protein that is substrate-specific and is required for virulence (Hooda, Lai et al. 2016, Huynh, Hooda et al. 2022).

However, none of the above-mentioned mechanisms explain the surface exposure of lipoproteins in *E. coli*. During my work, I investigated the mechanism RcsF is using to be translocated at the surface of *E. coli*. This topic will be discussed more in detail in the next chapter and the results section of this thesis.



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I.3 Envelope stress response system: an overview

I.3.1 General feature of envelope stress responses system in Gram-negative bacteria

Bacteria face a variety of challenges during the course of their cell cycle, including nutrition deprivation, oxidative stress, DNA damage, severe pH and temperature, antibiotic treatment, etc. Consequently, bacteria have evolved response systems to detect and overcome these multiple stresses (Grabowicz and Silhavy 2017). Throughout this thesis work, we had a particular interest in the cell envelope of Gram-negative bacteria. That is why in this section, I will introduce the five central envelope stress response systems present in *E. coli*: Cpx (conjugative pilus expression), Bae (Bacterial adaptive response), Rcs (Regulator of capsule synthesis), Psp (Phage shock protein), and σ^E (*rpoE*). A classification in two subgroups is possible: (1) the Cpx, Bae, and Rcs systems are based on a two-component system (TCS), while (2) the Psp and σ^E systems are not.

TCS are a universal solutions to transmit a signal across the cell envelope. The core components of TCS consist of a membrane-embedded sensor histidine kinase and a cytoplasmic response regulator (Zschiedrich, Keidel et al. 2016). Upon activation by a specific signal, the histidine kinase autophosphorylates, then the phosphoryl group is transferred to the response regulator, which becomes active and able to bind DNA. A response regulator is dedicated to the activation or inhibition of a specific set of genes; this regulation is called the “stress responses” (Zschiedrich, Keidel et al. 2016).

I.3.2 Overview of the five envelope stress response systems in *E. coli*

The **Cpx system** is a classical TCS, composed of CpxA as histidine kinase and CpxR as response regulator (Raivio, Leblanc et al. 2013). The Cpx is activated by a broad range of events such as the accumulation of misfolded proteins in the periplasm, PG defects, IM stress due to protein imbalance, and defects in lipoprotein sorting across the cell envelope (Laloux and Collet 2017). Upstream of the TCS system, CpxP works as a negative regulator of the response while NlpE (an OM lipoprotein) acts as a stress sensor that detects lipoprotein maturation and sorting defects as well as copper-induced stress (Delhay, Laloux et al. 2019, May, Lehman et al. 2019). Because of its broad role in protein maintenance and repair, the Cpx is considered to be one of the central envelope stress response systems.

The **Bae system** is another classical TCS composed of BaeS as histidine kinase and BaeR as response regulator (Raffa and Raivio 2002). The Bae system senses the presence of toxic compounds in the periplasm, such as nickel chloride, indole, flavonoids, and sodium tungstate. The system mainly upregulates the expression of multidrug efflux pumps as well as the expression of *spy* (spheroplast protein Y), a periplasmic chaperone involved in protein folding (Raffa and Raivio 2002, Leblanc, Oates et al. 2011, Quan, Koldewey et al. 2011).

In contrast with Cpx and Bae, the **Rcs phosphorelay** is more complex (Wall, Majdalani et al. 2018). Indeed, instead of a stress signal being directly transferred from the IM histidine kinase RcsC to the response regulator RcsB, it transits first via another IM histidine-kinase-like protein RcsD, then to RcsC (Takeda, Fujisawa et al. 2001). RcsC and RcsD are maintained in an inactive state by IgaA, an IM

inhibitor of the Rcs phosphorelay (Cho, Szewczyk et al. 2014, Hussein, Cho et al. 2018, Wall, Majdalani et al. 2020). RcsF, an OM lipoprotein, is responsible for the majority of Rcs stress sensing; by interacting with IgaA under stress, it alleviates the inhibition of IgaA on the downstream components of Rcs (Cho, Szewczyk et al. 2014, Hussein, Cho et al. 2018). RcsB forms either a homodimer (RcsB/RcsB) or an heterodimer (RcsB/RcsA), allowing fine-tuning the Rcs response (Wall, Majdalani et al. 2018). The Rcs system senses defects in LPS biogenesis, PG damage, perturbation in the OM integrity, and in lipoprotein maturation/trafficking (Majdalani and Gottesman 2005, Laubacher and Ades 2008, Callewaert, Vanoirbeek et al. 2009, Farris, Sanowar et al. 2010, Cho, Szewczyk et al. 2014, Konovalova, Mitchell et al. 2016). The Rcs system will be described in detail in Chapter II.

Psp is induced by various signals, all having in common that they conduct in severe IM perturbations, notably of a disruption of the IM proton motive force (Brissette, Russel et al. 1990, Flores-Kim and Darwin 2016). Under non-inducing conditions, PspF, the transcription factor, forms an inhibitor complex with the cytoplasmic protein PspA, which prevents it from regulating transcription. Under stress, the IM-bound proteins PspB and PspC, likely the sensors of the system, bind PspA, releasing PspF which then activates the *pspA* operon (Darwin 2005). Overall the Psp response helps restoring the proton motive force (Jovanovic, Engl et al. 2010).

σ^E is an essential alternative sigma factor of *E. coli* which, under non-inducing conditions, is sequestered on the cytoplasmic side of the IM by the IM protein RseA. (Rouviere, De Las Penas et al. 1995, De Las Penas, Connolly et al. 1997, Ades 2008). Under inducing conditions, a chain of proteolytic activity,

implicating IM protease RseP and periplasmic protease DegS that cleaves RseA, releases σ^E in the cytoplasm. The σ^E -bound RNA polymerase then promotes the transcription of genes encoding periplasmic chaperones, components of the BAM complex, and proteins involved in LPS assembly (Rhodius, Suh et al. 2006, Grabowicz and Silhavy 2017). The system is inhibited by RseB, a periplasmic protein that binds RseA, preventing the proteolytic activity (Cezairliyan and Sauer 2007, Lima, Guo et al. 2013). σ^E is activated during heat shock, hyperosmotic shock, metal ion exposure, and notably when there is an accumulation of misfolded or mis-translocated OMPs and LPS in the periplasm. Briefly, when unfolded OMPs accumulate in the periplasm, the periplasmic PDZ domain of DegS binds C-terminal residues of unfolded OMPs, which activate the proteolytic activity of DegS. In response, σ^E allows synthesis of small regulatory RNAs, which downregulate the synthesis of the major *E. coli* OMPs (OmpA, OmpC, OmpF, OmpX...etc.) (Rouviere, De Las Penas et al. 1995, Bianchi and Baneyx 1999, Egler, Grosse et al. 2005, Rhodius, Suh et al. 2006).

The Cpx, Bae, Rcs, Psp, and σ^E pathways are summarized in **Figure 11**

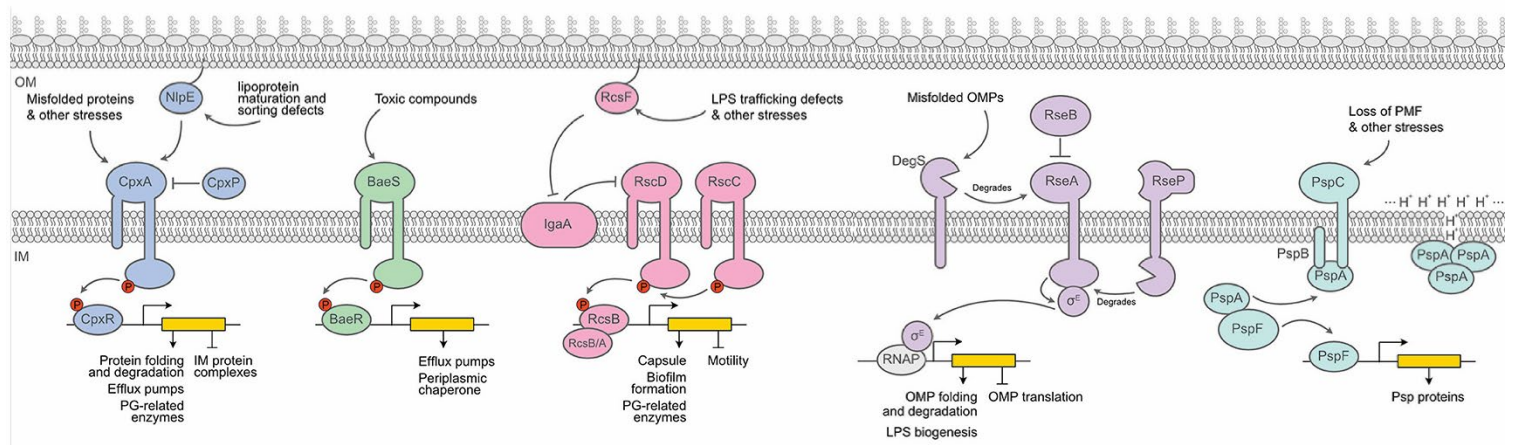


Figure 11. Overview of Cpx, Bae, Rcs, σ^E and Psp envelope stress response systems in *E. coli*. This illustrates the five main stress responses systems of the *E. coli* cell envelope. See text for details. Figure extracted from (Delhaye, Collet et al. 2019).

Together, the Cpx, Bae, Rcs, Psp, and σ^E systems are considered as the main the envelope stress response system present in *E. coli*. The diversity of cues sensed by these stress response systems shows how easily bacteria can modulate their cell envelope and ensure their survival in various harmful environments. Understanding the stress responses systems in detail will undoubtedly reveal targets and strategies that can be used to prevent and treat bacterial infections.

I.4 Conclusion

Throughout this chapter, I showed how important the cell envelope is for *E. coli* (and Gram-negative bacteria) growth and survival. The cell envelope constitutes the cell's border with its extracellular environment, plays a determining role in the cell shape, and protects the cell against a wide range of harmful conditions and toxic compounds. The integrity of the cell envelope is therefore crucial for *E. coli* survival. By fully understanding the cell envelope biogenesis, assembly, and maintenance, we will open the way to design new antimicrobial compounds needed to fight the rise of multiresistant bacteria.

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Chapter II: The Rcs system

II.1 Introduction

In the previous section, we briefly described the central envelope stress response systems (ESRs) of *E. coli*. Among them is the Rcs system, which is at the center of our work. In this chapter, I will summarize what is known about Rcs. The Rcs system is found in most *Enterobacteriaceae* and was initially identified as the positive regulator of colanic acid synthesis in *E. coli* (Gottesman, Trisler et al. 1985). Colanic acid is an exopolysaccharide secreted by *E. coli* which is loosely attached to its cell envelope (Goebel 1963). An overproduction of colanic acid results in a mucoid colony phenotype, which is often associated with Rcs activation. The Rcs system responds to a wide range of cell envelope stressors, including defects in LPS, PG damage, lipoprotein trafficking and maturation anomalies, oxidative stress, and exposure to cationic antimicrobial peptides (Majdalani and Gottesman 2005, Laubacher and Ades 2008, Callewaert, Vanoirbeek et al. 2009, Farris, Sanowar et al. 2010, Cho, Szewczyk et al. 2014, Farizano, Torres et al. 2014, Konovalova, Mitchell et al. 2016). The activation of the Rcs system fine-tunes the expression of genes involved, for instance, in motility (flagella or fimbriae), capsule, and biofilm production (colanic acid production). The Rcs system is seen as pivotal in biofilm maturity and enteric pathogenesis of *E. coli* (Clarke, Ferrieres and Clarke 2003, Majdalani, Heck et al. 2005, Castanie-Cornet, Cam et al. 2006).

In *E. coli*, the genes encoding the biofilm production are located on the *cps* operon (see further) and are downregulated under laboratory conditions. In 1984, while working on the *lon* operon, *Trisler et al.* discovered mutations that were leading to the mucoid phenotype (Trisler and Gottesman 1984). In an effort to understand the regulation involved in the mucoid phenotypes observed in *lon*- cells, they used insertional mutagenesis and identified the *cps* operon, and demonstrated *cps* regulation by the Lon ATP-dependent protease (Trisler and Gottesman 1984, Gottesman, Trisler et al. 1985). Later, the Gottesman group identified the *rcs* genes and demonstrated RcsA/B as a positive regulator of *cps* while RcsC is a negative one. They also showed that the Lon protease targets RcsA (Gottesman, Trisler et al. 1985). Based on sequence homology, quickly RcsC and RcsB were identified as likely members of a two-component system regulation pathway (Stout and Gottesman 1990). After gathering all this evidence by the *Gottesman* group, other researchers started to work actively on the Rcs system and, for instance, identified the Rcs system as a non-orthodox two-component system (see further).

II.2 The key players

The Rcs system is a non-orthodox two-component system (TCS) in which RcsC is the histidine-kinase and RcsB the response regulator. Additional components are present in the Rcs system, such as the histidine kinase-like RcsD, the Rcs inhibitor IgaA, the stress sensor RcsF, and a non-phosphorylatable response regulator RcsA.

RcsB and RcsA are the two downstream components of the Rcs system. RcsB forms homodimers or heterodimers with several proteins: RcsA (main partner), BglJ, MatA, and GadE, which then bind to a conserved motif in Rcs-regulated genes to activate or inhibit their transcription (Castanie-Cornet, Cam et al. 2010, Venkatesh, Kembou Koungni et al. 2010, Pannen, Fabisch et al. 2016, Wall, Majdalani et al. 2018). RcsB-RcsB and RcsB-RcsA complexes are Rcs dependent because RcsB phosphorylation is required for their function. The others auxiliary regulators are independent of RcsB phosphorylation and thus from the Rcs signaling cascade (Castanie-Cornet, Cam et al. 2010, Venkatesh, Kembou Koungni et al. 2010, Pannen, Fabisch et al. 2016).

Briefly, RcsB-BglJ activates the *bgl* genes involved in D-glucoside synthesis (nutrient uptake) (Venkatesh, Kembou Koungni et al. 2010). RcsB-MatA activates the *mat* operon involved in Mat/ECP fimbria biosynthesis. ECP stands for *Escherichia coli* common pilus (Pannen, Fabisch et al. 2016). Finally, the RcsB-GadE complex activates the *gad* operon related to acid resistance (Castanie-Cornet, Cam et al. 2010).

RcsB-RcsB positively regulates the small RNA *rprA* and negatively impacts *gadA* which encodes for the glutamate decarboxylase enzyme. *rprA* is required under osmotic shock for wild-type production of RpoS, also called σ^{38} , the master regulator of the general stress response in *E. coli* (Majdalani, Hernandez et al. 2002, Majdalani, Chen et al. 2004). Notably, overexpression of *rprA* increases acid tolerance in *E. coli*, decreases swarming motility, and promotes biofilm formation (Majdalani, Hernandez et al. 2002, Bak, Han et al. 2014). *gadA*, which is downregulated, encodes the glutamate decarboxylase, an enzyme involved in the glutamate-dependent acid resistance system, conferring resistance to acid conditions (Castanie-Cornet, Cam et al. 2010). Finally, RcsB-RcsA activates the *cps* operon and inhibits the *flhDC* operon. The *cps* operon is involved in colanic acid production, while *flhDC* regulates synthesis of the flagellum (Stout, Torres-Cabassa et al. 1991, Francez-Charlot, Laugel et al. 2003). The RcsB homodimers and heterodimers regulation is summarized in **Figure 12**.

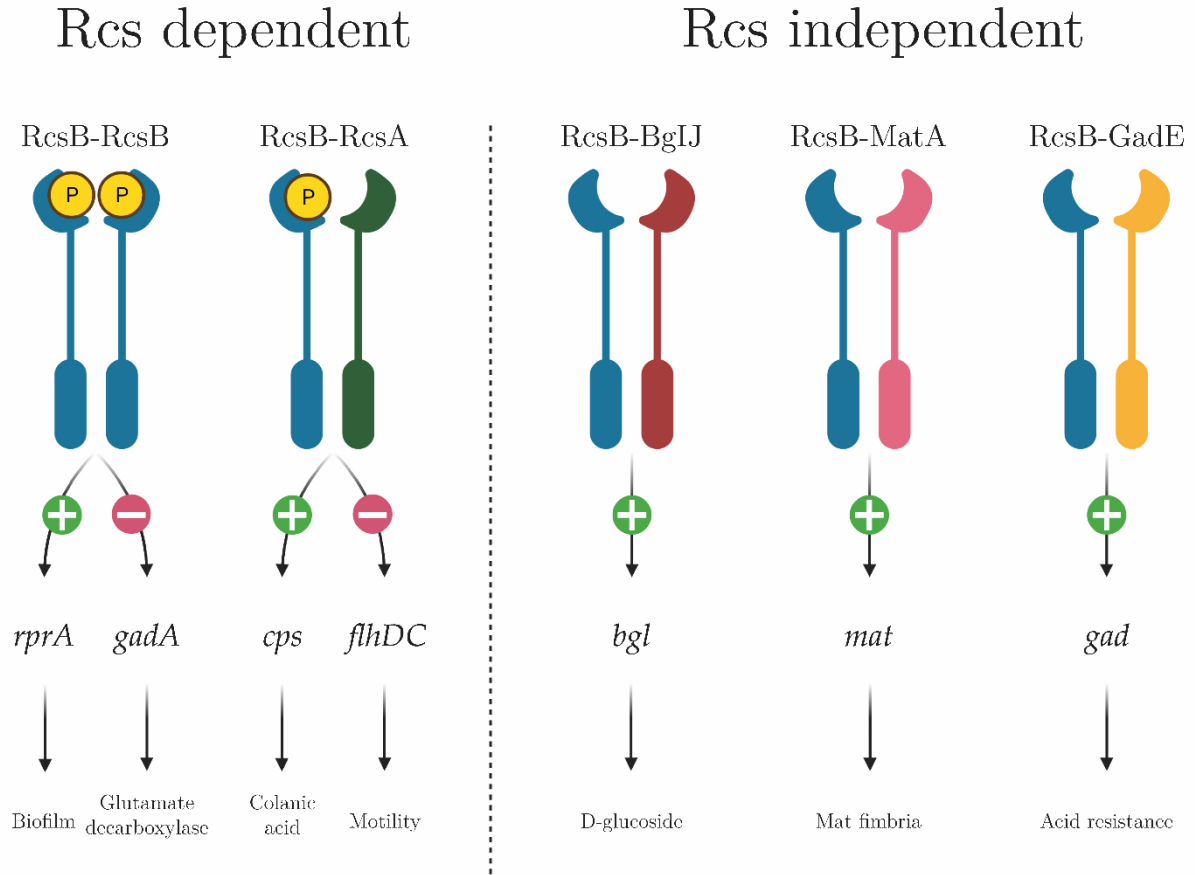


Figure 12. Regulation of RcsB homodimers and heterodimers. RcsB has two modes of regulation an Rcs-dependent one or an Rcs independent. In response to RcsB phosphorylation, RcsB can form RcsB-RcsB homodimers or RcsB-RcsA heterodimers while in absence of phosphorylation RcsB can form BglJ-RcsB, MatA-RcsB, and GadE-RcsB heterodimers. The RcsB homodimer or heterodimers then interact with a conserved motif in target genes to modulate their transcription, thereby regulating the physiological activities of bacteria. The figure was adapted from (Meng, Young et al. 2021).

RcsC and RcsD are IM proteins and act upstream of RcsB. Upon stress, RcsC autophosphorylates and activates the Rcs response. RcsC has both kinase and phosphatase activities, while RcsD lacks kinase activity (Majdalani and Gottesman 2005). It has been shown that Rcs activity is not regulated *via* RcsC but rather *via* RcsD, as deletion of the RcsC periplasmic loop is dispensable for

sensing the Rcs inducing signals (Wall, Majdalani et al. 2020). On the opposite, the RcsD periplasmic domain is in contact with IgaA, the Rcs repressor component. Even in the absence of an inducing signal, RcsB can be phosphorylated by phosphodonors present in the cytoplasm. That is why RcsC and RcsD also act as phosphatases to maintain a low level of phosphorylated RcsB in non-stress conditions (Clarke 2010, Hu, Chi et al. 2013).

IgaA is the IM negative regulator of the Rcs system, which in the absence of inducing Rcs conditions prevents RcsB phosphorylation. IgaA comprises five IM transmembrane domains, two cytoplasmic domains and a large periplasmic loop. IgaA is the only essential component of the Rcs system, as its deletion leads to lethal, constitutive Rcs activation (Dominguez-Bernal, Pucciarelli et al. 2004, Cho, Szewczyk et al. 2014, Hussein, Cho et al. 2018). In classical TCS, the systems regulators are usually small proteins located in the cytoplasm or in the periplasm (Tschauner, Hornschemeyer et al. 2014, Mitchell, Ismail et al. 2015). However, in Rcs, IgaA is a polytopic IM embedded proteins with large cytoplasmic and periplasmic domain likely involved in Rcs regulation (Dominguez-Bernal, Pucciarelli et al. 2004, Pucciarelli, Rodriguez et al. 2017). RcsF, by interacting with the periplasmic loop of IgaA, alleviates the Rcs pathway inhibition (Cho, Szewczyk et al. 2014, Hussein, Cho et al. 2018).

RcsF is a small lipoprotein localized in the OM which acts as the OM stress sensor in the Rcs pathway. Similar to ~50% of the OM localized lipoproteins (El Rayes, Szewczyk et al. 2021), RcsF harbors a positively charged N-terminal disordered linker followed by a globular domain. The globular domain of RcsF, also known as the signaling domain, is structured as two α -helix surrounding a four-stranded β -sheet and contains two non-consecutive disulfide bonds required for

folding (see **Figure 13**) (Leverrier, Declercq et al. 2011, Rogov, Rogova et al. 2011). RcsF senses almost all the inducing cues of Rcs and activates Rcs by interacting with IgaA under stress. A recent study also suggests that RcsF may interact with RcsC (Sato, Takano et al. 2017). As a lipoprotein, if its maturation or trafficking is impaired, RcsF will accumulate in the IM, interacting easily with the periplasmic loop of IgaA, activating Rcs. Note that RcsF is probably not the only stress sensor for the Rcs system, as an *E. coli* strain with an *rcsF* deletion still shows Rcs activation under certain conditions. As an example, an overexpression of DjlA (an IM protein functioning as co-chaperone with DnaK (Genevaux, Wawrzynow et al. 2001)) in a Δ *rcsF* background, activates the Rcs system (Shiba, Yokoyama et al. 2004).

A

```

      10      20      30      40      50
MRALPICLVA LMLSGCSMLS RSPVEPVQST APQPKAEPK PKAPRATPVR
      60      70      80      90     100
IYTNAEELVG KPFRDLGEVS GDSCQASNQD SPPSIPTARK RMQINASKMK
      110     120     130
ANAVLLHSCE VTSGTPGCYR QAVCIGSALN ITAK

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B

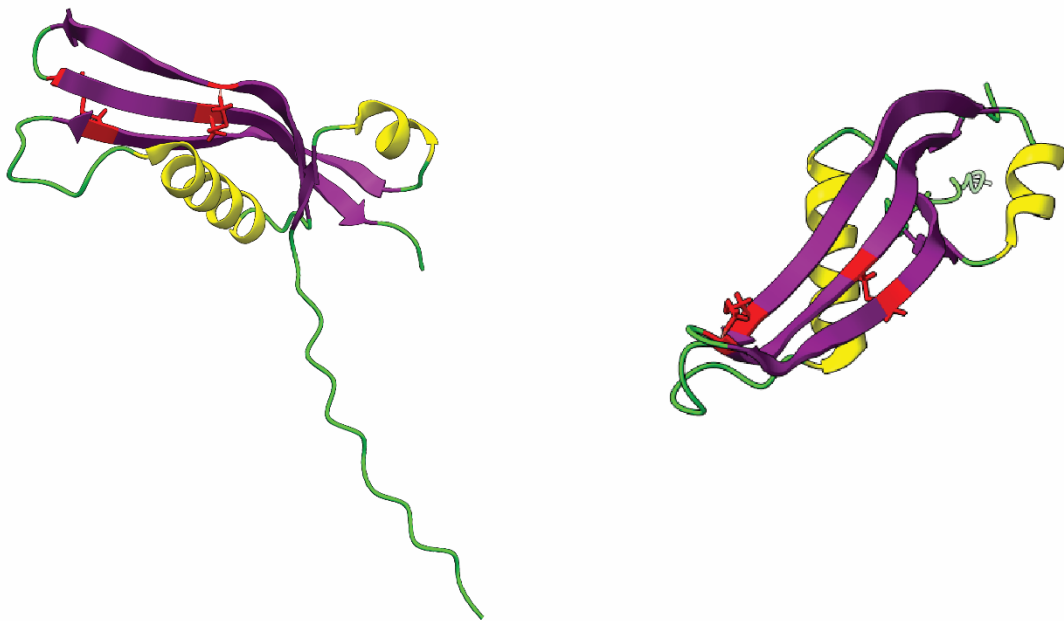


Figure 13. Structure of the lipoprotein RcsF. **A)** amino acid sequence of RcsF (UniprotKB: P69411), the signal sequence is colored in orange. **B)** Top and side view of a ribbon representation of RcsF structure solved by NMR (PDB: 2L8Y). RcsF displays two domains a long intrinsically disorder linker and a signaling domain composed of four-stranded β -sheets surrounded by two α -helices. The amino acid sequence and the ribbon representation are colored according to the secondary structure elements. the b-strands are colored in purple, the α -helix are in yellow and the coil regions are in green. The four cysteines involved in disulfide bonds are colored in red.

II.3 Signal transduction in the Rcs phosphorelay

Upon an extracytoplasmic stimulus, in a classical TCS, the histidine kinase autophosphorylates a histidine residue in its histidine-kinase domain in an ATP-dependent manner. The phosphoryl group is then transferred to the aspartate of the response regulator leading to specific gene regulations (Zschiedrich, Keidel et al. 2016).

The Rcs system is much more complex as the phosphoryl cascade follows a His-Asp-His-Asp pattern. Upon stress, the His⁴⁷⁹ of RcsC autophosphorylates via ATP hydrolysis, then the phosphoryl group is transferred to the Asp⁸⁷⁵ of RcsC itself. Only after the second phosphorylation step of RcsC, the phosphoryl group is transferred to His⁸⁴² of RcsD to be finally transferred to Asp⁵⁶ of RcsB, ultimately leading to the Rcs genes regulation (Chen, Takeda et al. 2001, Takeda, Fujisawa et al. 2001, Majdalani and Gottesman 2005, Clarke 2010).

IgaA interacts with RcsD via periplasmic and cytoplasmic loops restricting the passage of phosphoryl groups from RcsC to RcsD, inhibiting RcsC autophosphorylation. More specifically it has been shown that the first cytoplasmic domain of IgaA, which is predicted to be structurally similar to a protein motif found in domain involved in protein-protein interaction, was important for IgaA inhibitory activity (Hussein, Cho et al. 2018). It is only upon binding of RcsF on IgaA that the inhibition of the phosphoryl flow is alleviated (Wall, Majdalani et al. 2020). **Figure 14** summarizes the current view on the Rcs pathway.

It is established that formation of a complex between IgaA (200 copies/cell) and RcsF (~3,000 copies/cell) triggers Rcs. More specifically, the globular domain of RcsF interacts with the C-terminal periplasmic domain of IgaA which likely lead to conformational changes in IgaA and alleviates the IgaA inhibitory effects on the Rcs (Hussein, Cho et al. 2018). Thus, to dodge unwanted Rcs stress response, bacteria must prevent the IgaA and RcsF interaction. In the absence of stress; RcsF is occluded from IgaA by being exported to the OM, where it interacts with many OM partners (Cho, Szewczyk et al. 2014). Yet, more work are needed to fully understand the structural organization of the RcsF-IgaA complex.

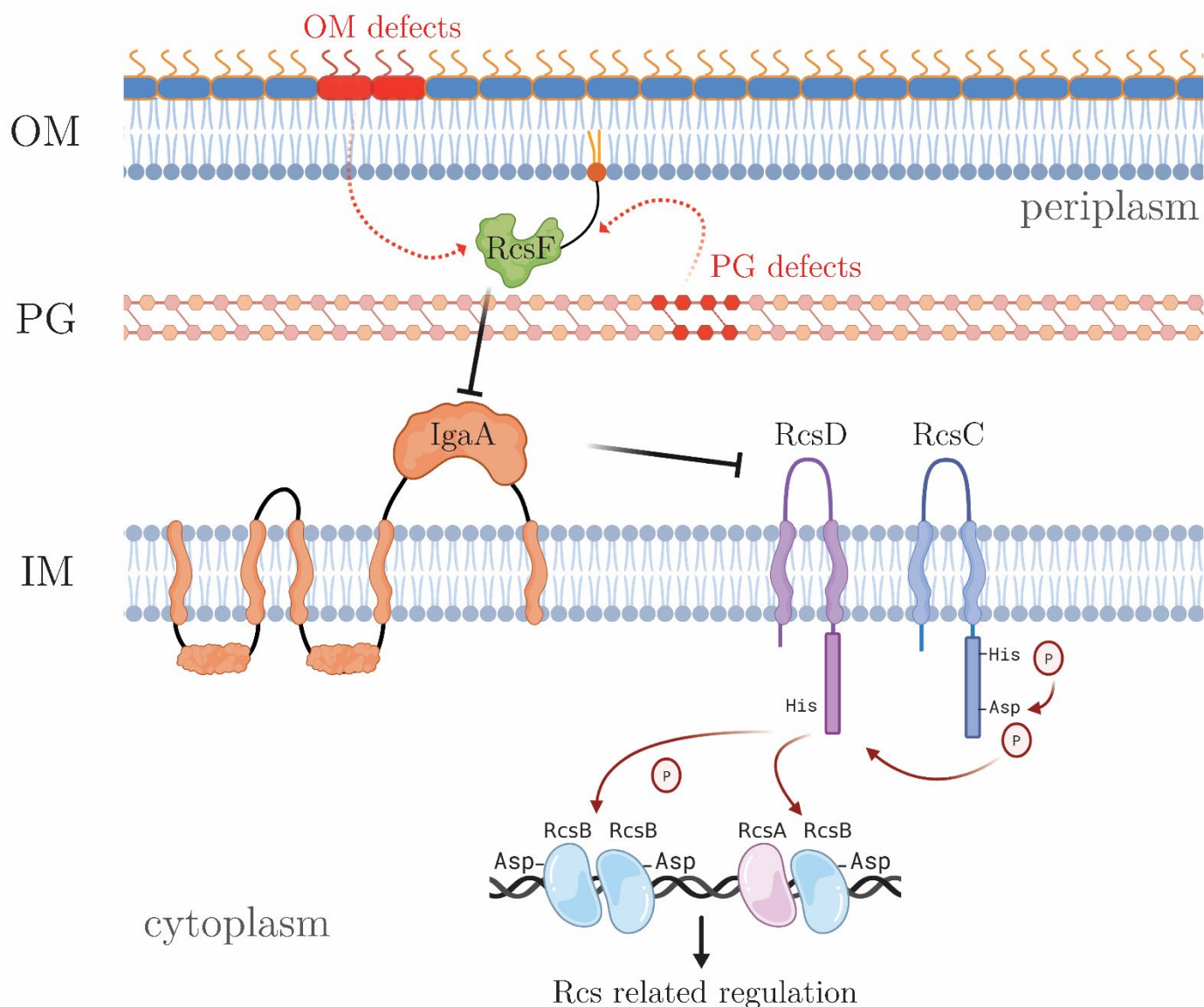
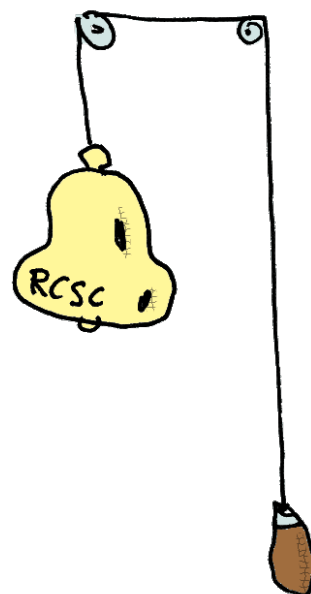


Figure 14. The Rcs regulation pathway. Six proteins composed the Rcs system. RcsF and IgaA are two upstream components of the cascade. RcsF is an OM lipoprotein that detects the majority of Rcs-inducing events. IgaA is an essential IM protein that interacts with RcsD to inhibit the Rcs pathway. Upon stress, RcsF interacts with the periplasmic domain of IgaA which relieves RcsD inhibition mediated by IgaA and activates the Rcs response. RcsA/B/C/D are the downstream components of the Rcs system. RcsC is the sensor kinase that autophosphorylates. The phosphoryl group (P) is then transferred from the histidine (His) and aspartate (Asp) residues of RcsC to RcsD and from RcsD to RcsB. Upon RcsB phosphorylation, RcsA and RcsB form homo- and hetero-dimers leading to activation and inhibition of sets of genes.



II.4 RcsF is a surface-exposed stress-sensor lipoprotein

Interestingly RcsF is the only *E. coli* lipoprotein that has been shown by different methods to reach the cell surface (Cho, Szewczyk et al. 2014, Konovalova, Perlman et al. 2014, Konovalova, Mitchell et al. 2016). Briefly, the lab of Silhavy combined biotinylation, surface proteolysis and antibody-based whole cell assays to conclude that at least a part of RcsF is surface-exposed, potentially its lipidated N-terminal positively charged linker (Konovalova, Perlman et al. 2014). On the other, our group identified RcsF on the surface using immunofluorescence microscopy and dot blots assays on intact cells, hypothesizing that the globular domain of RcsF would be exposed to the cell surface (Cho, Szewczyk et al. 2014).

The discovery of at least portions of RcsF being surface-exposed raises questions about the biological pathway that allows this lipoprotein to cross the OM. It led to the discovery of OMPs as both partners and potential vehicles for RcsF surface exposure. Indeed, BamA, OmpA, OmpC and OmpF have been shown to form complexes with RcsF (Cho, Szewczyk et al. 2014, Konovalova, Perlman et al. 2014, Konovalova, Mitchell et al. 2016, Rodriguez-Alonso, Letoquart et al. 2020).

According to the current model, RcsF is exported to the cell surface via the β -barrel assembly machinery (BAM). Supporting this model, *Rodríguez-Alonso et al.* recently reported the crystal structure of BamA in a complex with RcsF. In this structure, the RcsF globular domain is present in the lumen of BamA and interacts with regions proposed to undergo a lateral opening during β -barrel insertion. The BamA-RcsF structure suggests a push-pull mechanism for RcsF delivering to its OMPs partners (Tata and Konovalova 2019, Rodriguez-Alonso, Letoquart et al.

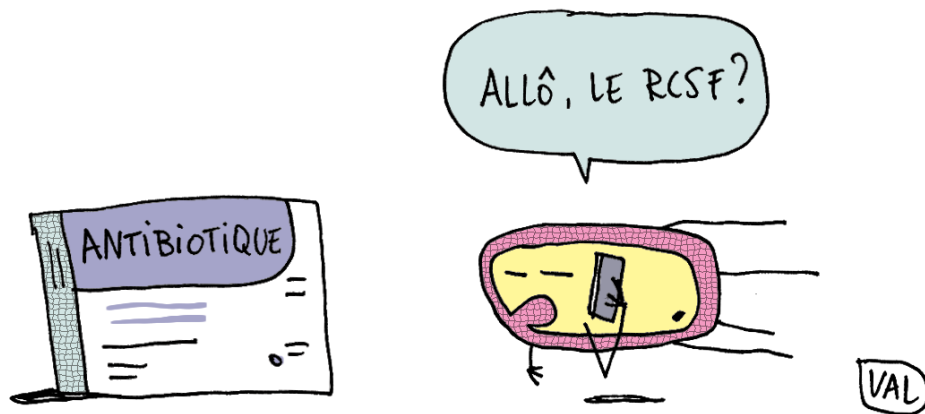
2020). The BamA-RcsF complex appears to be an intermediate and not a dead-end in the RcsF journey to the surface of *E. coli*, as the next step is for RcsF to be funneled with OMPs.

The discovery of RcsF in complex with BamA without being exposed to the surface suggests that RcsF partners and BAM clients proteins OmpC, OmpF, and OmpA are potential vehicles for RcsF surface exposure.

OmpC and OmpF are 16-stranded β -barrels harboring a central pore that can accommodate portions of RcsF (Basle, Rummel et al. 2006, Yamashita, Zhalnina et al. 2008). Both OmpC and OmpF are structurally similar and form trimers in the OM. Using site-specific photocrosslinking, *Konovalova et al.* discovered numerous residues in both RcsF domains (linker and globular domains) that interact with OmpC, and OmpF. They also identified two residues located inside the OmpF pore that interacts with RcsF, opening the way to a model of RcsF crossing the OM *via* the pore of OmpC/F (Konovalova, Perlman et al. 2014).

OmpA, which is structurally distinct from OmpC/F, is thought to adopt a two-domain structure conformation with an N-terminal 8-stranded β -barrel and a periplasmic globular domain (De Mot and Vanderleyden 1994, Pautsch and Schulz 1998, Pautsch and Schulz 2000, Park, Lee et al. 2012). In the two domains' conformation, the β -barrel of OmpA is too small to accommodate any regions of RcsF. Yet, site-specific photocrosslinking and chemical crosslinking identified OmpA as an interacting partner of RcsF (Cho, Szewczyk et al. 2014, Konovalova, Perlman et al. 2014).

Overall, the exact topology of the RcsF-OMPs complexes and their function in the Rcs system remain mysterious. Further investigations were needed, laying the ground for our work during this thesis (see Results).



II.5 How RcsF, when surface-exposed, signals to the Rcs system

RcsF is able to sense OM defects and to deliver the signal to IgaA. To avoid Rcs activation in the absence of stress, bacteria need to find a way to prevent RcsF from interacting with IgaA. We believe that this is the reason for RcsF to be surface exposed: it is a way to prevent IgaA and RcsF to interact. Furthermore, being displayed on the surface, RcsF is well positioned to monitor the LPS layer integrity. Yet, the way RcsF senses LPS and OM defects, as well as signals to the downstream Rcs components such defects remains elusive (Laloux and Collet 2017). So far, two models have been proposed:

In the **first model**, RcsF forms complexes with β -barrels (OmpA, OmpC, and OmpF) and employs its positively-charged and potentially surface-exposed N-terminal linker to sense the state of LPS lateral contacts (Konovalova, Perlman et al. 2014, Konovalova, Mitchell et al. 2016). When LPS lateral interactions are damaged (for instance by chemical compounds such as Polymixin B or other cationic peptides, or by LPS biosynthesis defects) conformational changes within RcsF-OMPs complexes can facilitate interaction of the globular domain of RcsF with Rcs downstream components to activate the stress response (Konovalova, Perlman et al. 2014, Konovalova, Mitchell et al. 2016).

In the **second model**, the BamA-RcsF complex is central to monitoring OM integrity. In the absence of stress, the BAM complex functions normally and continually assembles OMPs in the OM. By doing so, RcsF is surface-exposed via OMPs-RcsF complexes and is occluded from the IgaA periplasmic loop for which

RcsF has a strong affinity (Cho, Szewczyk et al. 2014, Hussein, Cho et al. 2018). Upon stress and defects in OM integrity, the BAM function is impaired. As a result of this, RcsF is not funneled to OMPs and accumulates in the periplasm, where it interacts with IgaA, releasing the inhibition of the Rcs system (Cho, Szewczyk et al. 2014).

II.6 Signal sensing and response in the Rcs system

Two types of stresses can lead to Rcs activation, environmental stress or intrinsic stress. Historically, the first environmental stress triggering Rcs that has been identified is osmotic upshift (Sledjeski and Gottesman 1996) that leads to the *cps* operon upregulation and the production of colanic acid. Other input signals related to envelope stress have now been uncovered. For instance, when lysozyme hydrolyzes peptidoglycan, the Rcs upregulates the transcription of lysozyme inhibitors in *E. coli*, minimizing the lysozyme damage to cells. (Hirakawa, Nishino et al. 2003, Laubacher and Ades 2008, Callewaert, Vanoirbeek et al. 2009). Oxidative stress has also been shown to activate the Rcs response in *Salmonella enterica* serovar Typhimurium; as such stress causes OM defects, the response is the transcription of *dps* gene. Dps (DNA protection during starvation) is a protein usually produced in stationary phase for the normal starvation response but also produced during oxidative stress to protect DNA by binding it in a non-specific manner (Almiron, Link et al. 1992, Dukan and Touati 1996, Martinez and Kolter 1997).

There are also intrinsic sources of stress that activate the Rcs response, including toxic substances produced by the cell metabolism, impaired translation due to the lack of specific amino acids, or mutations causing biogenesis alteration (Mitchell and Silhavy 2019). Mutations involved in the envelope biogenesis pathway are the primary Rcs triggers. For instance, mutations in the *lolA*, or *bamA* promoters (*bamA101*), as well as deletion of *ompA*, lead to Rcs activation (Cho, Szewczyk et al. 2014, Dekoninck, Letoquart et al. 2020). Deletion of *waaF*, which alters the LPS core are also sensed by RcsF (Ren, Wang et al. 2016) as well as, mutations in PBP (notably deletion of DacA, DacB, pbpG and AmpH) (Evans,

Kannan et al. 2013). Overall this shows how Rcs pathway is central for monitoring the envelope of *Enterobacteriaceae*.

II.7 Conclusion

Gram-negative bacteria rely on their cell envelope for survival, thus they created signal transduction systems to monitor and maintain envelope homeostasis. The Rcs pathway, found in most of the *Enterobacteriaceae*, detects a wide range of environmental cues mostly via the OM lipoprotein RcsF. In response, the Rcs system triggers a phosphorelay cascade that flows from RcsC to RcsD to finally activate the response regulator RcsB. RcsB regulates many distinct genes by generating homodimers and heterodimers, allowing an adaptive response of the cell envelope homeostasis which improves cell survival.

RcsF has been identified as being displayed at the surface of *E. coli* via an unknown mechanism. In parallel, RcsF has been found to interact with several OM partners such as BamA, OmpA, OmpC and OmpF. Previous work of our group exclude BamA as the vehicle of surface exposure but suggest instead the BamA-RcsF serve has an intermediate in the delivery of RcsF to the other OMPs partners.

The particular topology RcsF adopts in complex with OmpA, OmpC and OmpF remain largely unknown at the beginning of this thesis work and was at the center of my work. By understanding the RcsF-OMPs complexes we will gain insight into the OM defects sensing mechanism by the Rcs system.

A better understanding of the Rcs system will likely improve prevention, and treatment of bacterial infections.



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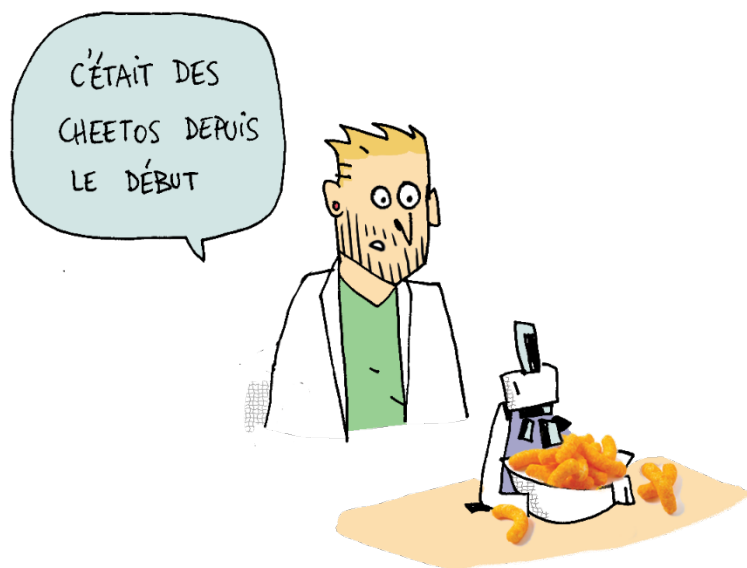
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Section II: Results

RÉSULTATS:



VAL

As presented in my Research Objectives (see page 11), we aimed to dissect the OmpA-RcsF interaction and to provide new insights into the role of the OmpA-RcsF into the Rcs stress response system.

This section contains the results of my thesis divided in two chapters. **Chapter III** consist in our article “Defining the function of OmpA in the Rcs stress response” which was published in September 2020 in the journal *eLife*. By gaining insight into the OmpA-RcsF complex, we elucidated a new function for OmpA in the Rcs regulation. Moreover, we proposed a model for OmpA-RcsF structure. **Chapter IV** reveal a flaw in the widely used site-specific photo-crosslinking methods that lead to artifactual identification of protein-protein interaction interface. We submitted this work in April 2022 to *Protein Science*.

Chapter III: Defining the function of OmpA in the Rcs stress response

Defining the function of OmpA in the Rcs stress response

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Abstract

OmpA, a protein commonly found in the outer membrane of Gram-negative bacteria, has served as a paradigm for the study of β -barrel proteins for several decades. In *Escherichia coli*, OmpA was previously reported to form complexes with RcsF, a surface-exposed lipoprotein that triggers the Rcs stress response when damage occurs in the outer membrane and the peptidoglycan. How OmpA interacts with RcsF and whether this interaction allows RcsF to reach the surface has remained unclear. Here, we integrated *in vivo* and *in vitro* approaches to establish that RcsF interacts with the C-terminal, periplasmic domain of OmpA, not with the N-terminal β -barrel, thus implying that RcsF does not reach the bacterial surface *via* OmpA. Our results suggest a novel function for OmpA in the cell envelope: OmpA competes with the inner membrane protein IgaA, the downstream Rcs component, for RcsF binding across the periplasm, thereby regulating the Rcs response.

Introduction

The cell envelope is the morphological hallmark of Gram-negative bacteria. It consists of an inner membrane (IM) surrounding the cytoplasm as well as an outer membrane (OM), an asymmetric bilayer with phospholipids in the inner leaflet and lipopolysaccharides in the outer leaflet {Silhavy, 2010 #2357}. The two membranes are separated by the periplasm, a compartment in which lies a thin layer of peptidoglycan. The cell envelope is essential for viability: the OM serves as a permeability barrier against toxic compounds present in the environment while the peptidoglycan provides shape and osmotic protection to cells {Okuda, 2016 #2268; Typas, 2012 #835; Egan, 2020 #2311}.

Given the functional and structural importance of the envelope, bacteria need to respond to breaches in envelope integrity in a fast and adequate manner. Bacteria have therefore evolved sophisticated signaling systems that monitor envelope integrity and respond to perturbations {Ruiz, 2005 #169; MacRitchie, 2008 #405; Delhay, 2019 #2312}. In *Escherichia coli* and the Enterobacteriaceae, the Rcs system detects damage to the OM and the peptidoglycan {Wall, 2018 #2333; Laubacher, 2008 #397; Farris, 2010 #391}. In response, Rcs modulates the expression of dozens of genes, including those involved in the biosynthesis of colanic acid, an exopolysaccharide that accumulates on the cell surface to form a protective capsule {Wall, 2018 #2333; Laloux, 2017 #1736}.

Rcs signal transduction involves a multi-step phosphorelay {Wall, 2018 #2333}. Under stress, the IM histidine kinase RcsC autophosphorylates, transfers the phosphoryl group to the IM protein RcsD and finally to the cytoplasmic response regulator RcsB. Rcs activity is modulated by two proteins that are not

part of the phosphorylation cascade: RcsF and IgaA. RcsF is an OM lipoprotein that senses most Rcs-inducing cues, while IgaA is an essential IM protein that down-regulates Rcs {Takeda, 2001 #415;Dominguez-Bernal, 2004 #795} by interacting with RcsD {Wall, 2020 #2309}. When perturbations occur in the peptidoglycan or in the OM, RcsF, while remaining anchored in the OM, reaches across the periplasm to interact with IgaA, leading this protein to alleviate its inhibition of the phosphorelay, turning on Rcs {Cho, 2014 #899;Hussein, 2018 #2192}. In the absence of stress, RcsF is occluded from IgaA by interacting with OM proteins. A complex between RcsF and BamA, the core component of the β -barrel assembly machinery (BAM), was identified {Cho, 2014 #899;Konovalova, 2014 #900} and its structure solved {Rodriguez-Alonso, 2020 #2316}. This complex forms as an intermediate {Cho, 2014 #899;Konovalova, 2014 #900}: delivery of unfolded OM β -barrels (OMPs) to BAM triggers the release of RcsF from BamA and its transfer to OMP partners {Rodriguez-Alonso, 2020 #2316}. Three abundant OMPs (OmpA, OmpC and OmpF) have been identified as RcsF partners {Cho, 2014 #899;Konovalova, 2014 #900}. Under stress conditions, newly synthesized RcsF molecules fail to interact with BamA {Cho, 2014 #899}: they remain in the periplasm, free to bind IgaA, triggering Rcs.

Crucially, whereas the general view is that OM lipoproteins are oriented toward the periplasm, previous work concluded that at least a portion of RcsF, a protein which is composed of an N-terminal disordered linker and a C-terminal globular domain required for signaling {Leverrier, 2011 #459;Rogov, 2011 #1186}, is exposed on the cell surface; OmpA, OmpC, and OmpF, but not BamA {Cho, 2014 #899}, were identified as potential vehicles for RcsF surface exposure {Cho, 2014 #899;Konovalova, 2014 #900;Konovalova, 2016 #1094}. In a topological model of RcsF surface exposure {Konovalova, 2014 #900}, the lipid moiety of RcsF

is anchored in the outer leaflet of the OM and the N-terminal disordered linker is exposed on the cell surface before being threaded through the lumen of the OMP partners. However, definitive evidence for this model is still lacking.

In addition, because OmpC, OmpF, and OmpA belong to two distinct structural groups, it is unclear whether RcsF interacts with its three OMP partners in a similar way. Indeed, OmpC and OmpF form 16-stranded β -barrels that associate into trimers in the OM. Because they form large β -barrels, they display a central pore {Basle, 2006 #2498; Yamashita, 2008 #2497; Radhakrishnan, 2010 #2054; Housden, 2013 #849} that is large enough to accommodate a disordered peptide such as the RcsF linker. The situation is less clear for OmpA: although this protein, the most abundant OMP in *E. coli*, has been studied for more than four decades, how it folds remains controversial. While some studies indicate that OmpA can also fold into a 16-stranded β -barrel with a large central pore {Singh, 2003 #2397; Stathopoulos, 1996 #1216}, the predominant view is that OmpA adopts a two-domain structure with an N-terminal 8-stranded β -barrel inserted in the OM {Pautsch, 1998 #1164} and a C-terminal, globular domain in the periplasm {De Mot, 1994 #2499; Park, 2012 #2179}. In this conformation, the β -barrel of OmpA is too small to accommodate a polypeptide. Thus, despite the tremendous work that has been done on OmpA, we do not know whether this protein adopts the large β -barrel structure when in complex with RcsF, or whether it folds into the predominant two-domain conformation. If the latter, where does RcsF bind OmpA?

To resolve these outstanding structural and mechanistic questions, here we dissected the OmpA-RcsF complex. By combining *in vivo* site-specific photo-crosslinking, targeted proteolysis, and nuclear magnetic resonance (NMR) titration, we established that OmpA adopts its two-domain structure when in complex with

RcsF and that it is the C-terminal, periplasmic domain—not the β -barrel—that interacts with the lipoprotein. In addition, we identified residues in RcsF and in OmpA that are involved in the interaction, thus providing information about the binding interface. Taken together, our results indicate that the topology of OmpA-RcsF is different from that of OmpC/F-RcsF; they also imply that RcsF does not use OmpA to reach the cell surface. This has important implications for how RcsF senses OM stress: if the linker of RcsF is not on the surface in the OmpA-RcsF complex, then OmpA-RcsF cannot serve to monitor the state of the lipopolysaccharide leaflet via direct interactions with lipopolysaccharide molecules, as previously proposed {Konovalova, 2016 #1094}. Finally, we determined the equilibrium dissociation constants of both the C-terminal domain of OmpA and the periplasmic domain of IgaA for RcsF and provide evidence suggesting that OmpA and IgaA compete for RcsF binding across the periplasm. Our results support a model in which OmpA serves as a buffer for RcsF, titrating it from IgaA, thereby fine-tuning Rcs activity.

Results

RcsF interacts with the C-terminal region of OmpA *in vivo*

The stress sensor lipoprotein RcsF was previously shown to be surface-exposed {Cho, 2014 #899;Konovalova, 2014 #900}, and OmpA was described as a possible vehicle for its surface exposure {Cho, 2014 #899;Konovalova, 2014 #900;Konovalova, 2016 #1094}. However, how OmpA folds when in complex with RcsF and whether this interaction allows RcsF to become surface-exposed remain to be determined.

To close this gap, we characterized the OmpA-RcsF interaction. In a previous study, we identified six RcsF residues (in the N-terminal disordered linker and at the tip of the signaling domain) as being part of the interaction interface between RcsF and OmpA {Cho, 2014 #899}. These residues were identified using a site-specific photo-crosslinking strategy in which a photoreactive, crosslinkable amino acid is inserted at specific positions in the protein of interest, with the help of an exogenous orthogonal tRNA/aminoacyl-tRNA synthetase pair {Chin, 2002 #1016}. We first sought to confirm and extend these results to more clearly define the binding interface in RcsF. Instead of using the hydrophobic crosslinker *p*-benzoyl-L-phenylalanine like before {Cho, 2014 #899}, we used *N*^ε-((3-(3-methyl-3*H*-diazirin-3-yl)propyl)carbamoyl)-*L*-lysine (DiZPK), a lysine analog with substantially higher photo-crosslinking efficiency than *p*-benzoyl-L-phenylalanine {Zhang, 2011 #806}. We selected 11 positions distributed along the RcsF sequence, including four (R21, Q28, Q33, R45) in the disordered linker and seven on the surface of the signaling domain (N54, Q79, R89, K98, E110, P116, Q121) (Figure 1). After UV illumination, a ~55 kDa band, the size of the OmpA (40 kDa)-RcsF

(14 kDa) complex, formed and was detected with an anti-RcsF antibody (Figure 1) for the following variants (in decreasing intensity): RcsF_{R89X}, RcsF_{P116X}, RcsF_{Q79X}, RcsF_{R45X}, RcsF_{K98X}, RcsF_{Q121X}, RcsF_{N54X}, and RcsF_{E110X}. The identity of the ~55 kDa band as OmpA-RcsF was further verified by showing that it did not form in $\Delta ompA$ cells (Figure 1 – figure supplements 1 and 2). Thus, as in our previous study {Cho, 2014 #899}, complex formation was observed when the photoactivatable amino acid was inserted in the linker (R45) or at the tip of the signaling domain (Q79 and P116). The complex also formed when DiZPK was incorporated in other regions of the signaling domain, such as α -helix 1 (N54), α -helix 2 (R89 and K98), β -strand 2 (E110), and β -strand 3 (Q121) (Figure 1). Noteworthy, residues Q79 , R89 and P116 are part of the binding interface between RcsF and the luminal wall of the BamA β -barrel in the recently published structure of the BamA-RcsF complex {Rodriguez-Alonso, 2020 #2316}. Taken together, these observations substantially enlarge the region of RcsF known to interact with OmpA. Of note, we observed that the RcsF_{R45X}, RcsF_{R89X} and RcsF_{K98X} variants formed a UV-dependent band migrating slightly higher than the OmpA-RcsF complex (Figure 1). Focusing on RcsF_{R89X}, we identified this band as a complex between RcsF and OmpC/F because it did not form when *ompR*, a transcription factor required for the production of OmpC and OmpF {Hall, 1979 #2313;Chubiz, 2011 #2314}, was deleted (Figure 1 – figure supplement 2).

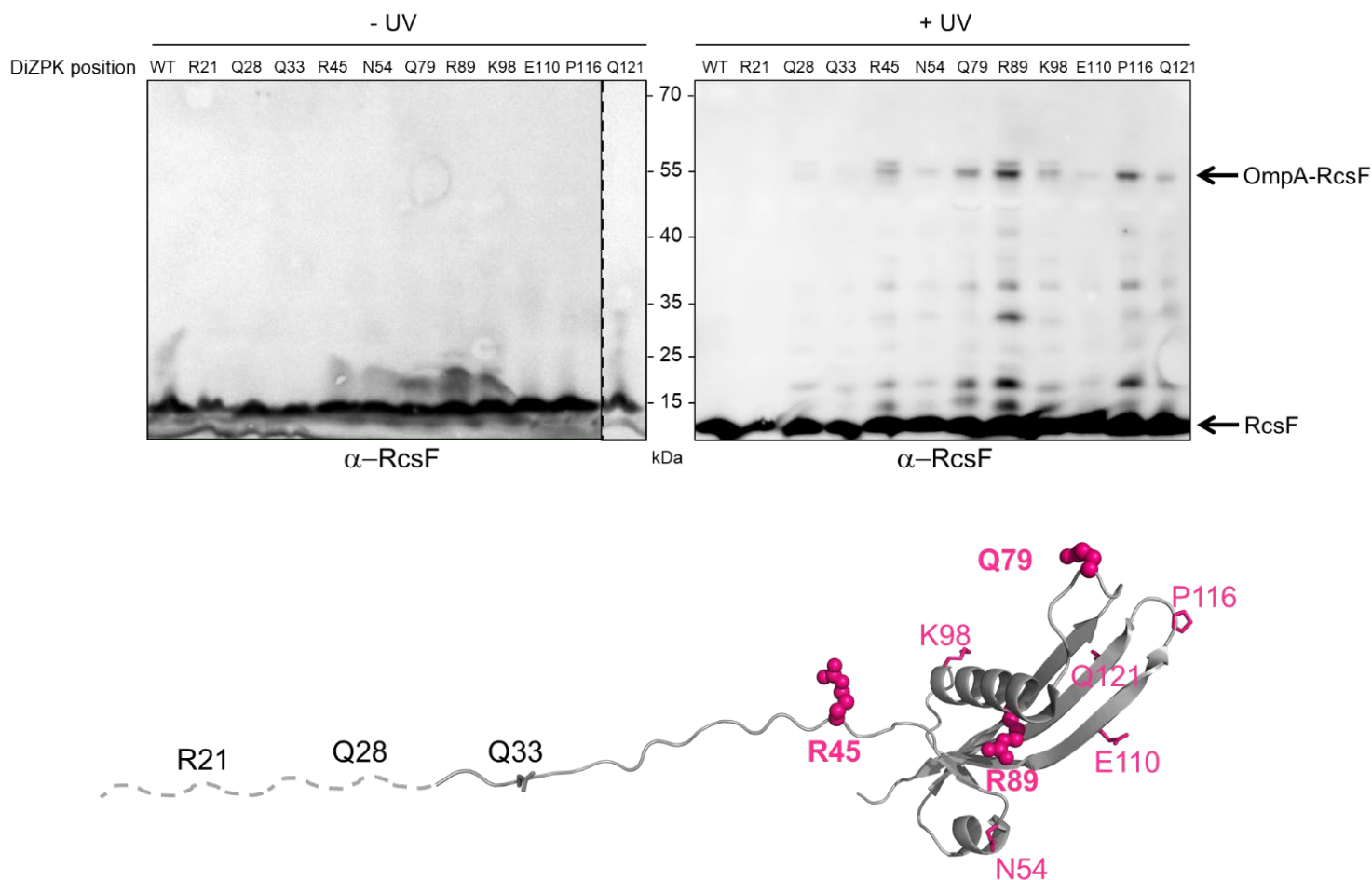


Figure 1. Defining the binding interface of RcsF on OmpA using *in vivo* site-specific photo-crosslinking. Upper panel: $\Delta rcsF$ cells expressing wild-type (WT) RcsF or DiZPK-containing RcsF variants (from pSC253) were irradiated with UV light (+) or not (-), and protein samples were immunoblotted with an anti-RcsF antibody. A 55 kDa band, corresponding to the size of the OmpA-RcsF complex, was observed for eight of the mutants (R45, N54, Q79, R89, K98, E110, P116, Q121). Lower panel: residues of RcsF were replaced by DiZPK to map the zone of interaction with OmpA. In this cartoon of the NMR structure of RcsF (PDB: 2L8Y), the truncated N-terminal portion of the protein is shown as a dashed line and the residues that were found to interact with OmpA appear in magenta. The side chains of the residues that were selected for further experiments are shown as spheres, and other side chains are represented as sticks.

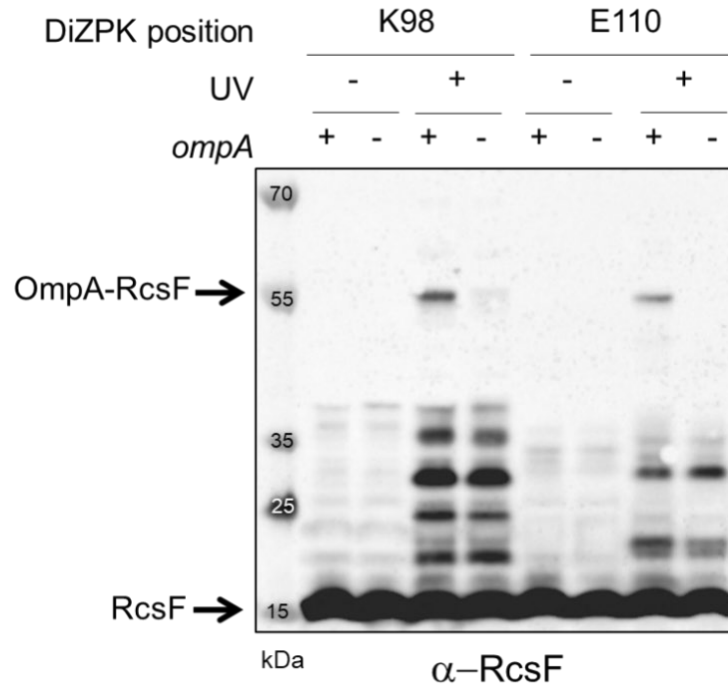


Figure 1 — figure supplement 1. The UV-dependent 55 kDa band is the **OmpA-RcsF complex**. To confirm the identification of the 55 kDa band as OmpA-RcsF, two DiZPK-containing RcsF variants (K98 and E110) were expressed from pSC253 in $\Delta rcsF$ and $\Delta rcsF\Delta ompA$ cells. Following UV exposure, the 55 kDa band only formed in cells in which OmpA was produced. Protein samples were immunoblotted with an anti-RcsF antibody.

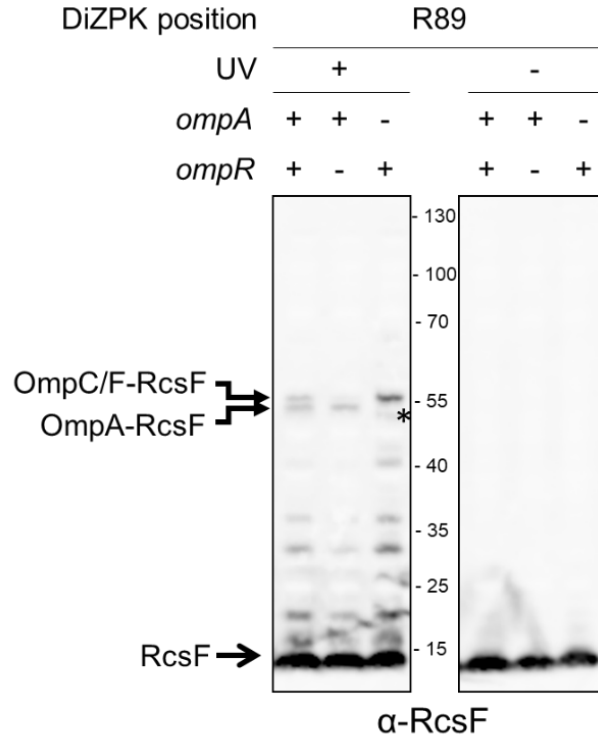


Figure 1 — figure supplement 2. The band migrating above the OmpA-RcsF complex is the OmpC/F-RcsF complex. A band migrating slightly higher than OmpA-RcsF was observed with the RcsF_{R89X} variant expressed from pSC253 in $\Delta rcsF$ cells following UV exposure. This band corresponds to the OmpC/F-RcsF complex because it did not form in cells lacking *ompR*, a transcription factor required for the production of OmpC and OmpF. The unknown band marked by * migrates lower than OmpA-RcsF.

We next sought to identify where RcsF binds OmpA. The general view is that OmpA consists of two domains, an 8-stranded β -barrel anchoring the protein in the OM and a soluble C-terminal domain located in the periplasm, where it binds the peptidoglycan {De Mot, 1994 #2499;Park, 2012 #2179} (Figure 2A). However, an alternative conformation has been proposed in which OmpA folds into a large, 16-stranded β -barrel {Singh, 2003 #2397;Stathopoulos, 1996 #1216} (Figure 2A). To gain insight into where RcsF binds OmpA and to characterize OmpA's conformation when in complex with RcsF, we first engineered an OmpA variant

with a thrombin cleavage site inserted after residue V189 (OmpA_{TH_189}), in the middle of the OmpA sequence (Figure 2B). Taking the two-domain structure as a reference, the cleavage site was inserted between the N- and C-terminal domains. We then selected three DiZPK-containing RcsF mutants (RcsF_{R45X}, RcsF_{Q79X}, RcsF_{R89X}) that formed a covalent complex with OmpA at high levels when exposed to UV light (Figure 1). These variants display DiZPK in three regions of RcsF: in RcsF_{R45X}, DiZPK is present at the end of the disordered linker, while RcsF_{Q79X} displays DiZPK in the large loop at the tip of the signaling domain and RcsF_{R89X} displays it on the central α -helix 2 (Figure 1). These variants were expressed in *E. coli* cells also producing OmpA_{TH_189}, and complex formation was induced with UV light (Figure 2C). For all three RcsF variants, digestion of the 55-kDa OmpA_{TH_189}-RcsF complex with thrombin yielded a ~35 kDa band that was recognized by both anti-RcsF and anti-His antibodies (Figure 2C, D). Given the presence of a His-tag in the C-terminus of OmpA (Materials and Methods), we concluded that RcsF (14 kDa) interacts with the C-terminal region of OmpA (~16 kDa) *in vivo*.

To further delineate the region of OmpA that is important for complex formation, a second OmpA mutant with a thrombin cleavage site inserted after residue I243 (OmpA_{TH_243}) was generated (Figure 2B). Like OmpA_{TH_189}, OmpA_{TH_243} (~10 kDa) could be photo-crosslinked to the three DiZPK-containing RcsF variants described above (Figure 2C, D). In all three cases, thrombin digestion of the OmpA_{TH_243}-RcsF complexes generated a smaller band (~30 kDa) that was recognized by anti-RcsF antibodies (Figure 2C, D). In contrast to OmpA_{TH_189}-RcsF, which underwent complete digestion, OmpA_{TH_243}-RcsF only underwent partial cleavage (Figure 2C, D), which probably reflected decreased accessibility of the cleavage site to the protease. This ~30 kDa band was also detected by anti-His antibodies (Figure 2D), indicating that the three tested residues of RcsF bind the

region of OmpA between residue I243 and the C-terminus.

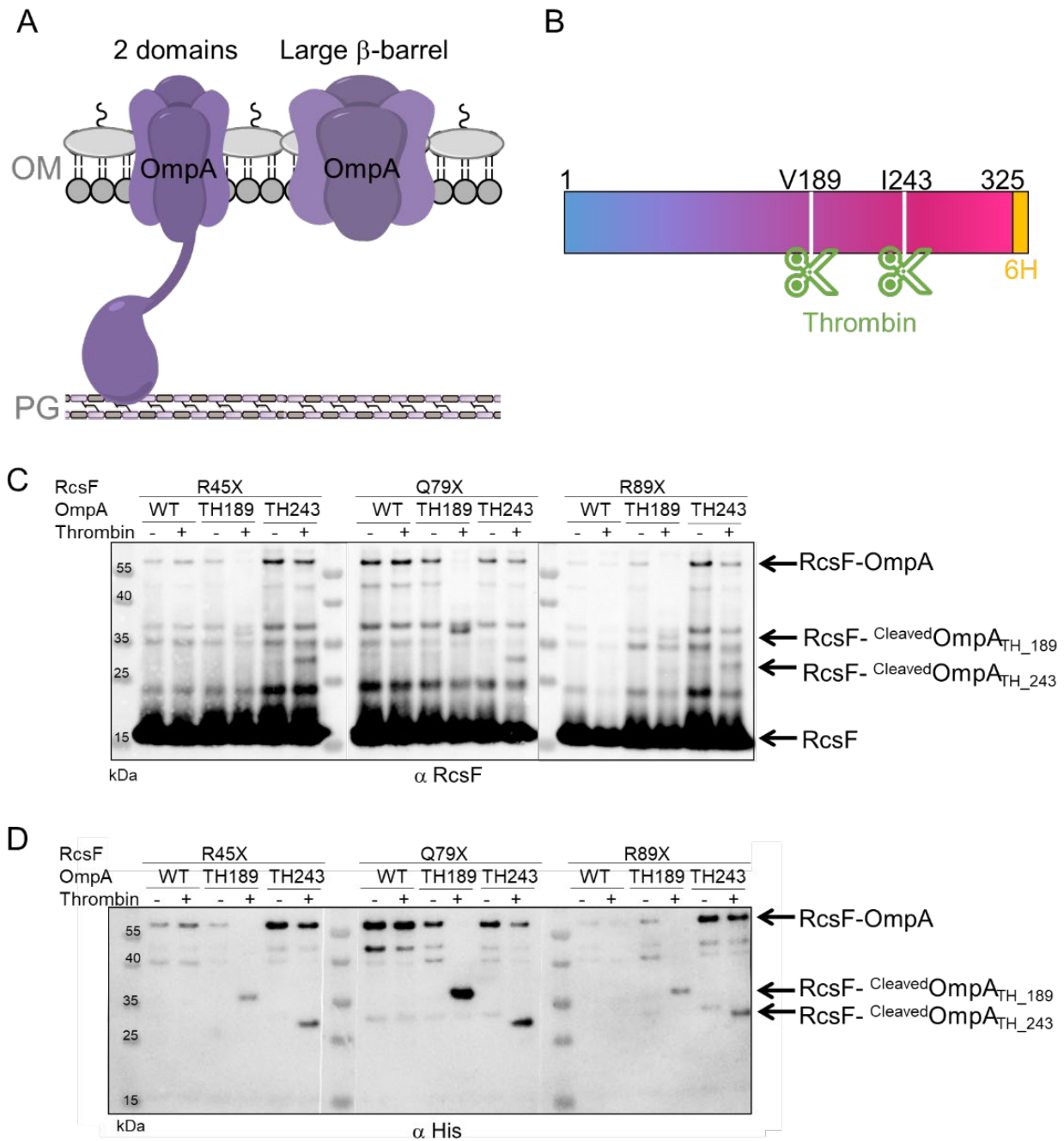


Figure 2. RcsF interacts with the C-terminal portion of OmpA *in vivo*.

(A) Schematic of the two conformations of OmpA. Left: the predominant view is that OmpA adopts a two-domain structure, with an N-terminal β -barrel embedded in the OM and a C-terminal periplasmic domain binding the peptidoglycan. Right: an alternative conformation in which OmpA folds into a large β -barrel has also been proposed {Singh,

2003 #2397;Stathopoulos, 1996 #1216}. **(B)** Schematic of OmpA variants containing a thrombin-specific cleavage site (scissors at positions V189 and I243) and a 6x-histidine tag (orange) at the C-terminus. **(C-D)** *In vivo* site-specific photo-crosslinking of RcsF. $\Delta rcsF$ cells co-expressing one of the DiZPK-containing RcsF variant (R45X, Q79X, or R89X) together with OmpA (wild-type, or with a thrombin site inserted at V189 or I243) were UV-irradiated. The RcsF variants were expressed from pSC253; OmpA, OmpA_{TH_189} and OmpA_{TH_243} were expressed from the chromosome. After immunoprecipitation with anti-RcsF, protein samples were incubated (+) or not (-) with thrombin and immunoblotted with an anti-RcsF **(C)** or an anti-His-tag antibody **(D)**. At least partial digestion of the ~55 kDa complex corresponding to OmpA-RcsF occurred with all three DiZPK-containing RcsF variants, yielding a band (RcsF^{-cleaved}OmpA_{TH_189} or RcsF^{-cleaved}OmpA_{TH_243}) migrating at lower molecular weights that was detected by both antibodies.

The C-terminal region of OmpA is necessary and sufficient for binding RcsF

The results above indicated that RcsF interacts with the C-terminal region of OmpA, raising the question of whether the N-terminal region also participates in this interaction. To probe this potential interaction directly, we generated an OmpA variant lacking the C-terminal moiety (OmpA₁₋₁₇₀) and tested whether it could be crosslinked to RcsF (Figure 3A). Chemical crosslinking was carried out with 3,3'-dithio-bis[sulfosuccinimidylpropionate] (DTSSP) {Cho, 2014 #899}. Whereas complexes formed in cells expressing wild-type OmpA, no complex was detected in cells producing OmpA₁₋₁₇₀ (Figure 3A). In addition, expression of OmpA₁₋₁₇₀ did not suppress the activation of the Rcs system (Figure 3A) that occurs in cells lacking OmpA and that results from the inability of RcsF to interact with this β -barrel {Cho, 2014 #899}.

To test whether the N-terminal domain of OmpA was indirectly required for the OmpA-RcsF complex to form, we generated a hybrid protein (OmpA_X) in which the C-terminal region of OmpA (OmpA₁₇₁₋₃₂₅), corresponding to the periplasmic domain in the two-domain structure, was fused to OmpX (Figure 3B). OmpX is a small 8-stranded β -barrel that constitutes a structural homolog of the N-terminal region of OmpA when it folds as a small β -barrel (the two β -barrels can be superimposed with a root mean square deviation (RMSD) of 2.49 Å; Figure 3 – figure supplement 1A). OmpX does not share sequence homology with the N-terminal region of OmpA (~25%) and can thus be considered as an OM anchor for the C-terminal region when fused to the latter as in OmpA_X (Figure 3B). We found that OmpA_X could be crosslinked to RcsF (Figure 3B) and that its expression fully suppressed Rcs activation (Figure 3B). Because we could not completely exclude

the unlikely possibility that OmpA_X could rearrange into a large β -barrel able to bind RcsF, we prepared an additional variant of OmpA (OmpA_{Pal}) in which the C-terminal domain (OmpA₁₇₁₋₃₂₅) was fused to the signal sequence and lipobox (for lipid modification; {Szewczyk, 2016 #1683}) of the OM lipoprotein Pal, thus converting the C-terminal domain of OmpA into a lipoprotein (Figure 3C). Remarkably, expression of OmpA_{Pal} led to the formation of OmpA_{Pal}-RcsF and substantially decreased Rcs activity (Figure 3C). In these experiments, we confirmed that the expression levels of OmpA₁₋₁₇₀, OmpA_X and OmpA_{Pal} were similar to those of the wild type (Figure 3 – figure supplement 2). Thus, the C-terminal region of OmpA is necessary and sufficient for the interaction with RcsF, and the N-terminal region is dispensable. Altogether, these results are consistent with the conclusion that OmpA adopts its two-domain structure—and not the large β -barrel conformer—when in complex with RcsF. We therefore conclude that RcsF interacts with the C-terminal, globular domain of OmpA and that this interaction takes place on the periplasmic side of the OM.

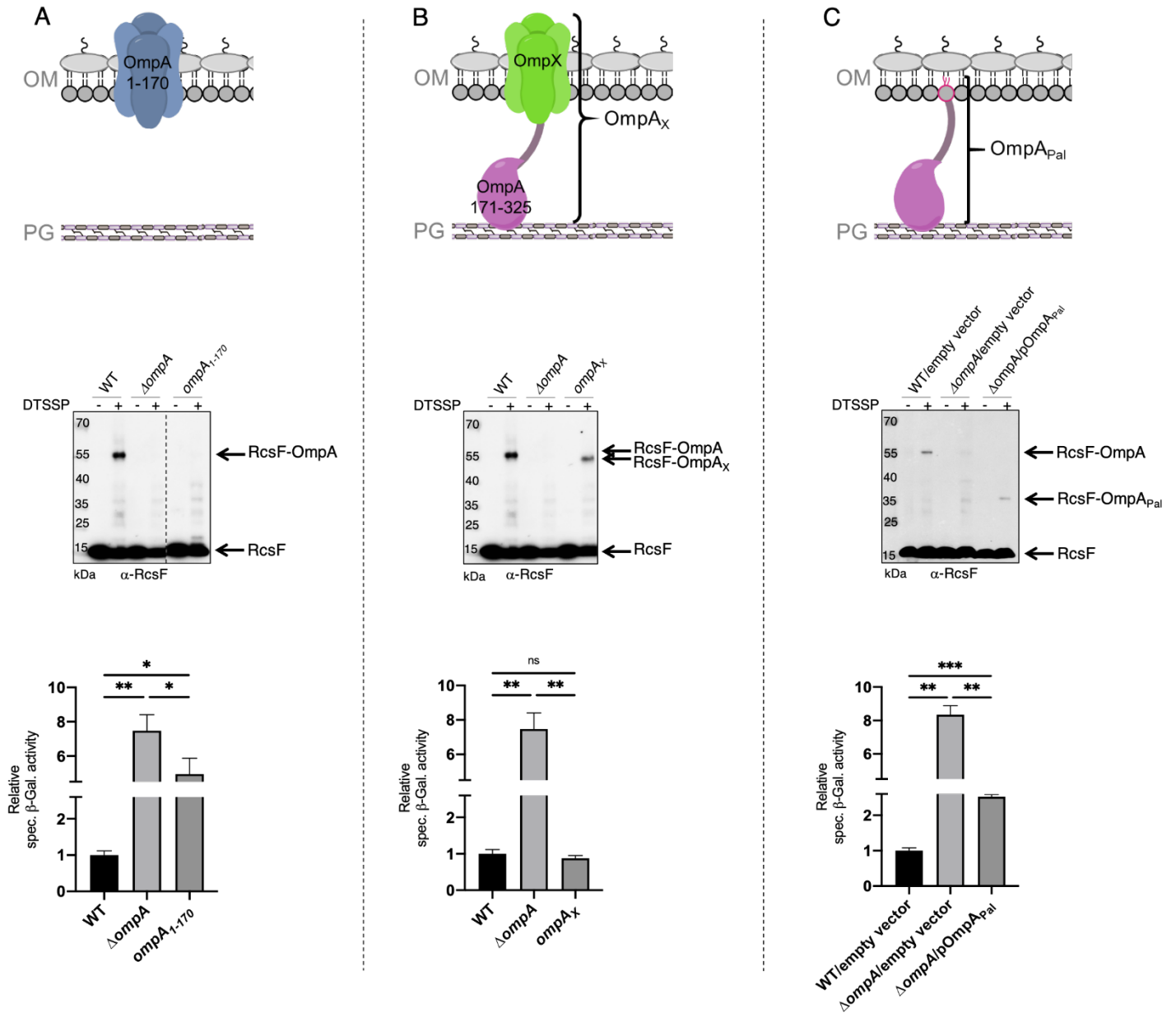


Figure 3. The periplasmic domain of OmpA is necessary and sufficient for the interaction with RcsF *in vivo*. (A) *Upper panel:* Schematic of the truncated OmpA variant corresponding to the 8-stranded β -barrel domain (OmpA₁₋₁₇₀). *Middle panel:* *In vivo* chemical crosslinking of RcsF to OmpA and OmpA₁₋₁₇₀. WT, $\Delta ompA$ and $\Delta ompA::ompA_{1-170}$ DH300 cells were incubated with or without 3,3'-dithio-bis[sulfosuccinimidylpropionate] (DTSSP). Proteins were immunoblotted with anti-RcsF (same for the middle panel in B and C). The OmpA-RcsF complex was detected in WT cells but not in cells expressing OmpA₁₋₁₇₀. *Lower panel:* β -galactosidase (β -gal) activity was measured using the

transcriptional *rprA-lacZ* fusion on the chromosome {Majdalani, 2002 #1116} using the same DH300 strains as in the middle panel (same for the lower panel in **B** and **C**). Deleting *ompA* induces Rcs, expression of OmpA₁₋₁₇₀ does not restore basal Rcs activity. (**B**) *Upper panel*: Schematic of the hybrid protein consisting of a fusion between the 8-stranded β -barrel OmpX and the periplasmic domain of OmpA (OmpA₁₇₁₋₃₂₅) (OmpA_X). *Middle panel*: *In vivo* chemical crosslinking of RcsF to OmpA and OmpA_X. WT, $\Delta ompA$ and $\Delta ompA::ompA_X$ cells were incubated with or without DTSSP. The RcsF-OmpA complex was detected in WT cells as well as in cells expressing OmpA_X (RcsF-OmpA_X). *Lower panel*: Deleting *ompA* induces Rcs, expression of OmpA_X restores basal Rcs activity. (**C**) *Upper panel*: Schematic of the C-terminal domain of OmpA (OmpA₁₇₁₋₃₂₅) fused to the signal sequence and lipobox of the OM lipoprotein Pal (OmpA_{Pal}). *Middle panel*: *In vivo* chemical crosslinking of RcsF to OmpA and OmpA_{Pal}. WT and $\Delta ompA$ harboring pDSW204, an empty vector, used as control, and $\Delta ompA$ cells harboring pKiD22, expressing OmpA_{Pal} from an IPTG-inducible promoter, were incubated with or without DTSSP. The OmpA-RcsF complex was detected in WT cells as well as in cells expressing OmpA_{Pal} (OmpA_{Pal}-RcsF). *Lower panel*: Deleting *ompA* induces Rcs, expression of OmpA_{Pal} substantially decreases Rcs activity. Mean (n=3) and standard deviation (error bars) are shown. Differences were evaluated with Student's *t* test (ns, not significant; *p<0.05; ***p<0.001).

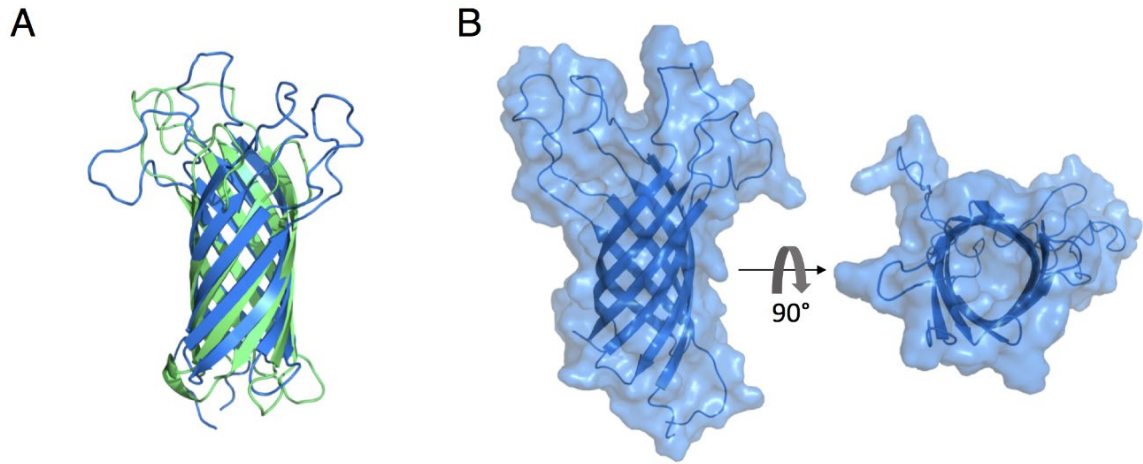


Figure 3 — figure supplement 1. The 8-stranded β -barrels of OmpA and OmpX are structurally similar and do not display an open channel through the membrane. (A) Superimposition of the structures of the membrane-embedded β -barrels of OmpA (blue; PDB: 1QJP; residues 1-170) and OmpX (green; PDB: 2M07). The two β -barrels, which have no sequence homology, can be superimposed with an RMSD of 2.49 Å. (B) Surface representation of the β -barrel domain of OmpA (PDB: 1QJP; residues 1-170). The right panel is rotated 90° around the horizontal axis from the left panel. In this conformation, OmpA does not display an open channel.

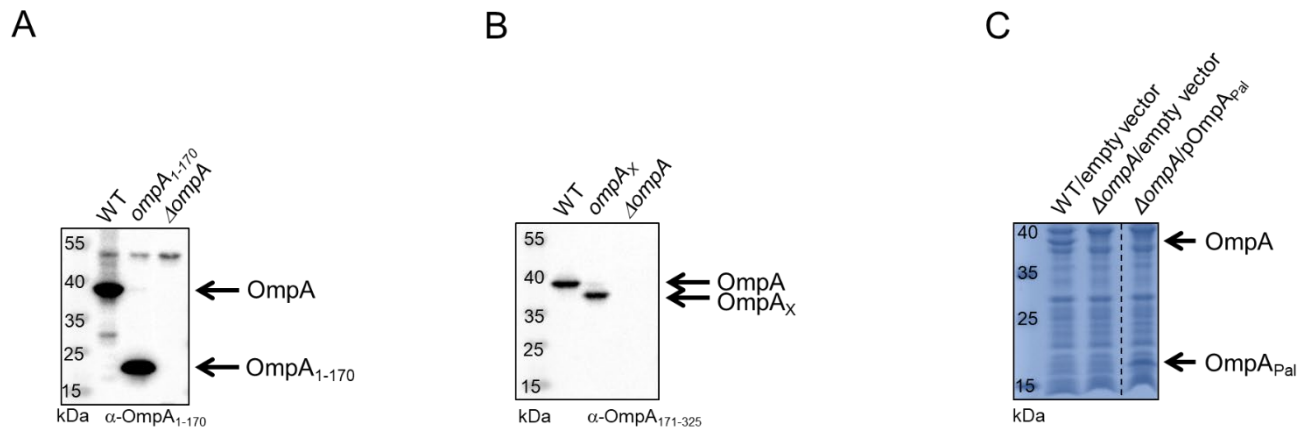


Figure 3 — figure supplement 2. The expression levels of the OmpA variants are similar to those of wild-type OmpA. (A,B) Cells expressing OmpA₁₋₁₇₀ (A) and OmpA_x (B) from the chromosome were harvested at mid-log phase ($OD_{600} = 0.4-0.6$) and

immunoblotted with antibodies specific for the periplasmic domain of OmpA (anti-OmpA₁₈₆₋₃₂₅) or the β -barrel domain (anti-OmpA₁₋₁₇₀) (Materials and Methods). (C) Cells harboring pKiD22 were harvested at mid-log phase ($OD_{600}=0.4-0.6$). Expression of OmpA_{Pal} was induced with IPTG (20 μ M). The membrane fraction was analyzed by SDS-PAGE and proteins stained with Coomassie Blue.

RcsF interacts with the periplasmic domain of OmpA *in vitro*

We next sought to validate our *in vivo* observations by probing the formation of a complex between RcsF and the soluble, periplasmic domain of OmpA (OmpA₁₈₆₋₃₂₅) *in vitro* using purified proteins. However, attempts to pull-down OmpA₁₈₆₋₃₂₅ with a soluble, His-tagged version of RcsF failed (data not shown), suggesting that the interaction between these two proteins was weak. Because NMR is a highly effective tool to investigate weak protein-protein interactions {Vaynberg, 2006 #2323}, we employed NMR titration experiments of ^{15}N -labeled OmpA₁₈₆₋₃₂₅ by RcsF. In this approach, the ^{15}N - ^1H 2D NMR spectra of OmpA were recorded upon addition of increasing amounts of RcsF (2 and 10 molar equivalents); when OmpA and RcsF interact, concentration-dependent perturbations in the NMR spectra appear. Upon addition of RcsF, several OmpA residues showed chemical shift variations in ^{15}N - ^1H 2D correlation experiments (Figure 4A, B). Most of the shifted residues (T240, G244, S245, D246, A247, G251, L252, K294 and A297; Figure 4B) were located near the tip of the periplasmic domain (Figure 4C, D), identifying this region, and in particular a flexible loop between β -strand 2 and α -helix 3, as part of the binding interface with RcsF. The importance of this loop for the OmpA-RcsF interaction was confirmed using site-specific photo-crosslinking: OmpA was strongly crosslinked to RcsF when DiZPK was introduced at residue D246, while weak complex formation was observed with OmpA_{R242X} and OmpA_{Y248X}

(Figure 4E). These results nicely fit with those obtained using OmpA_{TH_243} (Figure 2B) that identified the same region of the C-terminal domain of OmpA as part of the zone of interaction with RcsF. Thus, taken together, our results allow us to conclude that RcsF interacts with the C-terminal domain of OmpA in its globular conformation, not only *in vivo* but also *in vitro*.

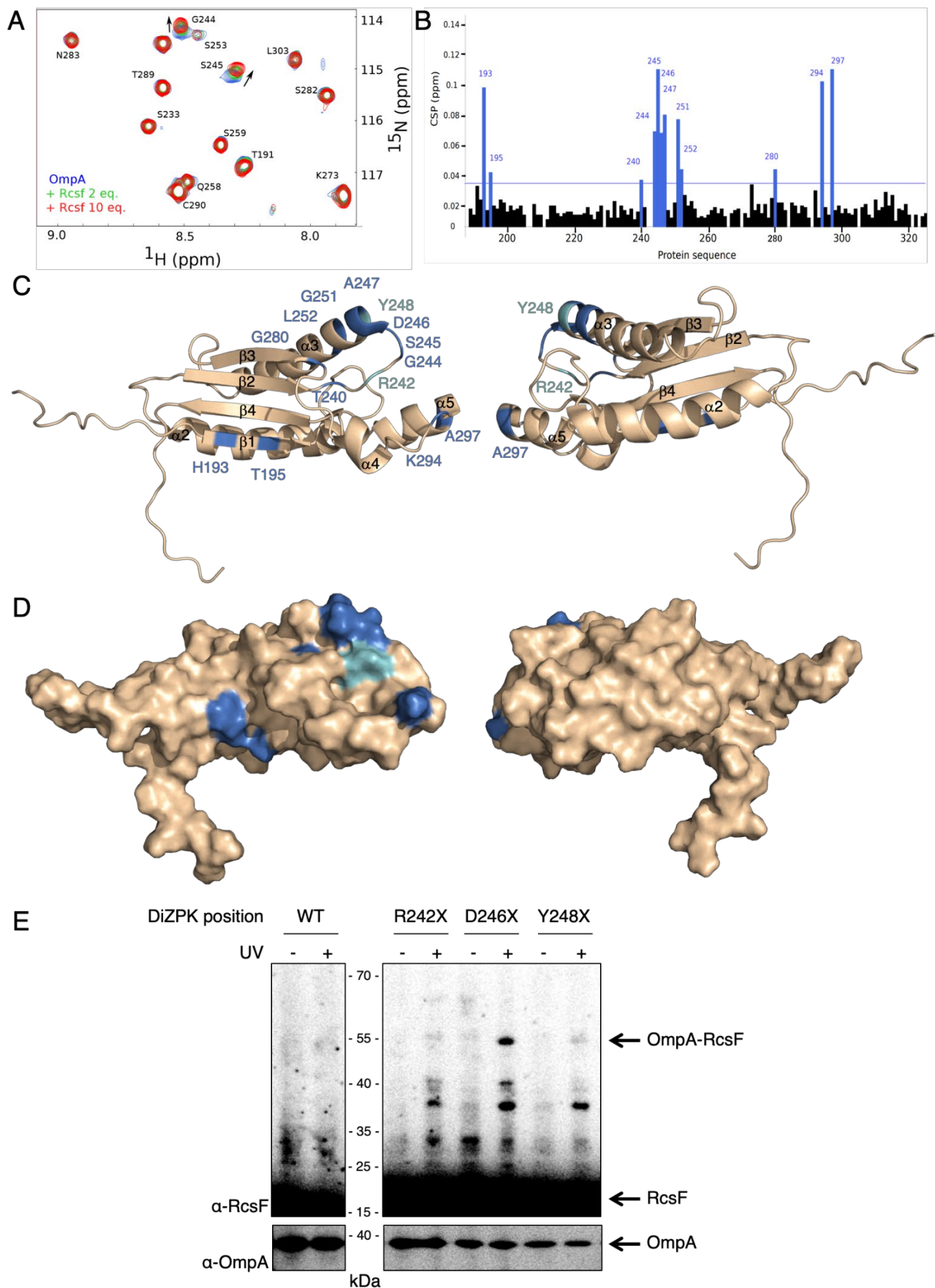


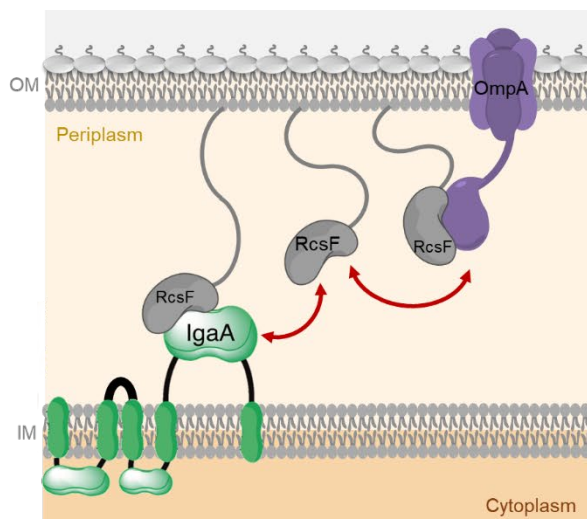
Figure 4. RcsF interacts with the periplasmic domain of OmpA *in vitro*. (A) An expanded region of the HSQC titration spectra of ^{15}N -labeled OmpA₁₈₆₋₃₂₅ with RcsF. Several residues in the $^1\text{H}/^{15}\text{N}$ BEST-TROSY-HSQC spectrum of OmpA (blue) show chemical shift perturbations (CSP) upon addition of RcsF at molar ratios of RcsF to OmpA of 2 (green) and 10 (red). Arrows indicate the direction of the chemical shift changes upon addition of RcsF to OmpA. (B) CSPs induced by the addition of RcsF to ^{15}N -labeled OmpA₁₈₆₋₃₂₅. Residues that showed CSP larger than two standard deviation are colored in blue. Cartoon (C) and surface (D) view of OmpA₁₈₆₋₃₂₅ (PDB: 2MQE): top view (on the left) and bottom view (on the right). The residues in OmpA that undergo a CSP larger than two standard deviation (as in panel B) appear as dark blue and are labeled. Most of these residues are located between β -strand 2 and α -helix 3. The residues R242 and Y248 that do not interact well with RcsF in (E) are colored in light blue. (E) To confirm the importance of the loop between β -strand 2 and α -helix 3 of OmpA for the interaction with RcsF, we used site-specific photo-crosslinking. $\Delta ompA rcsF^+$ cells expressing wild-type (WT) OmpA or three DiZPK-containing OmpA variants (OmpA_{R242X}, OmpA_{D246X}, and OmpA_{Y248X}) from pPR21 were irradiated with UV light (+) or not (-), and protein samples were immunoblotted with an anti-RcsF antibody. A strong 55 kDa band, corresponding to the size of the OmpA-RcsF complex, was observed with the OmpA_{D246X} variant, confirming the NMR data. Weak complex formation was also observed with OmpA_{R242X} and OmpA_{Y248X}. The expression levels of the OmpA variants were verified by immunoblotting (lower panel).

IgaA and OmpA likely compete for RcsF across the periplasmic space

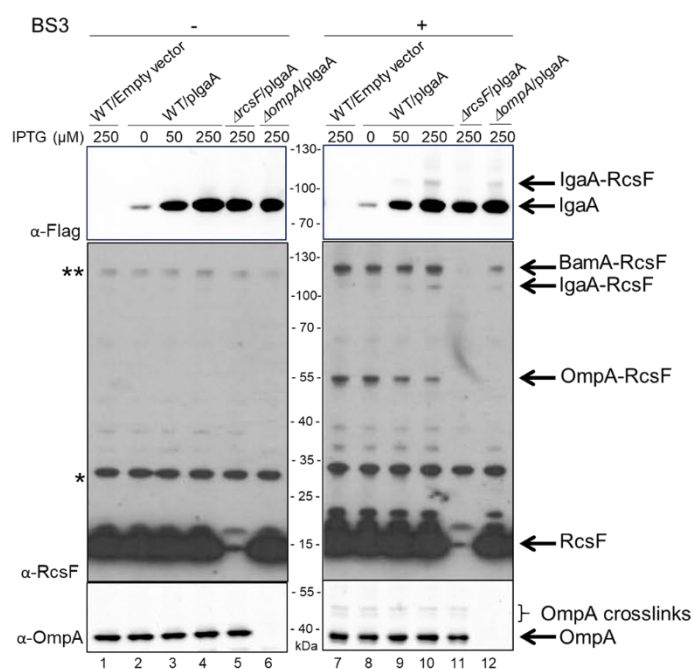
RcsF turns on the Rcs response by interacting with the periplasmic domain of the IM protein IgaA under stress {Hussein, 2018 #2192}. Here, we found that the interaction between RcsF and OmpA takes place in the periplasm (Figure 5A), thus suggesting that OmpA and IgaA compete for binding RcsF across this compartment. To investigate this hypothesis, we determined the effect of artificially increasing the IgaA concentration on the formation of the IgaA-RcsF and OmpA-RcsF complexes. In these experiments, a triple Flag-tagged, functional version of IgaA {Hussein, 2018 #2192} was expressed from an inducible plasmid. To monitor complex formation, we carried out chemical crosslinking using bis(sulfosuccinimidyl)suberate (BS3), a bifunctional crosslinker. Increasing the levels of IgaA led to changes in the IgaA-RcsF and OmpA-RcsF complexes that were inversely correlated: whereas overexpressing IgaA led to more IgaA-RcsF, it decreased the levels of the OmpA-RcsF complex (compare lanes 9 and 10 with lanes 7 and 8 in Figure 5B). Thus, IgaA and OmpA seem to compete for RcsF across the periplasm. Because IgaA (200 copies per cell; {Li G-W, 2014 #1742}) is far less abundant than OmpA (200,000 copies), even when overexpressed (we estimate that IgaA levels were increased 8-40 fold over baseline (Figure 5 – figure supplement 1; Materials and Methods) in the experiment above), these results suggested that IgaA has a substantially higher affinity for RcsF than OmpA. To probe this directly, we determined the affinity constants of the periplasmic domains of OmpA and IgaA for RcsF. Note that the periplasmic domain of IgaA interacts with RcsF *in vivo* and *in vitro* {Cho, 2014 #899;Hussein, 2018 #2192}). First, from the NMR shift data, we calculated the equilibrium dissociation constant (K_D) of OmpA₁₈₆₋₃₂₅ for RcsF as being 125 ± 85 μ M (Figure 5C). Second, using biolayer interferometry, we

measured that the periplasmic domain of IgaA has a K_D of 1.6 ± 0.3 nM for RcsF (Figure 5D). Thus, these values confirm that IgaA has substantially more affinity for RcsF than OmpA (see Discussion). Interestingly, we noted that the BamA-RcsF complex was not modified by the increased expression of IgaA (lanes 7-10 in Figure 5B), which is consistent with the fact that BamA has a much higher affinity for RcsF ($K_D \sim 400$ nM; {Rodriguez-Alonso, 2020 #2316}) than OmpA.

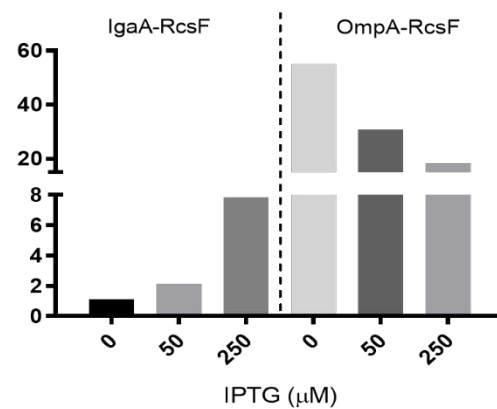
A



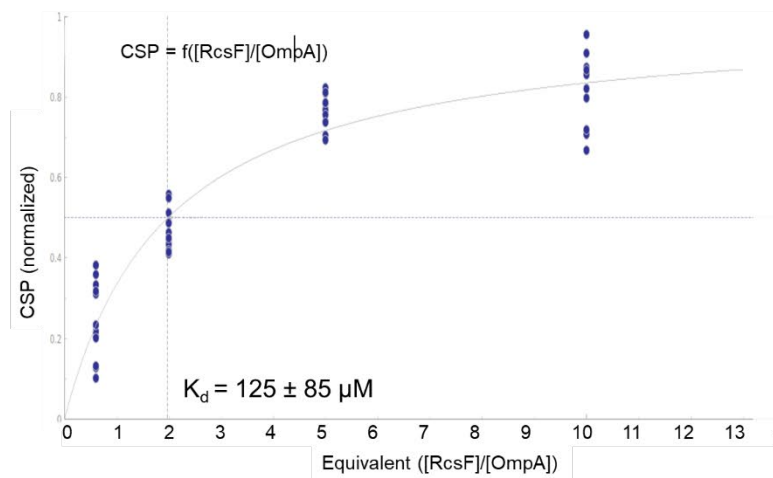
B



normalized signal intensity
(Arbitrary Unit)



C



D

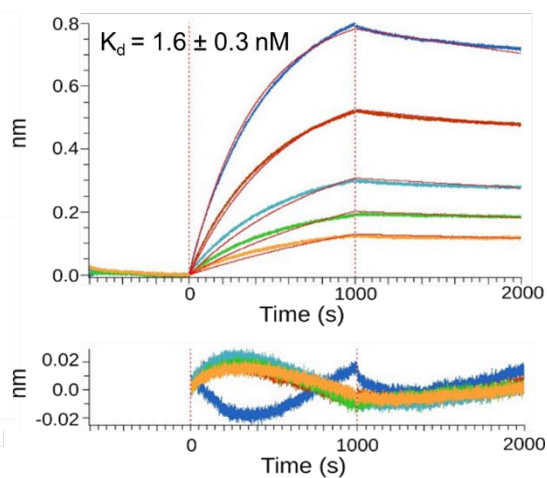


Figure 5. OmpA likely competes with the IM protein IgaA for RcsF. (A) Cartoon of the *E. coli* cell envelope, with IgaA in the IM and RcsF and OmpA in the OM. RcsF, an OM lipoprotein, interacts with OmpA. When in complex with RcsF, OmpA adopts its two-domain conformation, with an N-terminal β -barrel embedded in the OM and a C-terminal domain soluble in the periplasm. The periplasmic domain of OmpA interacts with RcsF, likely competing with IgaA for RcsF binding. Copy numbers are from {Li, 2014 #1288}. (B) Impact of over-expressing IgaA on the OmpA-RcsF complex (left). Cells harboring pSC231, an empty vector used as control, or pIgaA (pSC237, expressing IgaA-Flag₃ from an IPTG-inducible promoter) were harvested at mid-log phase and incubated without (lanes 1-6) or with (lanes 7-12) BS3. Protein samples were immunoblotted with α -Flag (upper panel), α -RcsF (middle panel), or α -OmpA₁₇₁₋₃₂₅ (lower panel) antibodies. IgaA-Flag₃ was expressed in WT (lanes 2-4 and 8-10), $\Delta rcsF$ (lanes 5 and 11), and $\Delta ompA$ cells (lanes 6 and 12) with the indicated IPTG concentrations. Quantitation of the IgaA-RcsF and OmpA-RcsF complexes detected by the anti-RcsF antibodies is shown (right panel).* and **, non-specific bands detected by the polyclonal α -RcsF antibody. (C) Plot of the chemical shift perturbation (CSP) measured on OmpA resonances as a function of the RcsF:OmpA ratio. Only the residues with significant CSP (colored in blue in Fig. 4B) are plotted and used to fit the K_D . (D) The interaction between RcsF and the periplasmic domain of IgaA was probed by biolayer interferometry (BLI). Sensortips carrying immobilized RcsF were dipped into increasing concentrations of IgaA (5.9, 8.9, 13.3, 20, 30 nM) from 0-1000 sec then into buffer (1000-2000 sec). Association and dissociation phases were fitted (red lines) to extract a K_D value. Residuals from the fits are shown at the bottom of the panel.

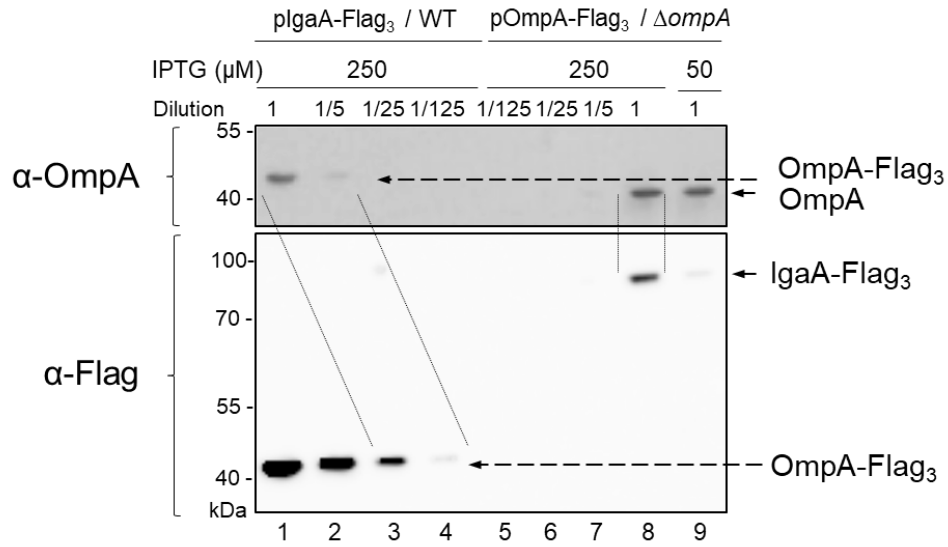


Figure 5 — figure supplement 1: Estimation of the concentrations of IgA and OmpA in the cell envelope. OmpA-Flag₃ was expressed from pOmpA-Flag₃ (pPR4) with 50 or 250 μ M IPTG in $\Delta ompA$ cells. IgA-Flag₃ was expressed from pIgaA-Flag₃ (pSC237) with 250 μ M IPTG in wild-type (WT) cells. Samples were serially diluted as indicated before SDS-PAGE. OmpA-Flag₃ (lanes 1-4) and OmpA (lanes 5-9) were detected with anti-OmpA (upper panel); OmpA-Flag₃ (lanes 1-4) and IgA-Flag₃ (lanes 5-9) were detected with anti-Flag (lower panel). Images are representative of experiments made in biological triplicate.

Discussion

OmpA is unlikely the vehicle allowing RcsF to reach the surface

OmpA was first purified from *E. coli* membranes in 1977 {Chai, 1977 #2324} and has served as a model for OMP assembly since then. Although the predominant view is that OmpA folds into a two-domain conformation, with an N-terminal 8-stranded β -barrel and a C-terminal periplasmic domain, an alternative conformation in which OmpA forms a single, 16-stranded β -barrel, has been proposed to also exist {Singh, 2003 #2397; Stathopoulos, 1996 #1216}. Here, we integrated *in vivo* (Figures 2, 3, 4 and 5) and *in vitro* (Figures 4 and 5) approaches to dissect the interaction between OmpA and the lipoprotein RcsF; altogether, our data establish that OmpA is in the two-domain conformation in the OmpA-RcsF complex and that it is the C-terminal, periplasmic domain of OmpA that interacts with RcsF. Using protein-protein docking and molecular dynamics simulations, we built a three-dimensional model taking into account the results of the cross-linking experiments; in this model, RcsF and the periplasmic domain of OmpA show a good surface complementarity, with a buried accessible surface of 1087 Å². In this model, all six residues mutated to DiZPK (RcsF_{R45X}, RcsF_{Q79X}, RcsF_{R89X}, OmpA_{R242X}, OmpA_{D246X}, and OmpA_{Y248X}) are situated in close proximity to residues from the binding partner (Figure 6A), in good agreement with the crosslinking data. Interestingly, the peptidoglycan-binding region of OmpA {Park, 2012 #2179} remains accessible and is oriented in the direction opposite to the N-terminal end of RcsF. Our conclusions have two important implications.

First, we and others previously showed that portions of RcsF are surface-exposed {Cho, 2014 #899; Konovalova, 2014 #900} and proposed OmpA, OmpC,

and OmpF as vehicles for surface exposure {Cho, 2014 #899;Konovalova, 2014 #900}. Understanding how RcsF reaches the surface is crucial: lipoprotein surface exposure is an emerging concept in *E. coli* and Enterobacteriaceae {Szewczyk, 2016 #1683;Konovalova, 2015 #2315} and how lipoproteins cross the OM remains to be clearly established. It was proposed that it is the N-terminal linker of RcsF that is exposed on the surface before being threaded through the lumen of OmpA and other OMPs {Konovalova, 2014 #900}. However, when OmpA adopts the two-domain conformation, as in the OmpA-RcsF complex, its β -barrel domain does not have an open channel (Figure 3 – figure supplement 1B) to accommodate the RcsF linker. We therefore conclude that RcsF does not reach the surface when in complex with OmpA, and predict that only OmpC and OmpF (which form large β -barrels) serve as vehicles for surface exposure. The fact that only a subset of the DiZPK-containing variants of RcsF that form a complex with OmpA also form a complex with OmpC/F (Figure 1) also supports the idea that the topology of OmpA-RcsF is different from that of OmpC/F-RcsF.

Second, a model was proposed in which RcsF, when in complex with OmpA, uses its positively charged, surface-exposed N-terminal linker to sense when interactions between lipopolysaccharide molecules are disturbed {Konovalova, 2016 #1094}. However, if the RcsF linker is not surface-exposed in the OmpA-RcsF complex, as we show here, then the function of OmpA-RcsF in Rcs needs to be re-evaluated (see below). Importantly, we found that lipopolysaccharide alterations caused by sub-lethal concentrations of polymyxin B induce Rcs in MG1655 cells lacking *ompA* (Figure 6 – figure supplement 1), in contrast to what was previously reported in another strain background (MC4100) {Konovalova, 2016 #1094}, which further questions the potential role of OmpA-RcsF in sensing lipopolysaccharide defects.

It will also be interesting to investigate the role of the BAM machinery in the assembly of OmpA-RcsF. It was reported that the formation of the OmpA-RcsF complex was likely mediated by BAM during the assembly of OmpA {Cho, 2014 #899;Konovalova, 2014 #900}. However, our finding that RcsF interacts with the C-terminal, periplasmic domain of OmpA, whose folding is unlikely to depend on BAM {Noinaj, 2017 #1749}, questions this conclusion. Further experiments, including pulse-chase experiments, will be carried out to clarify this point. Given that BAM both interacts with RcsF and assembles OmpA in the OM, we anticipate that interpreting the data will be challenging, and therefore these experiments are outside the scope of this publication.

OmpA functions as a buffer for RcsF

Finally, if OmpA does not allow RcsF to reach the surface, what could be the function of the OmpA-RcsF complex? On the basis of our results, we propose that the role of OmpA in Rcs is to modulate the activity of this system. Consider the equilibrium dissociation constants of the IgaA-RcsF and OmpA-RcsF complexes:

$$K_d^{\text{OmpA-RcsF}} = \frac{[\text{OmpA}][\text{RcsF}]}{[\text{OmpA-RcsF}]} \quad (1)$$

$$K_d^{\text{IgaA-RcsF}} = \frac{[\text{IgaA}][\text{RcsF}]}{[\text{IgaA-RcsF}]} \quad (2)$$

We estimate that OmpA's C-terminal domain is present in the periplasm at ~1 mM, RcsF at ~15 μ M, and IgaA at ~1 μ M (Materials and Methods). If we take into account these estimates, then the following equation can be derived from (1) and (2) (Materials and Methods):

$$[\text{IgaA} - \text{RcsF}] = \frac{K_d^{\text{ratio}}[\text{OmpA} - \text{RcsF}]}{1000 + K_d^{\text{ratio}}[\text{OmpA} - \text{RcsF}]} \quad (3)$$

$$\text{where } K_d^{\text{ratio}} = \frac{K_d^{\text{OmpA} - \text{RcsF}}}{K_d^{\text{IgaA} - \text{RcsF}}}.$$

Because OmpA is highly abundant, $[\text{OmpA}]$ can be considered constant. Thus, according to equation (1), the concentration of the OmpA-RcsF complex increases linearly with the concentration of RcsF in the periplasm (Figure 6 – figure supplement 2). From equation (3), we conclude that the concentration of IgaA-RcsF will also increase as a function of $[\text{RcsF}]$ in the periplasm. However, in this case, the increase is not linear, but is decelerated and follows a hyperbolic curve (Figure 6 – figure supplement 2), indicating that OmpA functions as a buffer for RcsF, negatively impacting its ability to activate Rcs. The buffering function of OmpA is nicely illustrated by the fact that exposure to low concentrations of polymyxin B causes higher Rcs induction in cells lacking *ompA* than in the wild type (Figure 6 – figure supplement 1).

We acknowledge that the ~80,000-fold difference in affinity for RcsF that we measured between IgaA and OmpA (Figure 5) does not seem to support the function of OmpA as a buffer for RcsF: with such a high affinity for RcsF, IgaA should always outcompete OmpA and formation of the IgaA-RcsF complex should be constitutive, which is not the case {Hussein, 2018 #2192}. To explain the apparent discrepancy between the *in vivo* observations and the *in vitro*

measurements, we propose that intracellular factors such as attachment of the periplasmic domains of these proteins to their respective membrane anchors (we used the soluble periplasmic domains of OmpA, IgaA and RcsF for the *in vitro* measurements) and binding of OmpA to the peptidoglycan {Reusch, 2012 #1182} modulate the affinity of OmpA and IgaA for RcsF *in vivo*, allowing them to actually compete for RcsF. It is remarkable that OmpA, a protein that had mostly been known for its role in stabilizing the OM, is also involved in the network controlling the activity of Rcs, one of the most complex signal transduction systems in bacteria {Cho, 2014 #899;Hussein, 2018 #2192;Laloux, 2017 #1736}, whose correct functioning is critical for success of commensal Enterobacteriaceae and virulence of pathogens.

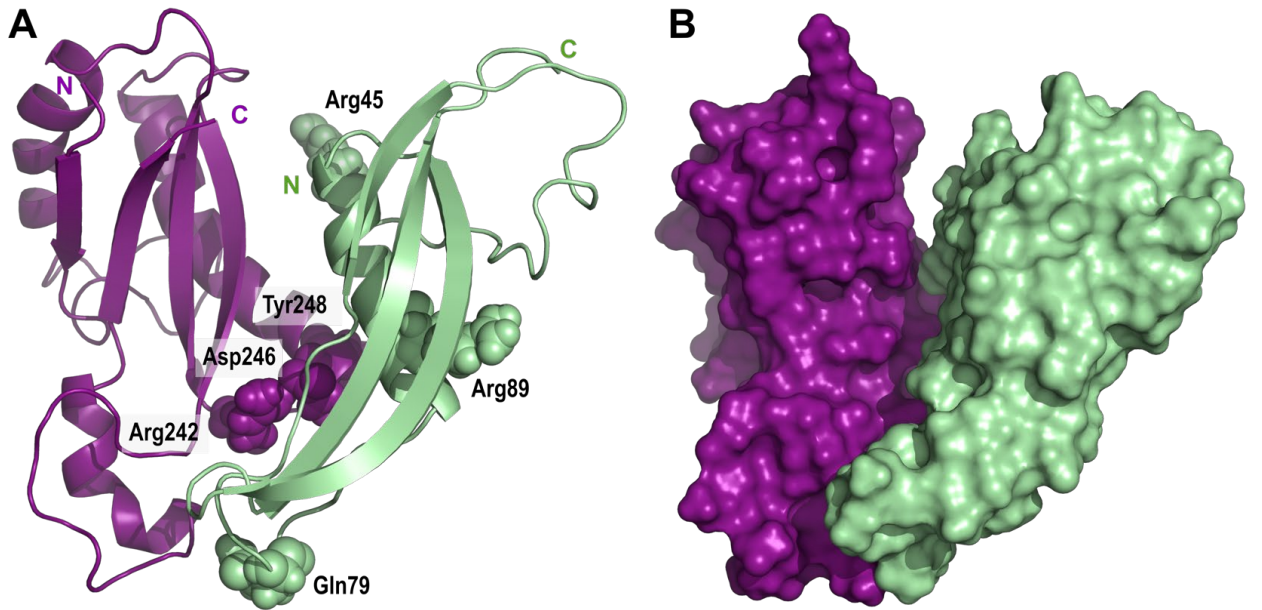


Figure 6. Model of the complex between RcsF and the C-terminal domain of OmpA.

(A) Cartoon and (B) surface representation of a complex between RcsF (residues 44-134, colored in light green) and OmpA (residues 190-315, colored in purple). The atoms from the six residues mutated to DiZPK (RcsF_{R45X}, RcsF_{Q79X}, RcsF_{R89X}, OmpA_{R242X}, OmpA_{D246X},

and OmpA_{Y248X}) are represented as spheres with the van der Waals radius. This model, which shows a good surface complementarity between the two proteins, was obtained by protein-protein docking with constraints from the crosslinking experiments and further refined by all-atom molecular dynamics (50 ns). The N-terminal residue of RcsF points out in the same direction as the extremities of the periplasmic domain of OmpA, i.e. towards the OM, and this binding mode is compatible with the interaction of the periplasmic domain of OmpA with the peptidoglycan.

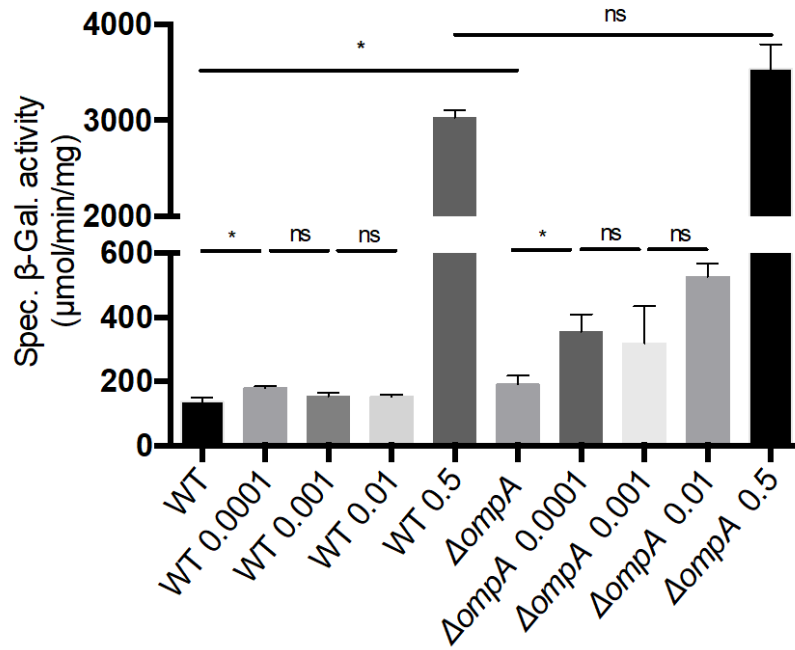


Figure 6 — figure supplement 1: The Rcs system responds to polymyxin exposure in cells lacking *OmpA*. Wild-type (WT; DH300, a MG1655 derivative {Majdalani, 2002 #1116}) and $\Delta ompA$ cells were treated with increasing concentrations of polymyxin B (0.0001 – 0.001 – 0.01 – 0.5 $\mu\text{g/mL}$) when they reached an OD_{600} of 0.4. Rcs activity was measured by a chromosomal *rprA::lacZ* fusion. Treatment with polymyxin B activated Rcs both in the WT and in the $\Delta ompA$ mutant. Mean ($n=3$) and standard deviation (error bars) are shown. Differences were evaluated with Student's *t* test (ns, not significant; $*p<0.05$).

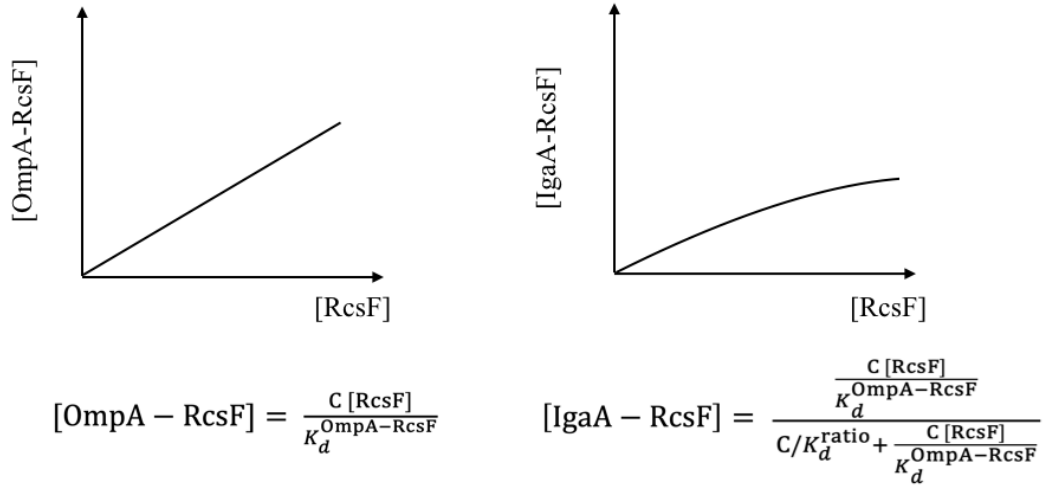


Figure 6 — figure supplement 2: OmpA functions as a buffer for RcsF. The concentration of OmpA $[\text{OmpA}]$ is high (1000 μM) and can therefore be considered constant ($[\text{OmpA}]=C$ in the equations above)). Left panel: the concentration of the OmpA-RcsF complex $[\text{OmpA-RcsF}]$ increases linearly with the concentration of RcsF in the periplasm. Right panel: the concentration of IgaA-RcsF $[\text{IgaA-RcsF}]$ increases as a function of $[\text{RcsF}]$ in the periplasm, but, in this case, the increase is decelerated and follows a hyperbolic curve. Thus, OmpA functions as a buffer for RcsF. See the Materials and Methods for more information.

Materials and Methods

Bacterial strains, primers, and plasmids

The bacterial strains and primers used in this study are listed in Supplementary Tables 1 and 2, respectively. The parental *E. coli* strain DH300 is a MG1655 derivative containing a deletion of the *lac* region; it also carries a chromosomal *rprA::lacZ* fusion at the λ phage attachment site to monitor Rcs activation {Majdalani, 2002 #1116}. The *ompA*, *ompR* and *rscF* deletion mutants were obtained by transferring the corresponding alleles from the Keio collection (kan^R) {Baba, 2006 #322} into DH300 {Majdalani, 2002 #1116} *via* P1 phage transduction, which was verified *via* PCR. To excise the kanamycin-resistance cassette, we used pCP20 as previously described {Datsenko, 2000 #438}. To insert *ompA-His* (encoding OmpA with six histidines at the C-terminus), *ompA_{TH189}-His*, *ompA_{TH243}-His*, and *ompX-ompA₁₇₁₋₃₂₅* on the chromosome at the *ompA* locus, we performed λ -Red recombineering {Yu, 2000 #1268} with the pSIM5-Tet plasmid {Koskiniemi, 2011 #2330}. First, a *cat-sacB* cassette encoding chloramphenicol acetyl transferase (*cat*) and *SacB*, a protein conferring sensitivity to sucrose, was amplified from strain CH1990 using primers “ompA_delCmSB F” and “ompA_delCmSB R”. The resulting PCR product shared 40 bp of homology to the 5' UTR and 3' UTR of *ompA* at its 5' and 3' ends, respectively, and was used for λ -Red recombineering {Yu, 2000 #1268}. We selected transformants for chloramphenicol resistance and verified that the *cat-sacB* cassette replaced *ompA* by sequencing across the junctions. The *cat-sacB* cassette was subsequently replaced by one of the versions of *ompA* (*ompA-His*, *ompA_{TH189}-His*, *ompA_{TH243}-His*, or *ompX-ompA₁₇₁₋₃₂₅*) using λ -Red recombineering and negative selection on sucrose-containing medium {Thomason, 2014 #2338; Gennaris, 2015 #1276}. *ompA-His*,

*ompA*_{TH189}-*His*, and *ompA*_{TH243}-*His* were amplified *via* PCR using primers “cmSB to OmpA_F” and “cmSB to OmpAhis_R” and plasmids pPR11, pPR11_{189thrombin}, and pPR11_{243thrombin} (see below) as templates, respectively. The PCR product of *ompX-OmpA*₁₇₁₋₃₂₅ was generated using primers “JLEo20-F-Chrom-OmpX” and “JLEo22-R-Chrom-OmpACt”, and pJLE17-OmpX-OmpACter as template. Strains were verified through DNA sequencing. To delete *ompA*₁₇₁₋₃₂₅, we performed λ -Red recombineering on the chromosome at the *ompA* locus in a way similar to the description above. Briefly, the kanamycin cassette from the strain SEN588 (Δ *ompA::kan*) was PCR-amplified using primers “ompAc_delKm F” and “ompAc_delKm R” to encompass the flanking regions of *ompA*₁₇₁₋₃₂₅. We selected transformants for kanamycin resistance.

The plasmids used in this study are described in Supplementary Table 3. pSC253, encoding RcsF, was constructed *via* digestion of pSC202 {Cho, 2014 #899} with *KpnI* and *HindIII* and insertion of the generated fragment into pBAD18 {Guzman, 1995 #323}. To generate the *rscF* variants containing an amber codon (TAG) at selected positions, site-directed mutagenesis was performed on pSC253 using primers described in Supplementary Table 2. To generate pPR11, *ompA-His* was PCR-amplified using primers “OmpA(NcoI) F” and “OmpA-his(XbaI) R” and chromosomal *E. coli* DNA as template. The PCR product was inserted into pDSW204 restricted with *NcoI* and *XbaI*, generating pPR11. To insert thrombin cleavage sites after residues 189 and 243 in OmpA, site-directed mutagenesis was performed using primer pair “189VPRGS thr_F” and “189VPRGS thr_R” and primer pair “243LVPR thr_F” and “243LVPR thr_R”, respectively, on pPR11, yielding pPR11_{189thrombin} [OmpA(189-Val-Val-Pro-Arg-Gly-Ser-Gln-190)] and pPR11_{243thrombin} [OmpA(243-Ile-Leu-Val-Pro-Arg-Gly-244)], respectively. pJLE17-OmpX-OmpA₁₇₁₋₃₂₅ (encoding OmpA_X) was constructed as follows. The two PCR

products corresponding to *ompX* and *OmpA*₁₇₁₋₃₂₅ were generated using primer pair “OmpX(NcoI)F” and “OmpX-OmpAc_R” and primer pair “OmpX-OmpAc_F” “JLEo16-R-XbaI-OmpACT”, respectively, and chromosomal *E. coli* DNA as template. To join the two PCR fragments, overlapping PCR was performed using primers “OmpX(NcoI)F” and “JLEo16-R-XbaI-OmpACT”, generating the PCR product encoding OmpA_X. Next, the PCR product of *OmpA*_X was digested with *NcoI* and *XbaI* and ligated with pDSW204 pre-digested with *NcoI* and *XbaI*, yielding pJLE17-OmpX-OmpACTer. pKiD22, expressing OmpA_{Pal}, was constructed as follows. The sequence encoding the signal sequence and lipobox of the lipoprotein Pal was PCR-amplified using primers “Palss(NcoI)F” and “Palss-OmpAc_R” and the sequence encoding OmpA₁₇₁₋₃₂₅ using primers “Palss-OmpAc_F” and “OmpA_stop_Flag₃(KpnI)R”. Chromosomal *E. coli* DNA was used as template. To join the two PCR fragments, overlapping PCR was performed using primers “Palss(NcoI)F” and “OmpA_stop_Flag₃(KpnI)R”. The PCR product encoding ssPal-OmpA₁₇₁₋₃₂₅ was digested with *NcoI* and *KpnI* and ligated with pDSW204, pre-digested with *NcoI* and *KpnI*, yielding pKiD22.

To generate pPR21, site-directed mutagenesis was performed on pPR11 using primers “OA_stopTGA_F” and “OA_stopTGA_R” to insert a stop codon upstream of the 6xHis tag. To generate the *ompA* variants containing an amber codon (TAG) at selected positions, site directed mutagenesis was performed on pPR21 using primers described in Supplementary Table 2. To obtain pKiD5, OmpA₁₈₆₋₃₂₅ with an N-terminal Strep-tag but no signal sequence was PCR-amplified using primers “KiDo14-F-NdeI-Strep-OmpACTD” and “KiDo15-R-SacI-Rbs-OmpACTD” and chromosomal *E. coli* DNA as template. The PCR product was digested with *NdeI* and *SacI* and inserted into pET21a. To prepare a version of pAM238 containing *lacF*^l, a *trc* promoter, and a triple Flag tag (Flag₃), we prepared

a PCR product using primers “lacIq NsiI_F” and “flag3-KpnI_R” and pMER77 as template {Hemmis, 2011 #2334}. This product was digested with *NsiI* and *KpnI* and ligated with pAM238 pre-digested with *PstI* and *KpnI*. To reduce the basal activity of the *trc* promoter, we modified the -10 region (from TATAAT to CATTAT) and the -35 region (from TTTACA to TTGACA), generating pSC231 {Weiss, 1999 #2333}. The coding sequences for OmpA and IgaA were obtained by PCR-amplification using primer pair “OmpA(NcoI) F” and “OmpA XbaI flag R” and primer pair “IgaA(NcoI) F” and “IgaA XbaI flag3 R”, respectively. Each product was digested with *NcoI* and *XbaI* and ligated into pSC231 pre-digested with the same enzymes, generating pPR4 and pSC237, respectively.

***In vivo* site-specific photo-crosslinking using DiZPK**

We used the site-specific photo-crosslinking method described previously {Cho, 2014 #899} with some modifications. To incorporate N^6 -((3-(3-methyl-3*H*-diazirin-3-yl)propyl)carbamoyl)-*L*-lysine (DiZPK) into RcsF, we used the pSup-Mb-DIZPK-RS plasmid encoding an evolved *Methanosarcina barkeri* pyrrolysyl-tRNA synthetase (PylRS) and an optimized tRNA^{Pyl}_{CUA} suppressor {Zhang, 2011 #806}. DH300 $\Delta rcsF$ (PL358) cells were co-transformed with pSup-Mb-DIZPK-RS and one of the plasmids containing an amber codon in *rcsF* in pSC253. Cells were grown in 3-(N-morpholino)propanesulfonic acid (MOPS) minimal medium supplemented with 0.2% glucose, 0.2% L-arabinose (MOPS-glucose/arabinose minimal medium), and 0.8 mM DiZPK (no supplement of other amino acids; see the reasons for using MOPS medium below) {Neidhardt, 1974 #2339}. Cell cultures were grown to an OD₆₀₀ of 1-1.2 and 1 mL of samples was irradiated with UV light at 365 nm or left unirradiated for 10 min. Cells were precipitated with

trichloroacetic acid (TCA), and the pellets were washed with acetone and solubilized in 60 μ L of SDS-sample buffer before further analysis. We used a similar method to incorporate DiZPK into OmpA with minor modifications; DH300 $\Delta ompA$ (PR46) cells were co-transformed with pSup-Mb-DIZPK-RS and one of the plasmids containing an amber codon in *ompA* in pPR21. Cells were grown in LB medium supplemented with 0.2% L-arabinose, 200 μ M IPTG and 1 mM DiZPK. Cell cultures were grown to an OD₆₀₀ of 1 and 0.75 mL of samples was irradiated with UV light at 365 nm or left unirradiated for 10 min. Cells were precipitated with TCA, and the pellets were washed with acetone and solubilized in 100 μ L of SDS-sample buffer before further analysis.

In previous experiments incorporating *p*-benzoyl-L-phenylalanine into RcsF, we used LB as the growth medium {Cho, 2014 #899}. However, we found that the expression levels of the DiZPK-containing RcsF mutant proteins were substantially lower when cells were grown in LB (data not shown); in contrast, the expression levels of RcsF were greatly enhanced in MOPS-glucose/arabinose minimal medium (data not shown). Therefore, we used MOPS-glucose/arabinose minimal medium for all photo-crosslinking experiments involving DiZPK-containing RcsF variants.

Synthesis of DiZPK

DiZPK was synthesized as described previously {Zhang, 2011 #806}.

Immunoprecipitation of RcsF-containing complexes and thrombin cleavage

Protein samples in SDS-sample buffer (without reducing agent) were denatured for 15 min at 65 °C and 15 min at 95 °C with vigorous shaking. Next, the samples were diluted in 750 μ L of KI buffer (50 mM Tris-HCl [pH 8], 2% Triton X-100, 150 mM NaCl, 1 mM EDTA) and centrifuged at 4 °C for 3 min at 12,000 $\times g$ to harvest the PG. Photo-crosslinked RcsF complexes were immunoprecipitated by adding 1 μ L of undiluted guinea pig anti-RcsF antibody {Cho, 2014 #899} and 10 μ L of protein A/G magnetic beads (PierceTM); samples were incubated for 1 h on a wheel at room temperature. After three washes with 500 μ L of KI buffer, RcsF complexes were eluted with 20 μ L of glycine buffer (100 mM glycine [pH 1.5], 0.1% Triton X-100) after 10 min of incubation at room temperature. Proteins samples were neutralized with 2 μ L of 1.5 M Tris-HCl [pH 8.8] and diluted with 18 μ L of KI buffer before SDS-PAGE and immunoblotting or thrombin cleavage. For thrombin cleavage, 20 μ L of the elution samples were incubated for 1 h at room temperature with 1 μ L of thrombin (thrombin from bovine plasma, Sigma). Samples were analyzed *via* SDS-PAGE and immunoblotting as indicated in the figure legends.

Immunoblotting and antibodies

Protein bands were transferred from the gels onto nitrocellulose membranes (Millipore) using a semi-dry electroblotting system. The membranes were blocked with 5% skim milk. The rest of the immunoblot steps were performed using standard protocols. Signal from antibody binding was visualized by detecting chemiluminescence from the reaction of horseradish peroxidase with luminol. Polyclonal RcsF antibodies were purified against the carboxy-terminal domain of RcsF as previously described {Cho, 2014 #899} and used at a dilution of 1:20,000 in 1% skim milk in 50 mM Tris-HCl [pH 7.6], 0.15 M NaCl and 0.1% Tween 20 (TBST). Since we found that the anti-OmpA antibody only recognizes the periplasmic domain (data not shown), we used an antibody directed against loop 4 of the β -barrel of OmpA to detect OmpA₁₋₁₇₀. The anti-OmpA antibodies are gifts from the Lloubes and Bernstein laboratories {Cascales, 2002 #1517; Hussain, 2018 #2328}. These antibodies were used at dilutions of 1:20,000 and 1:10,000, respectively. The anti-His antibody conjugated to horseradish peroxidase (Qiagen) was used at a dilution of 1:5,000. The anti-Flag M2 monoclonal antibody (F1804, Sigma) was used at a dilution of 1:20,000.

***In vivo* 3,3'-dithio-bis[sulfosuccinimidylpropionate] and bis(sulfosuccinimidyl)-suberate (BS3) crosslinking**

DTSSP and BS3 (CovaChem) are bifunctional primary amine crosslinkers; DTSSP contains a disulfide bond in its spacer arm. *In vivo* crosslinking has been performed as described previously {Cho, 2014 #899}. The media used are MOPS-glucose minimal medium {Neidhardt, 1974 #2339} (Figure 3) and LB (Figure 5)

β -galactosidase assay

E. coli cells were grown in MOPS-glucose minimal medium {Neidhardt, 1974 #2339} or LB to mid-log phase ($OD_{600} = 0.4-0.6$). β -galactosidase activity was measured as described previously {Zhang, 1995 #2346}.

Expression and purification of RcsF, OmpA₁₈₆₋₃₂₅ and of the periplasmic domain of IgaA

Expression and purification of RcsF with a C-terminal His-tag were performed as previously described {Leverrier, 2011 #459}. *E. coli* BL21(DE3) cells harboring pKiD5 expressing N-terminal Strep-tagged OmpA₁₈₆₋₃₂₅ were grown at 37°C in M9-glucose minimal medium containing 1 g/L $^{15}\text{NH}_4\text{Cl}$ (99%, ^{15}N ; Eurisotop) to uniformly label the protein with ^{15}N . The expression was induced by adding 1 mM IPTG at an OD_{600} of 0.5. After a 5h induction, cells were harvested by centrifugation and resuspended in 20 mL of lysis buffer (20 mM Tris-HCl, 500 mM NaCl [pH8], containing a protease inhibitor cocktail (Complete®, Roche)). Re-suspended cells were stored at -20 °C. Frozen cells were thawed on ice

and lysed by two passages through a French pressure cell at 1,500 psi. The soluble fraction was isolated after centrifugation for 1h at 40,000 x *g* at 4 °C. The supernatant was filtered through 0.45 µm filters and loaded onto a 5 mL Strep-Tactin column (IBA, Lifesciences), previously equilibrated in buffer A (50 mM NaH₂PO₄, 100 mM NaCl [pH7.0]). After a washing step with buffer A, elution was performed with buffer A supplemented with 2.5 mM D-desthiobiotin. The sample was then further purified using size-exclusion chromatography on a HiLoad 16/60 Superdex 75 column (GE Healthcare) using buffer A. To express IgaA₃₆₁₋₆₅₄ with a C-terminal His-tag, *E. coli* SHuffle T7 cells harboring pSC211 were grown at 37°C in LB. Expression of the protein was induced by adding 1 mM IPTG at an OD₆₀₀ of 0.5. After a 5h induction, cells were harvested by centrifugation and resuspended in 20 mL of lysis buffer. Re-suspended cells were stored at -20 °C. Frozen cells were thawed on ice and processed as explained above for Strep-tagged OmpA₁₈₆₋₃₂₅. IgaA₃₆₁₋₆₅₄ was purified using Ni-NTA agarose beads (5 mL; IBA Lifescience), previously equilibrated in buffer B (20 mM NaH₂PO₄, 150 mM NaCl [pH7.5]). After washing the resin with buffer B supplemented with 20 mM imidazole, proteins were eluted with five column volumes of buffer B supplemented with 200 mM imidazole. As a final purification step, a size-exclusion chromatography was performed using a HiLoad 16/60 Superdex 200 column (GE Healthcare) with buffer B. Purity was checked *via* SDS-PAGE with Coomassie Staining and concentration was performed using Vivaspin Turbo apparatus (Sartorius) with a 5 kDa molecular weight cut-off.

NMR titration experiments of ^{15}N -labelled OmpA₁₈₆₋₃₂₅ with RcsF

50 μM ^{15}N -labelled OmpA₁₈₆₋₃₂₅ in buffer A at 25°C was titrated with increasing concentrations of unlabelled RcsF in buffer A to reach 2 and 10 RcsF/OmpA₁₈₆₋₃₂₅ molar ratios. To follow ^{15}N - ^1H resonances chemical shifts perturbations, ^{15}N - ^1H BEST-TROSY-HSQC correlation experiments were recorded at 25°C using Bruker AVANCE spectrometer operating at 700 MHz proton frequency equipped with a TCI cryoprobe {Favier, 2011 #2330}. OmpA₁₈₆₋₃₂₅ assignments were transposed from BMRB entry 25030. Chemical shift perturbations (CSP) were calculated on a per-residue basis for the highest substrate-to-protein ratio as described previously {Egan, 2018 #2331}. Spectra were processed with Topspin 3.57 (Bruker) and analyzed with ccpnmr 3 (<https://www.ccpn.ac.uk>).

Western blot analysis

Quantitation of the intensity of the bands was done by optical densitometry and analyzed using ImageQuant TL processing software (ImageQuant TL v8.1.0.0, GE Healthcare). After background correction, the values were normalized by the intensity of non-specific bands detected by the polyclonal antibody.

Biolayer interferometry

Biolayer Interferometry Experiments (BLI) were recorded on an OctetRED96e (ForteBio) using streptavidin (SA) biosensors (ForteBio). To biotinylate RcsF, RcsF-His (5mg/mL in 0.1 M MES [pH 5.5]) was incubated with Biotin-LC-Hydrazide (1.25 mM, final concentration; Sigma) and N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide (EDC; 6.5 mM, final concentration) during 2h at 22°C under agitation. Biotinylated RcsF was dialysed against buffer C (10 mM Hepes, 150 mM NaCl [pH7.5]) and immobilized at 5 µg/mL onto SA tips in buffer C supplemented with 0.02% Tween-20 to reach ~3.5 nM of immobilization level. RcsF-loaded biosensors were dipped into different concentrations of the periplasmic domain of IgaA (5.9 nM to 30 nM) in buffer C at 23°C. Kinetics were recorded with 1000 sec association and 1000 sec dissociation phases, and repeated 4 times with 10mM HCl pulses (18 sec in total) used for regeneration between cycles. Sensorgrams were subtracted for contribution of buffer alone and binding of non-functionalized biosensors. Kinetic analysis of the data was performed using 1:1 interaction model in the ForteBio data analysis software. K_D obtained from 4 independent injection series were averaged and produced a K_D of 1.6 nM with a standard deviation of 0.3.

Estimation of the expression levels of IgaA

Overexpressed IgaA has a triple Flag tag (IgaA-Flag₃). To compare the expression levels of IgaA to those of OmpA, we generated a triple Flag-tagged version of OmpA (OmpA-Flag₃); this variant was expressed from an IPTG-inducible plasmid in the *ompA* strain. If we compare the intensity of the signal corresponding to OmpA-Flag₃ detected either by the anti-OmpA or the anti-Flag antibodies, we estimate

the anti-Flag antibodies to be ~ 25 times more sensitive than the anti-OmpA antibodies (lanes 1-4 in Figure 5 – figure supplement 1). With this value in hand, we can now compare the expression levels of IgaA-Flag₃ to those of untagged OmpA produced from the chromosome and detected with the anti-OmpA antibodies (lanes 5-9 in Figure 5 – figure supplement 1); because the intensities of OmpA and IgaA-Flag₃ in lane 8 are similar, we estimate that IgaA-Flag₃ is 25 times less abundant than OmpA when IgaA is expressed with 250 μ M IPTG; likewise, we estimate the levels of IgaA-Flag₃ to be $\sim 1/125$ (0.8%) of those of OmpA when IgaA is expressed with 50 μ M IPTG. Because the concentration of OmpA is ~ 1 mM, we estimate the concentration of overexpressed IgaA to be 40 and 8 μ M, respectively.

Molecular modelling

HADDOCK 2.4 web server {van Zundert, 2016 #1; Wassenaar, 2012 #2} was used for protein-protein docking using the structures 2MQE for OmpA-CTD {Ishida, 2014 #1082} and 2L8Y for RcsF {Rogov, 2011 #1186}. Six residues identified from the crosslinking experiments were considered as active during the docking calculations: 242, 246 and 248 for OmpA and 45, 79 and 89 for RcsF. The resulting clusters were inspected visually, and the one compatible with the interaction between the periplasmic domain of OmpA and the peptidoglycan {Park, 2012 #2179} was selected for further refinement using molecular dynamics. Molecular dynamics simulations were carried out with GROMACS version 2020.1 {Abraham, 2015 #3} using the OPLS-AA {Jorgensen, 1996 #4} force field. Each system was energy-minimized until convergence using a steepest descents algorithm. Molecular dynamics with position restraints was then performed (50 ps NVT and 50 ps NPT), followed by the production run of 50 ns. During the position restraints and production runs, the V-rescale and Parrinello-Rahman methods were

used for temperature and pressure coupling, respectively. Electrostatics were calculated with the particle mesh Ewald method. The P-LINCS algorithm was used to constrain bond lengths, and a time step of 2 fs was used throughout.

Estimation of the concentrations of OmpA, RcsF and IgaA in the periplasm

According to {Li, 2014 #1288}, each cell contains ~200,000 molecules of OmpA in rich media, which corresponds to $\sim 3.448 \times 10^{-19}$ mol (200,000/Avogadro constant). If we consider the volume of an *E. coli* cell to be 10^{-18} m³ (10^{-15} L) and compare it to a cube with 1 μ m edges (*E. coli* has 0.5 μ m in width and 2 μ m in length; EcoliWiki ecoliwiki.net/colipedia/index.php/Escherichia_coli), we calculate that the cellular concentration of OmpA is $\sim 3.448 \times 10^{-4}$ M. The volume of the envelope being ~30% of the total cell volume {Stock, 1977 #2332}, we calculate that the concentration of the C-terminal domain of OmpA in the periplasm is ~1 mM. If we perform the same calculations for RcsF and IgaA (~3,000 and ~200 copies/cell, respectively), we find that their periplasmic concentrations are ~15 μ M and ~1 μ M, respectively.

OmpA functions as a buffer for RcsF

The equilibrium dissociation constants for the OmpA-RcsF and IgaA-RcsF complexes are:

$$K_d^{\text{OmpA-RcsF}} = \frac{[\text{OmpA}][\text{RcsF}]}{[\text{OmpA-RcsF}]} \quad (1)$$

$$K_d^{\text{IgaA-RcsF}} = \frac{[\text{IgaA}][\text{RcsF}]}{[\text{IgaA-RcsF}]} \quad (2).$$

If we divide (1) with (2), we obtain:

$$\frac{K_d^{\text{OmpA-RcsF}}}{K_d^{\text{IgaA-RcsF}}} = \frac{[\text{OmpA}][\text{IgaA-RcsF}]}{[\text{IgaA}][\text{OmpA-RcsF}]} \quad (3).$$

If we replace $\frac{K_d^{\text{OmpA-RcsF}}}{K_d^{\text{IgaA-RcsF}}}$ by K_d^{ratio} , then we can rearrange equation (3) to

$$\frac{[\text{IgaA-RcsF}]}{[\text{IgaA}]} = \frac{K_d^{\text{ratio}}[\text{OmpA-RcsF}]}{[\text{OmpA}]} \quad (4).$$

Under physiological conditions, we estimate the concentration of IgaA to be $\sim 1 \mu\text{M}$ (see above). Thus, because $[\text{IgaA}] + [\text{IgaA-RcsF}] = 1 \mu\text{M}$, equation 4 can be successively rearranged to

$$\frac{[\text{IgaA-RcsF}]}{1 - [\text{IgaA-RcsF}]} = \frac{K_d^{\text{ratio}}[\text{OmpA-RcsF}]}{[\text{OmpA}]} \quad (5), \quad \frac{1 - [\text{IgaA-RcsF}]}{[\text{IgaA-RcsF}]} = \frac{[\text{OmpA}]}{K_d^{\text{ratio}}[\text{OmpA-RcsF}]} \quad (6),$$

$$\frac{1}{[\text{IgaA-RcsF}]} = \frac{[\text{OmpA}]}{K_d^{\text{ratio}}[\text{OmpA-RcsF}]} + 1 \quad (7), \quad \frac{1}{[\text{IgaA-RcsF}]} = \frac{[\text{OmpA}] + K_d^{\text{ratio}}[\text{OmpA-RcsF}]}{K_d^{\text{ratio}}[\text{OmpA-RcsF}]} \quad (8),$$

yielding:

$$[\text{IgaA} - \text{RcsF}] = \frac{K_d^{\text{ratio}}[\text{OmpA-RcsF}]}{[\text{OmpA}] + K_d^{\text{ratio}}[\text{OmpA-RcsF}]} \quad (9).$$

In equation (1), $[\text{OmpA}]$, which is $\sim 1000 \mu\text{M}$, can be considered as a constant.

Therefore, equations (1) and (9) become:

$$[\text{OmpA} - \text{RcsF}] = \frac{1000 [\text{RcsF}]}{K_d^{\text{OmpA-RcsF}}} \quad (10)$$

$$[\text{IgaA} - \text{RcsF}] = \frac{K_d^{\text{ratio}}[\text{OmpA-RcsF}]}{1000 + K_d^{\text{ratio}}[\text{OmpA-RcsF}]} =$$

$$\frac{[\text{OmpA-RcsF}]}{1000/K_d^{\text{ratio}} + [\text{OmpA-RcsF}]} \quad (\text{equation 3 in the discussion}) = \frac{\frac{1000 [\text{RcsF}]}{K_d^{\text{OmpA-RcsF}}}}{1000/K_d^{\text{ratio}} + \frac{1000 [\text{RcsF}]}{K_d^{\text{OmpA-RcsF}}}}$$

(11).

Thus, from equation (11), we conclude that whereas $[\text{OmpA-RcsF}]$ increases linearly to $[\text{RcsF}]$, $[\text{IgaA-RcsF}]$ increases proportionally, but not linearly, to $[\text{RcsF}]$.

Thus, OmpA functions as a buffer for RcsF.

Analysis of protein structures

Protein structures were downloaded from the Protein Data Bank (<http://www.rcsb.org>; PDB codes are indicated) and visualized using PyMOL Molecular Graphics System (Version 2.3.4, Schrödinger, LLC). FASTA protein sequences were downloaded from Uniprot (<http://www.uniprot.org/>).

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Competing interests

The authors declare no competing interests.

Supplementary Tables

Supplementary Table 1. Strains used in this study.

Strains	Relevant Genotype or Features	Source or notes
XL1-Blue	<i>endA1 gyrA96(nal^R) thi-1 recA1 relA1 lac glnV4'</i> F' [::Tn10 <i>proAB</i> ⁺ <i>lacI</i> ^q Δ(<i>lacZ</i>)M15] <i>hsdR17</i> (r _K ⁻ m _K ⁺)	Stratagene
BL21 (DE3)	<i>E. coli</i> str. B F ⁻ <i>ompT gal dcm lon hsdS_B</i> (r _B ⁻ m _B ⁻) λ(DE3 [<i>lacI lacUV5-T7p07 ind1 sam7 nin5</i>]) [<i>malB</i> ⁺] _{K-12} (λ ^S)	Novagen
SHuffle T7	<i>fhuA2 lacZ::T7 gene1 [lon] ompT ahpC gal</i> λatt::pNEB3-r1-cDsbC (<i>SpecR, lacI_q</i>) Δ <i>trxB</i> <i>sulA11 R(mcr-73::miniT-0--TetS)2 [dcm] R(zgb-</i> <i>210::Tn- --TetS) endA1 Δgor Δ(mcrC-</i> <i>mrr)114::IS10</i>	New England Biolabs (NEB)
DH300	<i>rprA-lacZ</i> MG1655 (<i>argF-lac</i>) <i>U169</i>	{Majdalani, 2002 #1116}
Keio collection single mutants	Δ <i>rcsF</i> :: <i>kan</i> , Δ <i>ompA</i> :: <i>kan</i>	{Baba, 2006 #322}
PL358	DH300 Δ <i>rcsF</i>	{Cho, 2014 #899}
SEN588	DH300 Δ <i>ompA</i> :: <i>kan</i>	{Cho, 2014 #899}
PR46	DH300 Δ <i>ompA</i>	this study
SEN589	PL358 Δ <i>ompA</i> :: <i>kan</i>	{Cho, 2014 #899}
SEN524	PL358 Δ <i>ompR</i> :: <i>kan</i>	this study
SEN550	DH300 pSC237	this study

SEN860	DH300 $\Delta ompA::cat, sacB$	this study
SEN861	PL358 $\Delta ompA::cat, sacB$	this study
SEN1194	SEN860 $ompA-6\times His$	this study
SEN905	SEN861 $ompA-6\times His$	this study
SEN900	SEN860 $ompA_{TH189}-6\times His$	this study
SEN901	SEN860 $ompA_{TH243}-6\times His$	this study
SEN962	SEN900 $\Delta rcsF::kan$	this study
SEN964	SEN901 $\Delta rcsF::kan$	this study
SEN856	DH300 $\Delta ompA_{171-325}::kan$	this study
SEN857	PL358 $\Delta ompA_{171-325}::kan$	this study
SEN858	DH300 $\Delta ompA_{171-325}$	this study
SEN859	PL358 $\Delta ompA_{171-325}$	this study
SEN1089	PL358 pSUP-Mb_DiZPK-RS and pSC253	this study
SEN1091	PL358 pSUP-Mb_DiZPK-RS and pSC253(Q79X)	this study
SEN1094	PL358 pSUP-Mb_DiZPK-RS and pSC253(P116X)	this study
SEN1109	PL358 pSUP-Mb_DiZPK-RS and pSC253(K98X)	this study
SEN1113	PL358 pSUP-Mb_DiZPK-RS and pSC253(E110X)	this study
SEN1115	PL358 pSUP-Mb_DiZPK-RS and pSC253(Q121X)	this study
SEN1129	PL358 pSUP-Mb_DiZPK-RS and pSC253(R21X)	this study
SEN1130	PL358 pSUP-Mb_DiZPK-RS and pSC253(Q28X)	this study

SEN1131	PL358 pSUP-Mb_DiZPK-RS and pSC253(Q33X)	this study
SEN1134	PL358 pSUP-Mb_DiZPK-RS and pSC253(R45X)	this study
SEN1136	PL358 pSUP-Mb_DiZPK-RS and pSC253(N54X)	this study
SEN1141	PL358 pSUP-Mb_DiZPK-RS and pSC253(R89X)	this study
SEN1275	SEN589 pSUP-Mb_DiZPK-RS and pSC253(R89X)	this study
SEN1276	SEN524 pSUP-Mb_DiZPK-RS and pSC253(R89X)	this study
SEN1689	SEN589 pSUP-Mb_DiZPK-RS and pSC253(K98X)	this study
SEN1690	SEN589 pSUP-Mb_DiZPK-RS and pSC253(E110X)	this study
SEN1378	PR46 pSUP-Mb_DiZPK-RS and pPR21	this study
SEN1381	PR46 pSUP-Mb_DiZPK-RS and pPR21(R242X)	this study
SEN1552	DH300 pSC231	this study
SEN1562	PR46 pSC237	this study
SEN1565	PL358 pSC237	this study
PR44	PR46 pPR4	this study
JLE60	SEN860 $\Delta ompA::ompX-ompA_{171-325}$	this study
JLE61	SEN861 $\Delta ompA::ompX-ompA_{171-325}$	this study
KiD003	BL21(DE3) pKiD5	this study
KiD083	PR46 pSUP-Mb_DiZPK-RS and pPR21(D246X)	this study
KiD084	PR46 pSUP-Mb_DiZPK-RS and pPR21(Y248X)	this study
KiD123	DH300 pKiD22	this study

KiD124	PR46 pKiD22	this study
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Supplementary Table 2. Primers used in this study.

Name	Sequence (5' to 3')
OmpA(NcoI) F	gagaCCATGGgaaaaaagacagc
OmpA-his (XbaI) R	gagaTCTAGAtcagtggtggtggtggtggtgagcctgcggctgagttac
ompA_delCmSB F	atattcatggcgtatTTTGGATGATAACGAGGCGCAAAAAAAAATGAGACGTTGATCGGCACG
ompA_delCmSB R	aaaaccccgccagcagcggggTTTTTctaccagacgagaacATCAAAGGGAAGAACTGTCCA
cmSB to OmpA_F	atattcatggcgtatTTTGGATGATAACGAGGCGCAAAAAATGAAAAAGACAGCTATCGCG
cmSB to OmpAhis_R	aaaaccccgccagcagcggggTTTTTctaccagacgagaacTTAGTGGTGGTGGTGGTGGTGAGCCTG
ompAc_delKm F	gcactcgccggacaacggcatgctgagcctgggtgttctaccgtttcTAAattccggggatccgtcgacc
ompAc_delKm R	gcagcggggTTTTTctaccagacgagaacttaagcctgcggctgagttactgtaggctggagctgcttcg
OmpX(NcoI)F	GAGccatgggtAAAAAAATTGCATGTCTTTC
OmpX-OmpAc_F	GTTGGTTACCGCTTCggtcagggcgaagca
OmpX-OmpAc_R	gccctgaccGAAGCGGTAACCAACACCGGC
JLEo16-R-XbaI OmpACt	GGGtctagactaagcctgcggctgagttacaacg
JLEo20-F-Chrom-OmpX	atattcatggcgtatTTTGGATGATAACGAGGCGCAAAAAATGAAAAAATTGCATGTC
JLEo22-R-Chrom-OmpACt	aaaaccccgccagcagcggggTTTTTctaccagacgagaacctaagcctgcggctgagttacaacg
ompA -100	cgccaggggtgctcggcataagccg

ompA +100	tcttctctgaagcaggatctgcaggc
189VPRGS thr_F	gctccggcaccggaagtaGTGCCGCGCGGATCCcagaccaagca cttcactc
189VPRGS thr_R	gagtgaagtgcttggctctgGGATCCGCGCGGCACtacttccggtgc cggagc
243LVPR thr_F	ggttacaccgaccgcataCTGGTGCCGCGCggttctgacgcttaca c
243LVPR thr_R	gttgtaagcgtcagaaccGCGCGGCACCAGgatgcggtcggtgtaac c
KiDo14-F-NdeI-Strep-OmpACTI	GGGcatATGTGGAGCCACCCGCAGTTCGAAAAAgca ccggaagtacagacc
KiDo15-R-SacI-Rbs-OmpACTD	GGGGAGCTCGGCTATATCTCCTTCTTAAAGTTAA ACAAAATTGCGGCCGCttaagcctgcggctgagttac
lacIq NsiI_F	CGAAGCGGCATGCATTTAC
flag3-KpnI_R	gagagggtaccCTACTTGTCATCGTCATC
IgaA(NcoI) F	gagaCcatggggAGCACCATTGTG
IgaA XbaI flag3 R	gggTCTAGAttcgataaggctttctgaagggg
OA_stopTGA_F	actcagccgcaggctTGAcaccaccaccaccac
OA_stopTGA_R	gtggtggtggtggtgTCAagcctgcggctgagt
Palss(NcoI)F	gagCCatgGGAcaactgaacaaagtgctg
Palss-OmpAc_F	GCATGTTCTTCCgaagcagc
Palss-OmpAc_R	ggagctgcttcGGAAGAACATGCCGCAAT
OmpA_stop_Flag3(KpnI)R	ggtaccCTACTTGTCATCGTCATCCTTGTAATCggatc aagcctgcggctgagttac
AA62_RcsF_R21x_F	ctgttccatgttaagcTAGtcccctgtcgaacc

AA63_RcsF_R21x_R	ggttcgacaggggaCTAgcttaacatggaacag
AA64_RcsF_Q28x_F	ctgtcgaacccgttTAGagcactgcaccccagc
AA65_RcsF_Q28x_R	gctggggtgcagtgcCTAaacgggttcgacag
AA66_RcsF_Q33x_F	CAAagcactgcacccTAGccgaaagcggagc
AA67_RcsF_Q33x_R	gctccgctttcggCTAagggtgcagtgcTTG
AA72_RcsF_N54x_F	cCGAatttataccTAGgcagaagaattagtcggc
AA73_RcsF_N54x_R	gccgactaattcttctgcCTAaggtataaatTCGg
AA74_RcsF_R89x_F	cattccaaccgcaTAGaagcggatgcaaatcaacg
AA75_RcsF_R89x_R	cgttgatttgcacccgcttCTAtgcggttggaatg
RcsF_R45x_F	aaaccgaaagcgccgTAGgccacgccggtccga
RcsF_R45x_R	tcggaccggcgtggcCTAcggcgctttcggttt
RcsF_Q79x_F	tgccaggcctctaataAGgactctccgcgagc
RcsF_Q79x_R	gctcggcggagagtcCTAattagaggcctggca
RcsF_K98x_F	atgcaaatcaacgcctctTAGatgaaagccaatgctgta
RcsF_K98x_R	tacagcattggctttcatCTAagaggcggtgatttgc
RcsF_E110x_F	ttactgcatagctgcTAGgtcaccagcggtagc
RcsF_E110x_R	cgtaccgctggtgacCTAgcagctatgcagtaa
RcsF_P116x_F	gtcaccagcggtagcTAGggctgctatcgtagc
RcsF_P116x_R	ctgacgatagcagccCTAcgtaccgctggtgac
RcsF_Q121x_F	acgccaggctgctatcgtTaggctgtatgtatcggtt

RcsF_Q121x_R	aaccgatacatacagcctAacgatagcagcctggcgt
RcsF_R242x_F	ctgggttacaccgacTAGatcggttctgacgt
RcsF_R242x_R	agcgtcagaaccgatCTAgtcggtgtaaccag
KiDo87_OmpA_D246x_F	catcggttctTAGgcttacaaccagg
KiDo88_OmpA_D246x_R	gttgtaagcCTAagaaccgatgcgg
KiDo89_OmpA_Y248x_F	ttctgacgctTAGaaccagggtc
KiDo90_OmpA_Y248x_R	cctgggttCTAagcgtcagaaccg

Supplementary Table 3. Plasmids used in this study.

Plasmids	Features	Source or notes
pDSW204	IPTG-regulated modified P_{trc} , ampicillin	{Weiss, 1999 #2333}
pAM238	IPTG-regulated P_{lac} , pSC101-based, spectinomycin	{Gil, 1991 #1056}
pBAD18	Arabinose regulation, ampicillin	{Guzman, 1995 #323}
pSup-Mb-DIZPK-RS	PylRS, tRNA ^{Pyl} _{CUA} opt, p15A origin, chloramphenicol	{Zhang, 2011 #806}
pSIM5-tet	pSC101-based, $repA^{ts}$, tetracycline	{Koskiniemi, 2011 #2330}
pCP20	FLP^+ , λ cI857 ⁺ , λ _{PR} Rep ^{ts} , ampicillin, chloramphenicol	{Cherepanov, 1995 #1015}
pSC202	pAM238 with RcsF	{Cho, 2014 #899}
pSC253	pBAD18 with RcsF	this study
pPR11	pDSW204 with OmpA-6 $\times$ His	this study
pPR11 _{189thrombin}	pDSW204 with OmpA(189-Val-Val-Pro-Arg-Gly-Ser-Gln-190)-6 $\times$ His	this study
pPR11 _{243thrombin}	pDSW204 with OmpA(243-Ile-Leu-Val-Pro-Arg-Gly-244)-6 $\times$ His	this study
pJLE17-OmpX-OmpA ₁₇₁₋₃₂₅	pDSW204 with OmpA _X	this study
pKiD5	pET21a with strep-OmpA ₁₈₆₋₃₂₅	this study

pMER77	pDSW204 with Flag ₃	{Hemmis, 2011 #2334}
pSC231	pAM238 with LacI ^q , the modified P _{trc} , and Flag ₃	this study
pPR4	pSC231 with OmpA	this study
pSC237	pSC231 with IgaA	this study
pPR21	pDSW204 with OmpA	this study
pKiD22	pDSW204 with OmpA _{Pal}	this study

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Chapter IV: Hydrophobic pockets in proteins are molecular magnets for the photo-crosslinking amino acid *p*Bpa.

Hydrophobic pockets in proteins are molecular magnets for the photo-crosslinking amino acid *pBpa*

Running title: *A note of caution when using cross-linking with pBpa*

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Abstract

Diverse methods exist to investigate protein interaction networks. Site-specific photo-crosslinking is an increasingly popular technique in which a photoactivable amino acid, such as *p*-benzoyl-L-phenylalanine (*p*Bpa), is genetically inserted in a protein of interest to trap interacting partners. The *Escherichia coli* β -barrel protein OmpA forms a complex with the outer membrane lipoprotein RcsF. To test the current model for the OmpA-RcsF interaction, we inserted *p*Bpa at selected sites in RcsF and identified where the RcsF variants bind to OmpA. Unexpectedly, we found that all *p*Bpa-containing RcsF variants bind to the same hydrophobic pocket in OmpA, regardless of the location of *p*Bpa in RcsF. Altering the chemical properties of this pocket decreased its ability to bind *p*Bpa, without preventing the formation of the OmpA-RcsF complex. Remarkably, the hydrophobic pocket of OmpA was found to bind *p*Bpa when inserted in other proteins from the bacterial cell envelope. Altogether, our results indicate that the hydrophobic pocket of OmpA functions as a molecular magnet attracting *p*Bpa regardless of its location in partner proteins, therefore precluding the reliable identification of interaction interfaces on OmpA using photo-crosslinking with *p*Bpa. Because similar hydrophobic pockets with affinity for *p*Bpa are likely to exist in proteins other than OmpA, great care should be taken when mapping the zone of interaction between two proteins using *p*Bpa-based site-specific photo-crosslinking.

KEYWORDS: OmpA, RcsF, protein-protein interaction, β -barrel, photoaffinity

Introduction

Proteins do not function in isolation but rather as components of complex networks involving multiple proteins. A large array of methods is available to investigate the countless protein interactions that take place in living cells and control biological processes {Santin, 2019 #2325}. An increasingly popular method is site-specific photo-crosslinking in which an artificial, photo-crosslinkable amino acid is genetically incorporated into a protein of interest in response to the amber codon (TAG) with the help of an exogenous orthogonal tRNA/aminoacyl-tRNA synthetase pair {Kauer, 1986 #2349;Young, 2010 #2329;Chin, 2002 #1016} (**Figure 1A**). Upon UV irradiation, the unnatural amino acid forms a covalent bond with protein partner(s) present nearby, which can be identified using mass spectrometry. Commonly-used photoactivable amino acids are *p*-benzoyl-L-phenylalanine (*p*Bpa), a phenylalanine analog, and *N*^ε-((3-(3-methyl-3H-diazirin-3-yl)propyl)carbamoyl)-L-lysine (DiZPK), a lysine analog. Site-specific photo-crosslinking, which was originally developed in *Escherichia coli* {Chin, 2002 #2351}, has now been applied to *Saccharomyces cerevisiae* {Chin, 2003 #2352} and mammalian cells {Hino, 2005 #2353;Liu, 2007 #2354;Wang, 2007 #2355}. The success of this method stems from the fact that it offers several advantages over techniques based on conventional chemical crosslinkers. For example, because *p*Bpa and DiZPK only react with residues that are in very close proximity (between 3 to 14 Å) {Farrell, 2005 #2331;Yang, 2016 #2332}, the risk of trapping non-specific interactions is lowered. In addition, because any residue in the protein of interest can be replaced by an artificial amino acid, the interaction interface between this protein and its partner(s) can be precisely established.

In the framework of our research on proteins involved in the assembly and maintenance of the *E. coli* outer membrane, we used a *p*Bpa-based site-specific photo-crosslinking approach to map the interface between the β -barrel OmpA and the lipoprotein RcsF. This research led us to discover that *p*Bpa has an intrinsic affinity for an hydrophobic pocket present in OmpA, which can lead to the identification of non-specific, artifactual interaction sites between proteins of interest and OmpA. As *p*Bpa-based site-specific photo-crosslinking is increasingly used, even in organisms other than *E. coli*. As hydrophobic pockets with *p*Bpa affinity are likely to exist in proteins other than OmpA, it is advisable to interpret the data obtained using *p*Bpa with caution.

Results and Discussion

RcsF variants harboring *p*Bpa at three different positions cross-link to the same site in OmpA.

One of our goals is to understand how the outer membrane lipoprotein RcsF activates the Rcs stress signaling pathway when the integrity of the *E. coli* cell envelope is decreased {Wall, 2018 #2333}. RcsF forms a complex with the periplasmic domain of the abundant β -barrel protein OmpA (**Figure 1(B-C-D)**) {Konovalova, 2014 #900;Cho, 2014 #899;Dekoninck, 2020 #2324}. This complex is important for fine-tuning Rcs activity {Dekoninck, 2020 #2324}. In a recent study, we used DiZPK to map the binding interface between OmpA and RcsF. Informed by the results from the site-specific photo-crosslinking, as well as from NMR titration and targeted proteolysis experiments, we used protein-protein docking and molecular dynamics simulations to build a structural model for the OmpA-RcsF complex (**Figure 1(D)**) {Dekoninck, 2020 #2324}.

Here, we decided to challenge the structural model above by introducing *p*Bpa at selected positions in RcsF (**Figure S1**) and mapping the cross-links using liquid chromatography tandem mass spectrometry (LC-MS/MS). We first selected RcsF_{K40X} and RcsF_{Q79X} for LC-MS/MS analysis because these variants show strong UV-dependent cross-linking with OmpA (**Figure S1**). For the experiments, we used a fully functional {Cho, 2014 #899} version of RcsF with a His-tag at the C-terminus (**Figure S2(A)**). After UV illumination, samples were purified using Ni-magnetic beads and analyzed by SDS-PAGE (**Figure S2(B)**). The UV-crosslinked RcsF-OmpA complexes were then cut from the gel, digested with trypsin and analyzed by LC-MS/MS (**Figure S2(C) and S3**). Crosslinks were mapped in the

MS dataset by the software “Crossfinder”, which we developed specifically for this purpose {Mueller-Planitz, 2015 #2343;Forne, 2012 #2344}.

Unexpectedly, both RcsF_{K40X} and RcsF_{Q79X} covalently crosslinked to the same target peptide GIPADK (**Figure S2(C) and S3**). This peptide is located in the periplasmic domain of OmpA (**Figure 1(C-D)**), where it forms two hydrophobic holes with a nose-like bulged region (**Figure 1(E)**). This result was surprising because K40 and Q79 are distantly located from each other in the structure of RcsF: K40 is present in a disordered linker found at the N-terminus of the protein, while Q79 is on a loop located at the tip of the globular domain (**Figure 1(B)**) {Rogov, 2011 #1186;Leverrier, 2011 #459}. Also, according to our model {Dekoninck, 2020 #2324}, Q79 should not bind to the GIPADK region (**Figure 1(D)**). Intrigued by these results, we decided to map the cross-links with a third RcsF variant, RcsF_{G60X}, in which *p*Bpa is inserted in a site opposite of Q79 in the globular domain of RcsF (**Figure 1(B)**). To our great surprise, RcsF_{G60X} also photo-crosslinked to the same GIPADK motif in OmpA (**Figure 1(D), S4(A-B) and S5**).

The GIPADK motif forms a flexible, hydrophobic pocket able to accommodate *p*Bpa.

The finding that *p*Bpa, regardless of its location in RcsF, binds the GIPADK motif suggests that this motif has an intrinsically high affinity for *p*Bpa. Also, ligand-protein docking experiments indicate that the GIPADK motif should be able to accommodate a *p*Bpa-containing peptide (**Figure S6(A)**). We thus mutated the GIPADK motif to find out how this would affect the formation of UV-crosslinked complexes between OmpA and the *p*Bpa-containing RcsF variants.

Instead of introducing random mutations that could disrupt the structure of OmpA and prevent formation of the OmpA-RcsF complex, we replaced the GIPADK motif by two sequences borrowed from Pal and YiaD (GVSADQ and GVDASR, respectively), two *E. coli* lipoproteins that are structural homologs of the C-terminal domain of OmpA (**Figure S6(B)**) {Parsons, 2006 #1163;Tachikawa, 2011 #2336}.

The genes encoding OmpA_{Pal} and OmpA_{YiaD} were inserted on the chromosome in place of *ompA*. In contrast to $\Delta ompA$ mutant cells, bacteria producing OmpA_{Pal} and OmpA_{YiaD} had a wild-type phenotype in terms of activation of the Rcs system and permeability of the cell envelope (**Figure S7**), indicating that OmpA_{Pal} and OmpA_{YiaD} are functionally equivalent to the wild-type protein. Importantly, both OmpA_{Pal} and OmpA_{YiaD} remained able to form complexes not only with wild-type RcsF (**Figure S7(A)**) but also with the *pBpa*-containing variant RcsF_{Q79X}, as observed by DTSSP cross-linking (**Figure 2(A)**). Thus, mutating the GIPADK motif did not alter the properties of OmpA nor its ability to interact with RcsF.

We then tested whether OmpA_{Pal} and OmpA_{YiaD} were still able to form UV-dependent complexes with the *pBpa*-containing RcsF_{Q79X} variant. As shown in **Figure 2(A)**, we observed a significant decrease in UV-dependent complex formation with both OmpA_{Pal} and OmpA_{YiaD}; we noted that the decrease was particularly severe with OmpA_{Pal}. Importantly, UV-dependent RcsF-OmpA_{Pal} and RcsF-OmpA_{YiaD} complexes were observed when a DiZPK-containing RcsF_{Q79X} variant was produced. Thus, mutating residues involved in the formation of the GIPADK motif displayed by OmpA does not prevent formation of the OmpA-RcsF complex but specifically alters photo-crosslinking with the *pBpa*-containing RcsF

variant, most likely by changing the structural features of the GIPADK region and decreasing its affinity for *p*Bpa. Supporting this, molecular dynamics showed that replacing the GIPADK motif by GVSADQ (OmpA_{Pal}) or GVDASR (OmpA_{YiaD}) decreases the flexibility of the region and makes its surface more negatively charged (**Figure 2(B)**).

The GIPADK pocket of OmpA attracts *p*Bpa when inserted into other proteins.

Strikingly, we found that OmpA (harboring its native GIPADK hydrophobic pocket) attracts *p*Bpa not only in RcsF but also in other proteins. First, during our work on the OmpC-Mla system, which is important for maintaining outer membrane lipid asymmetry {Malinverni, 2009 #2337;Chong, 2015 #2328}, we replaced multiple residues of the periplasmic protein MlaC with *p*Bpa. Interestingly, two MlaC variants (MlaC_{Q157X} and MlaC_{T86X}) were found to crosslink to OmpA upon UV irradiation (**Figure S8(A-D)**). Substitution of the GIPADK motif of OmpA with the corresponding sequences from Pal and YiaD significantly reduced the formation of these complexes (**Figure 3(A)** and **Figure S8(E)**), thus strongly suggesting that, akin *p*Bpa-variants of RcsF, MlaC_{Q157X} and MlaC_{T86X} both crosslinked to the GIPADK motif of OmpA. Second, *Ranava et al.* {Ranava, 2021 #2327} recently reported the formation of UV-dependent complexes between OmpA and *p*Bpa-containing variants of DolP, an outer membrane lipoprotein; they even identified the GIPADK pocket as being part of the interaction interface. Accordingly, we found that the UV-dependent DolP-OmpA interaction was substantially decreased when a *p*Bpa-containing DolP variant was produced in cells expressing OmpA_{Pal} or OmpA_{YiaD} (**Figure 3(B)**). Finally, during our work on intrinsically-disordered linkers that are displayed by half *E. coli* outer membrane lipoproteins {El Rayes, 2021 #2326}, we observed the formation of UV-dependent complexes between *p*Bpa-containing variants of YedD and OmpA (**Figure 3(C)**). Here also, we observed less complex formation when the crosslinking experiments were repeated in cells expressing the OmpA mutants (**Figure 3(C)**).

Conclusion

Altogether, our results indicate that the hydrophobic pocket of OmpA functions as a molecular magnet attracting *p*Bpa regardless of its location in partner proteins, therefore precluding the reliable identification of interaction interfaces on this β -barrel. Because similar hydrophobic pocket with affinity for *p*Bpa are likely to exist in proteins other than OmpA, great care should be taken when mapping the zone of interaction between two proteins by *p*Bpa-based site-specific photocrosslinking. If the results indicate that the *p*Bpa-interacting interface is hydrophobic, confirming the data by using a different set of mutants would be advised.

Material and Methods

Bacterial strains and plasmids.

The bacterial strains and primers used in this study are listed in **Table S1** and **Table S3**, respectively. The parental *E. coli* strain DH300 is an MG1655 derivative that is deleted for the *lac* region and carries a chromosomal *rprA_P::lacZ* fusion at the λ phage attachment site to monitor Rcs activation {Majdalani, 2002 #1116}. Deletion mutants generated for this work (except the one for *ompA*, which was generated as explained below) were obtained by transferring the corresponding alleles from the Keio collection (kan^R) {Baba, 2006 #322} into DH300 or MG1655 {Majdalani, 2002 #1116} by P1 phage transduction and verified by PCR. To excise the resistance cassette, we used pCP20 as previously described {Datsenko, 2000 #438}. To insert *ompA-His* (encoding OmpA with six histidines at the C-terminus), *ompA_{Pal}-His*, and *ompA_{YiaD}-His* on the chromosome at the *ompA* locus, we performed λ -Red recombineering {Yu, 2000 #1268} with the use of the pSIM5-Tet plasmid {Koskiniemi, 2011 #2330}. First, a *cat-sacB* cassette, encoding chloramphenicol acetyl transferase (*cat*) and *SacB*, a protein conferring sensitivity to sucrose, was amplified from strain CH1990 using primers *ompA_delCmSB F* and *ompA_delCmSB R*. The resulting PCR product shared a 40-bp homology to the 5' UTR and 3' UTR of *ompA* at its 5' and 3' ends, respectively, and was used for λ -red recombineering {Yu, 2000 #1268}. We selected transformants for chloramphenicol resistance and verified that the *cat-sacB* cassette replaced the *ompA* gene by sequencing across the junctions. The *cat-sacB* cassette was subsequently replaced by one of the versions of *ompA* (*ompA-His*, *ompA_{Pal}-His*, or *ompA_{YiaD}-His*) using λ -Red recombineering and negative selection on sucrose-containing medium {Thomason, 2014 #2338; Gennaris, 2015 #1276}. *ompA_{His}*, *ompA_{Pal-His}* or *ompA_{YiaD-His}* were amplified by PCR using primers “cmSB to

OmpA_F” and “cmSB to OmpA_R” and plasmids pPR11, pPR11_{Pal}, and pPR11_{YiaD} (see below) as a template, respectively. The strains were finally verified by sequencing.

The plasmids used in this study are shown in **Table S2**. To generate pPR11, *ompA_{-His}* was PCR-amplified using OmpA(NcoI) F and OmpA-his(XbaI) R as primers and chromosomal *E. coli* DNA as template. The PCR product was then inserted into pDSW204 restricted with NcoI and XbaI, generating pPR11. To replace the GIPADK motif of OmpA by GVSADQ from Pal or GVDASR from YiaD, site-directed mutagenesis was performed using primers pal_GVSADQ_F and pal_GVSADQ_R, or yiaD_GVDASR_F and yiaD_GVDASR_R, respectively, on pPR11 yielding pPR11_{Pal} and pPR11_{YiaD}. pSC252, encoding RcsF-Flag-His, was constructed by digestion of pJS11{Cho, 2014 #899} with KpnI and HindIII and insertion of the generated fragment into pBAD18 {Guzman, 1995 #323}. To generate the *rcsF* variants containing an amber codon (TAG) at selected positions, site-directed mutagenesis was performed on pSC202 {Cho, 2014 #899}, pJS11 {Cho, 2014 #899}, and pSC252 using primers RcsF1-27. To generate pKiD41, DolP with a triple C-terminal FLAG tag (DolP-triple Flag) was PCR-amplified using primers KiDo231 and KiDo232, with flanking regions overlapping the pBAD18 sequence, and chromosomal *E. coli* DNA as template. In parallel, we amplified the pBAD18 plasmid using primers KiDo233 and KiDo234, with flanking regions overlapping the DolP-triple Flag. We used the HiFi DNA assembly kit (NEB) to join the two fragments. To generate the *dolP* variant containing an amber codon (TAG; DolP_{V41X}), site-directed mutagenesis was performed on pKiD41 using primers KiDo223 and KiDo224. To generate pAG310, *yedD* was PCR-amplified using AG175 and AG176 as primers and chromosomal *E. coli* DNA as template. The PCR product was then inserted into pAM238 restricted with KpnI and PstI,

generating pAG310. To generate the *yedD* variants containing an amber codon (TAG; YedD_{L47X} and YedD_{E51X}), site-directed mutagenesis was performed on pAG310 using primers AG293-296.

For the study with MlaC, the parental strain used is MC4100 {Casadaban, 1976 #321}. The gene deletion in this strain was performed using P1 transduction as above. pET23/42-*mlaC-His* (**Table S3**), which was used for the expression of MlaC, was constructed as described in {Chong, 2015 #2328} for the MlaA expression plasmid.

***In vivo* site-specific photo-crosslinking using the *pBpa*-containing variants of RcsF, DolP, YedD and MlaC.**

We used the site-specific photo-crosslinking method described by Okuda *et al.* {Okuda, 2012 #1156} with some modifications. To incorporate *p*-benzoyl-L-phenylalanine (*pBpa*) into RcsF, DolP, and YedD, we used the pEVOL plasmid encoding an evolved *Methanocaldococcus jannaschii* aminoacyl-tRNA synthetase and an optimized tRNA suppressor {Young, 2010 #2329}. For RcsF, PL358, SEN861, SEN905, SEN1166, SEN1167 cells were co-transformed with pEVOL and one of the plasmids containing an amber codon in *rcsF*. For DolP, sKiD328-331 cells were co-transformed with pEVOL and the plasmid containing an amber codon in *dolP*. Cells were grown in MOPS-glucose minimal medium supplemented or not with *pBpa* (final concentration of 0.8 mM; see the reasons for the use of MOPS medium below). When an OD₆₀₀ of 0.75 was reached, samples (1 ml for the experiment shown in **Figure S1**; 10 ml for the identification of the cross-linked peptides in **Figure S2 and S4**) were irradiated with UV light at 365 nm or not for 10 min. Cells were then precipitated with trichloroacetic acid (TCA), the pellets

were washed with acetone and finally solubilized in 50 μ L of SDS-sample buffer before SDS-PAGE and immunoblot analyses.

In our previous study, we used LB as a growth medium {Cho, 2014 #899}. However, we found that the *pBpa*-containing RcsF mutants were less expressed from pSC202 and pJS11 than wild-type RcsF encoded on the chromosome (data not shown). Since the expression levels of RcsF greatly affect complex formation with OmpA {Cho, 2014 #899}, we decided to increase the expression levels of the *pBpa*-containing RcsF variants to reach levels similar to those of the wild type expressed from the chromosome, which was achieved by growing the cells in MOPS minimal medium with 0.2% glucose {Neidhardt, 1974 #2339} (data not shown). Noteworthy, RcsF_{G60X} was expressed from pSC252 to reach levels similar to RcsF_{K40X} and RcsF_{Q79X} in MOPS minimal medium.

To express the *pBpa*-containing YedD variants, AG320 and AG762 cells were co-transformed with pEVOL and one of the plasmids containing an amber codon in *yedD*. Cells were grown in LB supplemented with *pBpa* (final concentration of 0.8mM) and photo-cross-linking was carried out as above.

To incorporate *pBpa* into MlaC, plasmid pSup-BpaRS-6TRN, which encodes the orthogonal tRNA and aminoacyl synthetase pair derived from *M. jannaschii*, and plasmid pET23/42-*mlaC-His* (amber mutant) were both used to transform cells MLA000 and CZS015. Cells were grown in LB supplemented with *pBpa* (final concentration of 1 mM) and photo-cross-linking was carried out as above.

***In vivo* site-specific photo-crosslinking using DiZPK for the study with RcsF.**

We used the site-specific photo-crosslinking method described by Dekoninck *et al.* {Okuda, 2012 #2288;Dekoninck, 2020 #2324}

Immunoblot analysis and antibodies.

Protein bands were transferred —using a semi-dry electroblotting system— from the gels onto nitrocellulose membranes (Millipore) when analysis was carried out to detect RcsF, DolP, YedD and OmpA or onto polyvinylidene fluoride membranes (Bio-Rad) to detect MlaC. The membranes were blocked by 5% skim milk (RcsF, DolP, YedD and OmpA) or 1X casein blocking buffer (Sigma; MlaC). The rest of the immunoblot steps were performed in the standard format. The specific signals from the antibody binding were visualized by detecting the chemiluminescence from the reaction of horseradish peroxidase (HRP) with luminol. The polyclonal RcsF antibodies, purified or not against the carboxy-terminal domain of RcsF as previously described {Cho, 2014 #899}, were used for **Figure 2**, **S1** and **S7**, respectively. The anti-OmpA antibody is a gift from the Llobes laboratory {Cascales, 2002 #1517}. Polyclonal YedD antibodies were generated by immunizing rabbits with YedD-6xHis (CER Group, Marloie, Belgium). The anti-Flag antibody was purchased from Sigma and used at a dilution of 1:10,000. The anti-His antibody (pentahistidine) conjugated to HRP (Qiagen) was used at a dilution of 1:5,000. Other antibodies were used at a dilution of 1:20,000.

Purification of the UV-crosslinked complexes for the study with RcsF.

The photo-crosslinked complex between RcsF-Flag-hexa His tag and OmpA was purified using Nickel affinity chromatography as in {Cho, 2014 #899}. After elution of the proteins with a high imidazole concentration, the samples were incubated for 10 min at 95°C in the presence of 10 mM tris(2-carboxyethyl)phosphine (Sigma) to reduce disulfide bonds. 10 mM *N*-ethylmaleimide (Sigma) was then added to alkylate free cysteines. The samples were analyzed by SDS-PAGE, and the proteins were stained using « Coomassie staining solution » (Thermo Scientific). The protein bands that were observed in the UV-treated samples migrating at the size of the RcsF-OmpA complex were cut. The corresponding area in the non-UV treated sample (where no band was observed) was also cut, as well as the bands corresponding to free RcsF and free OmpA (in the non-UV treated sample). Note that the Ni-affinity purified RcsF-OmpA complex migrates as two bands as observed previously {Cho, 2014 #899}. The fast-migrating band (~42 kDa) likely contains folded OmpA, which is known to migrate faster than denatured OmpA.

Purification of the UV-crosslinked complexes for the study with MlaC.

pBpa-incorporated MlaC expressing cells were grown in 1.5 L LB. The cells were harvested by centrifuging the cultures at 4,700 x *g* for 20 min and then resuspended in 40 mL TBS (20 mM Tris-HCl pH 8.0, 300 mM NaCl). 20 mL of the culture was transferred into a Petri dish and subjected to irradiation with UV light at 365 nm for 30 min. The other 20 mL culture was not subjected to UV irradiation.

To both cultures, 1 mM PMSF, 100 µg/mL lysozyme and 50 µg/mL DNase were added. The resuspended cells were lysed through a single passage of French Press (French Press G-M, Glen Mills) at 20,000 psi. Cell debris were removed by centrifugation at 4,700g x *g* for 10 min at 4°C. The supernatant was subjected to ultra-centrifugation (Model Optima L-100K, Beckman Coulter) at 100,000 x *g* for 1 h at 4°C to separate the membrane and soluble fractions. The membrane pellet was extracted with 4 mL TBS containing 5 mM MgCl₂, 10% glycerol, and 1% dodecylmaltoside (DDM) and incubated by rocking for 2.5 h on ice. The extracted membrane pellet was then incubated with 1 mL TALON cobalt resin (Clontech), which had been equilibrated with 2.5 mL of TBS wash buffer, containing 20 mM imidazole, by rocking for 30 min on ice. The resin mixtures were loaded onto gravity columns (Bio-Rad). The columns were washed with 10 x 2 mL TBS wash buffer and the cross-linked complex was eluted from the column with 2.5 mL elution buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 200 mM imidazole) containing 5 mM MgCl₂, 10% glycerol and 0.05% DDM. The eluates were concentrated to 100 µL by centrifuging at 3,700 x *g* at 4°C. 2X SDS-reducing buffer was added to the concentrated eluates. The samples were then boiled at 100°C for 5 minutes and loaded onto the gradient gels. After SDS-PAGE, the gels were visualized by silver staining (Silver Quest, Invitrogen) or subjected to immunoblot analysis. Excised bands were sent for tandem-MS analysis at the Taplin MS facility, Harvard Medical School.

Mapping the *p*Bpa-cross-linked peptides by mass spectrometry.

Gel bands were destained and digested with trypsin as described previously {Wilm, 1996 #2340;Shevchenko, 2000 #2341} and resuspended in 10 µL 0.1% formic acid. For LC-MS/MS purposes, samples were desalted off-line using C18

Stagetips, injected in an Ultimate 3000 RSLCnano system (Thermo) and then separated in a 15cm analytical column C18 nanocolumn (75µm ID home-packed with ReproSil-Pur C18-AQ 2.4 µm from Dr. Maisch) with a 40 or 60 min gradient from 5 to 60% acetonitrile in 0.1% formic acid. The effluent from the HPLC was directly electrosprayed into an LTQ-Orbitrap XL as described before ¹⁰. For automation purposes, each individual Thermo binary raw file was transformed to a dta file using Decon2LS {Zimmer, 2006 #2342} or to mzXML using msconvert (Proteowizard Tools proteowizard.sourceforge.net/tools.shtml) and processed with Crossfinder {Mueller-Planitz, 2015 #2343;Forne, 2012 #2344} with the following settings: MS1 and MS2 mass accuracy, within 15 ppm; enzyme, trypsin; allowed number of missed cleavage sites, three; fixed modifications, *N*-ethylmaleimide on cysteine; variable modifications, oxidation on methionine; number of top MS2 peaks considered per 50 Da mass window, twelve.

Protein identification by mass spectrometry.

Gel bands were digested with trypsin, and the peptides were analyzed by LC-MS/MS in a micro electrospray ionization ion trap mass spectrometer (LTQ XL, Thermo Scientific, San José, CA) as described {Pyr Dit Ruys, 2012 #2345}. The mass spectrometer was operated in the data-dependent mode and switched automatically between MS, Zoom Scan for charge state determination, and MS/MS collision energy of 35% for sequence information. This process was repeated for the five most abundant ions. Dynamic exclusion was enabled to allow analysis of co-eluting peptides. For peptide identification, peak lists were generated using the application spectrum selector in the Proteome Discoverer 1.4 package. The resulting peak lists were searched using Sequest against an *E. coli* protein database obtained from Uniprot. Peptide matches were filtered using the q-value and Posterior Error

Probability calculated by the Percolator algorithm ensuring an estimated false positive rate below 5% and manually validated. For the identification of the proteins which were photo-crosslinked with MlaC, the excised bands were sent for tandem-MS analysis at the Taplin MS facility, Harvard Medical School.

***In vivo* DTSSP cross-linking.**

In vivo cross-linking with 3,3'-dithiobis[sulfosuccinimidylpropionate] (DTSSP) (CovaChem) was performed as described {Cho, 2014 #899}.

β -galactosidase assay.

β -galactosidase activity was measured as described {Zhang, 1995 #2346}.

Sequence alignment.

Clustal Omega {Sievers, 2021 #2347; Sievers, 2021 #2347} was used for the amino acid sequence alignment. FASTA protein sequences were downloaded from Uniprot (<http://www.uniprot.org/>).

Homology modeling.

The structures of the Pal and YiaD mutants were generated from the structure of the periplasmic domain of OmpA (PDB 2MQE) using the *swapaa* command from UCSF Chimera {Pettersen, 2004 #2348}.

Molecular docking.

The three-dimensional structure of Gly-*p*Bpa-Gly was generated using CORINA (version 3.60, Molecular Networks GmbH, Erlangen, Germany) starting from the corresponding SMILES string. Docking calculations were performed using the GOLD software {Verdonk, 2003 #2323} from CCDC's Discovery_2021 suite and the GoldScore scoring function, with the OmpA_{CTD} structure (PDB 2MQE) as receptor and the binding sites defined as a 20 Å radius around the C α atom of Asp291.

Molecular dynamics.

Molecular dynamics simulations were performed with GROMACS version 2020.3 {Van Der Spoel, 2005 #2322} using the OPLS-AA force field {Kaminski, 2001 #2319}. The protein was centered in a cubic periodic box, with at least 1.0 nm on each side. The simulation box was then filled with TIP4P water molecules and the system neutralized with Na⁺ and Cl⁻ ions until reaching the physiological ionic strength (150 mM). Each system was energy-minimized until convergence using a steepest descents algorithm. Molecular dynamics was then performed for 50 ns with position restraints on backbone, with the exception of residues 287-292 corresponding to the mutated hexapeptide, which were unrestrained. The Parrinello–Rahman method was used for pressure coupling {Parrinello, 1981 #2321} and the temperature was coupled using the Nosé–Hoover method at 300 K {Nosé, 1984 #2320}. Electrostatics were calculated with the particle-mesh Ewald method {Essmann, 1995 #2316}. The P-LINCS algorithm {Hess, 2008 #2317} was used to constrain bond lengths, and a time step of 2 fs was used throughout. All

frames from each simulation were clustered to extract the most representative conformation.

Acknowledgments.

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Competing interests.

The authors declare no competing interest.

Figures

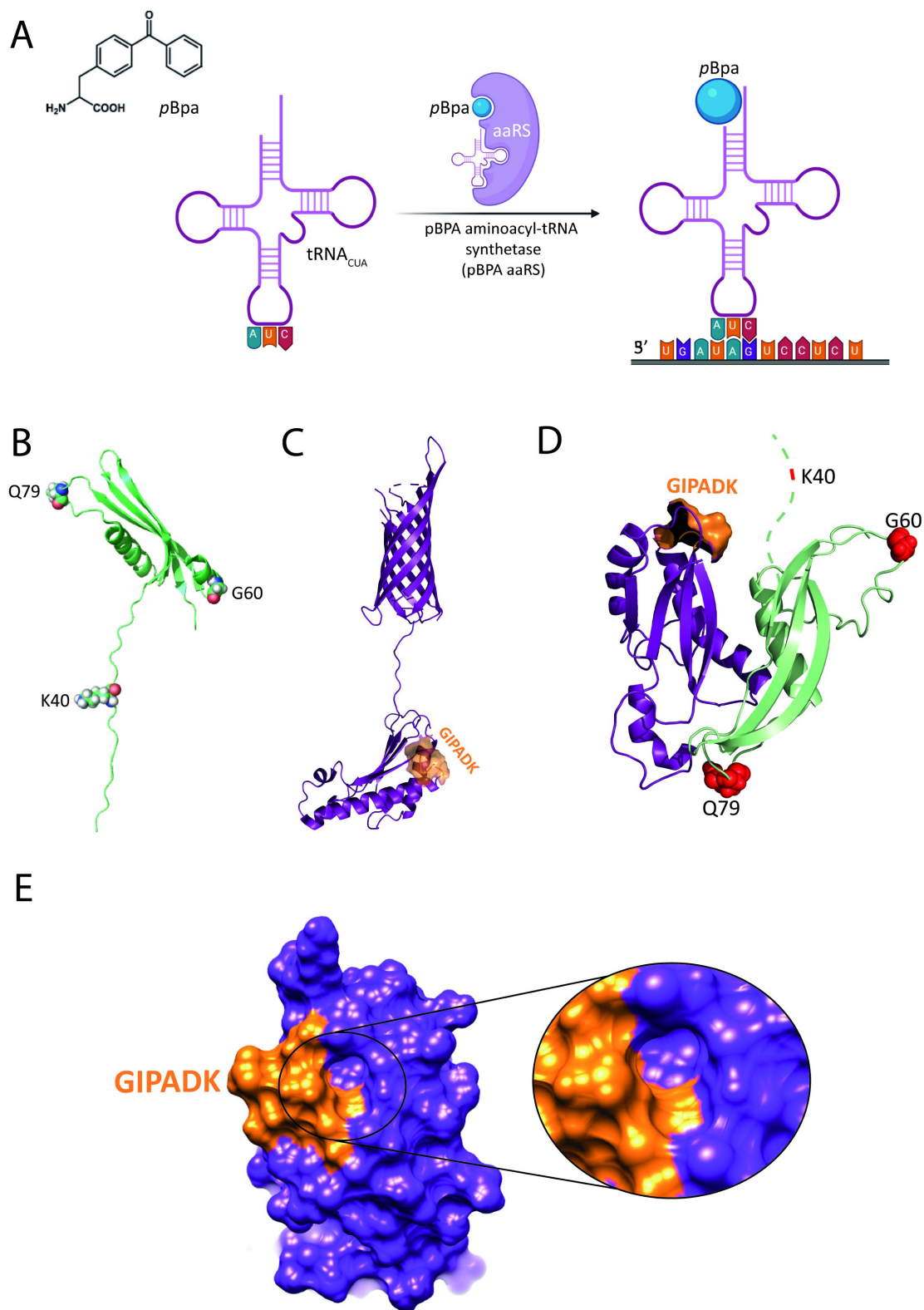


Figure 1. *pBpa*-mediated photo-crosslinking of RcsF to OmpA. (A) How to genetically incorporate *pBpa* into a protein. The structure of *pBpa* is shown on the left. *pBpa* (supplemented in the growth medium) can be incorporated in the protein of interest

in response to the amber nonsense codon (TAG) via an orthogonal suppressor tRNA (tRNA_{CUA}), which is charged by a *pBpa* aminoacyl-tRNA synthetase (aaRS). Any given codon in the gene encoding the protein of interest can be replaced by the amber codon using classical methods of genetic engineering. **(B)** A structural model of *E. coli* RcsF (PDB:2L8Y) showing the three residues selected for replacement by *pBpa* and LC-MS/MS analysis. **(C)** Structural model of *E. coli* OmpA. Shown are the X-ray crystal structures of the amino-terminal β -barrel domain (PDB:1QJP) and of the periplasmic, soluble domain of OmpA (PDB:2MQE). The GIPADK peptide found to photo-crosslink with different RcsF variants is shown in orange. **(D)** Cartoon representation of a structural model {Dekoninck, 2020 #2324} for the complex between the periplasmic domain of OmpA (purple) and RcsF (green). The three residues replaced by *pBpa* in RcsF (K40, G60 and Q79) are colored in red. The GIPADK motif of OmpA is shown in orange. **(E)** Top view of a surface representation of the periplasmic domain of OmpA (PDB:2MQE). The GIPADK peptide, which forms two hydrophobic holes with a nose-like bulged region, is colored in orange and shown in the zoomed in area.

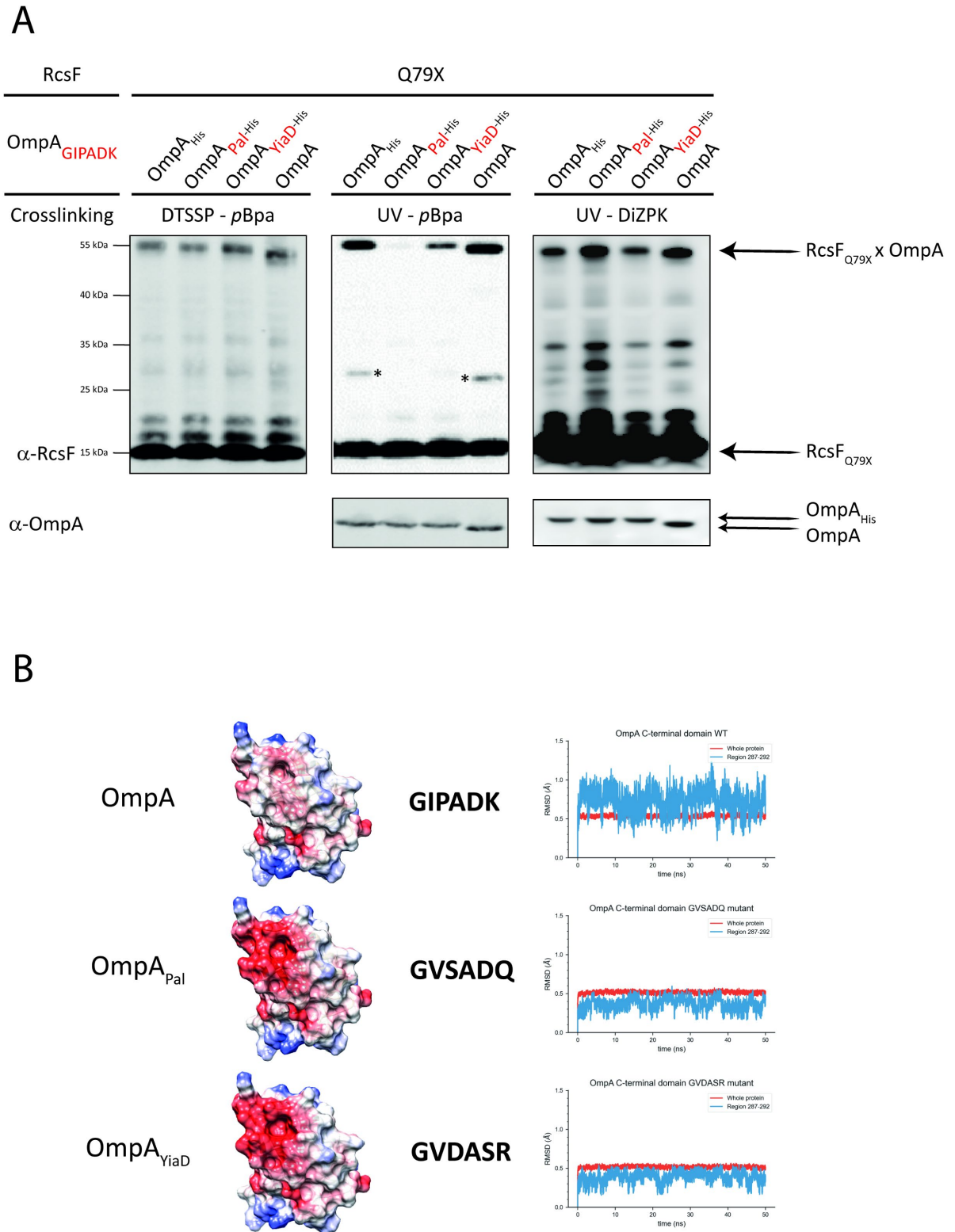
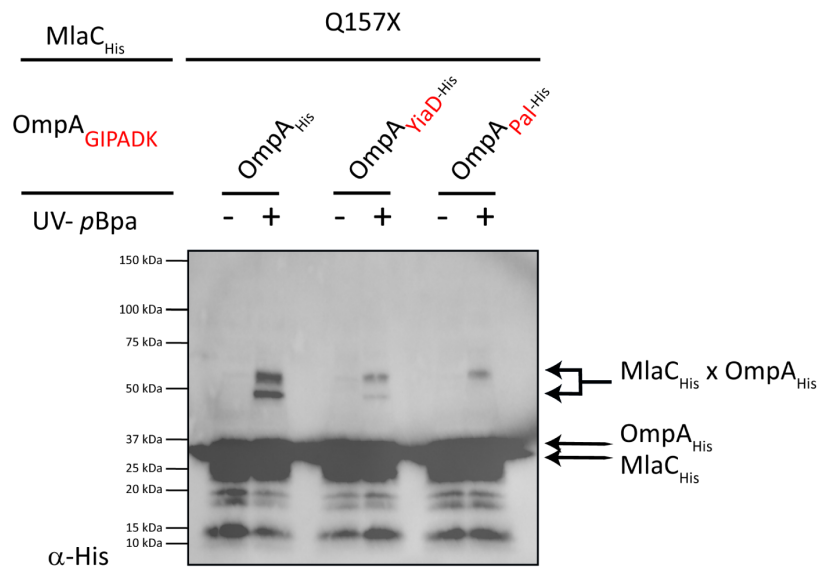


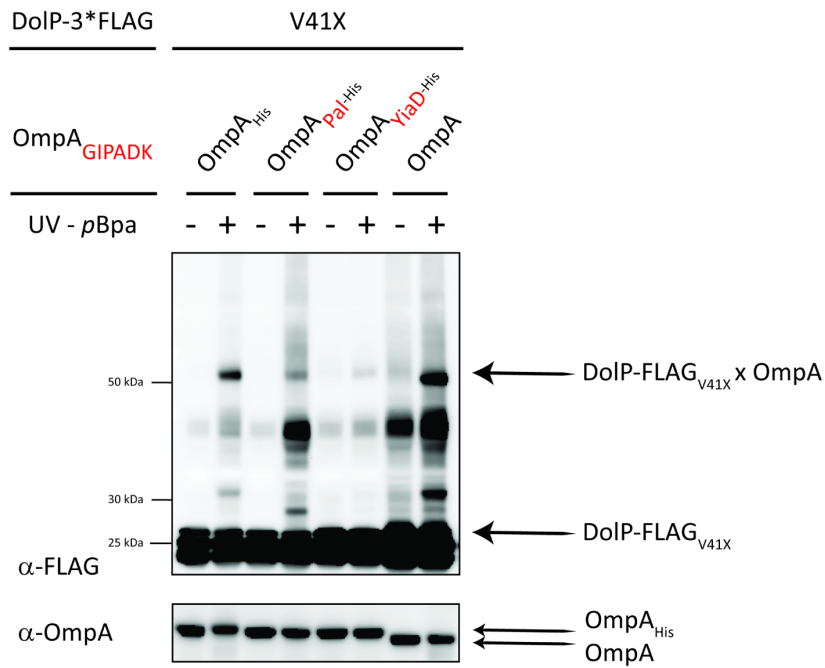
Figure 2. Changing the structural features of the GIPADK motif of OmpA alters its interaction with *pBpa*-containing RcsF variants. (A) *In vivo* cross-linking of RcsF_{Q79X} with OmpA_{His}, OmpA_{Pal-His}, and OmpA_{YiaD-His}. RcsF_{Q79X} was expressed from a plasmid (a pSC202 derivative for *pBpa* and a pSC252 derivative for DiZPK) in

$\Delta rcsF$ cells and detected by immunoblotting with an anti-RcsF antibody (upper panels). The expression levels of OmpA_{His}, OmpA_{Pal-His}, and OmpA_{YiaD-His} were verified by immunoblotting with an anti-OmpA antibody (lower panels). Left panel: DTSSP was used to cross-link *pBpa*-containing RcsF_{Q79X} to OmpA_{His}, OmpA_{Pal-His} or OmpA_{YiaD-His}. OmpA without a C-terminal His-tag was used as a control. Middle panel: Cells were irradiated with UV to cross-link *pBpa*-containing RcsF_{Q79X} in cells expressing the three OmpA variants and the wild type. The samples in the middle panel were also used in the experiment shown in the left panel (DTSSP *pBpa*). Right panel: cells were irradiated with UV to cross-link DiZPK-containing RcsF_{Q79X} in cells expressing the three OmpA variants and the wild type. In these experiments, the genes encoding OmpA and the two mutant proteins fused to a C-terminal His-tag (OmpA_{His}, OmpA_{Pal-His}, and OmpA_{YiaD-His}) were inserted on the chromosome in place of *ompA*. **(B)** Left panel: Electrostatic potential visualization of wild-type OmpA, OmpA_{Pal} and OmpA_{YiaD}. Neutral, negatively charged, and positively charged residues are colored in white, red, and blue, respectively. The surface of the periplasmic domain of OmpA_{Pal} and OmpA_{YiaD} is more negatively charged than that of the wild type. Right panel: Root-mean-square deviation (RMSD) plots from molecular dynamics simulations of wild-type OmpA_{WT}, OmpA_{Pal} and OmpA_{YiaD}. The region between residues 287 and 292 (corresponding to the GIPADK motif in wild-type OmpA) is shown in blue. This region is less flexible in OmpA_{Pal} and OmpA_{YiaD}.

A



B



C

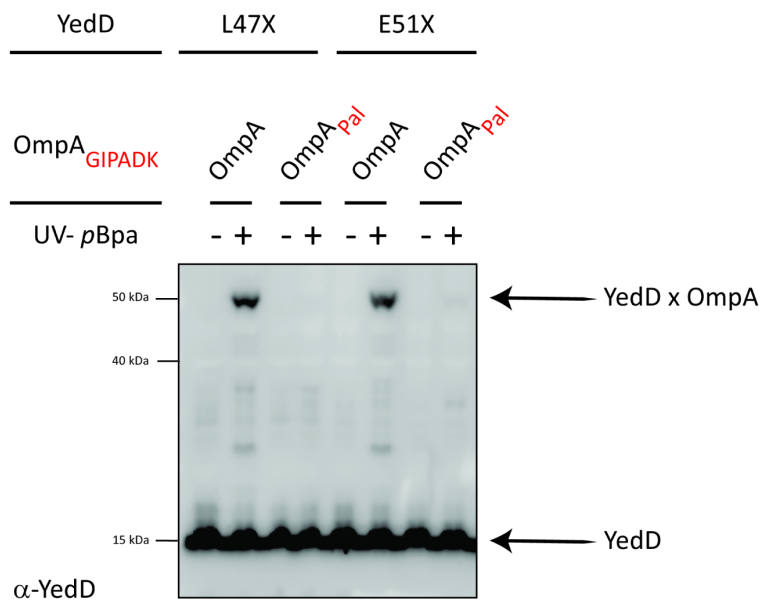


Figure 3. Artfactual interaction between *pBpa*-containing MlaC, DolP and YedD variants with the GIPADK motif in OmpA. (A) The *pBpa*-containing MlaC_{Q157X} variant was expressed from a plasmid (a pET23/42-*mlaC-His* derivative) in cells encoding OmpA_{His}, OmpA_{YiaD-His}, or OmpA_{Pal-His}. MlaC was detected by immunoblotting with an anti-His antibody. (B) The *pBpa*-containing DolP_{V41X}-triple FLAG variant was expressed from a plasmid (a pKiD41derivative) in cells encoding OmpA_{His}, OmpA_{YiaD-His}, OmpA_{Pal-His} or OmpA. DolP and OmpA were detected by immunoblotting with an anti-FLAG antibody or an anti-OmpA antibody (lower panel), respectively. (C) The *pBpa*-containing YedD_{L47X} and YedD_{E51X} variants were expressed from a plasmid (pAM238-yedD derivatives) in cells encoding OmpA or OmpA_{Pal}. YedD was detected by immunoblotting with an anti-YedD antibody. All samples were exposed to UV or not.

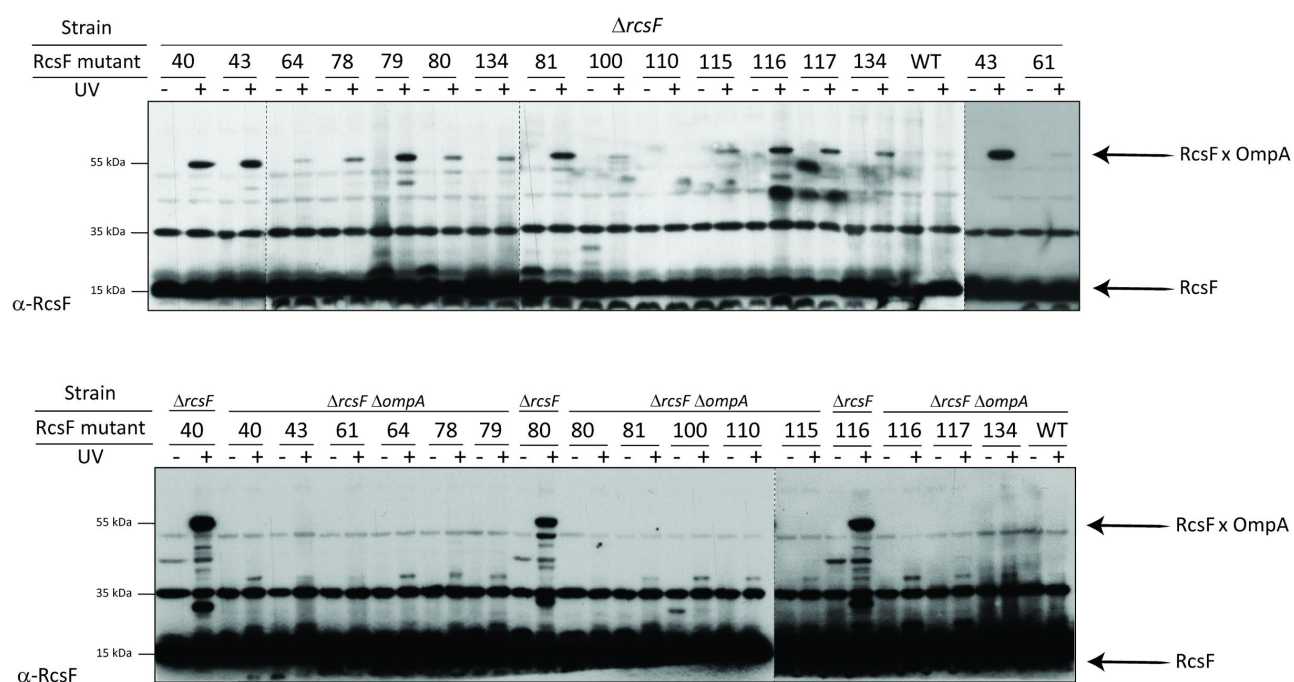
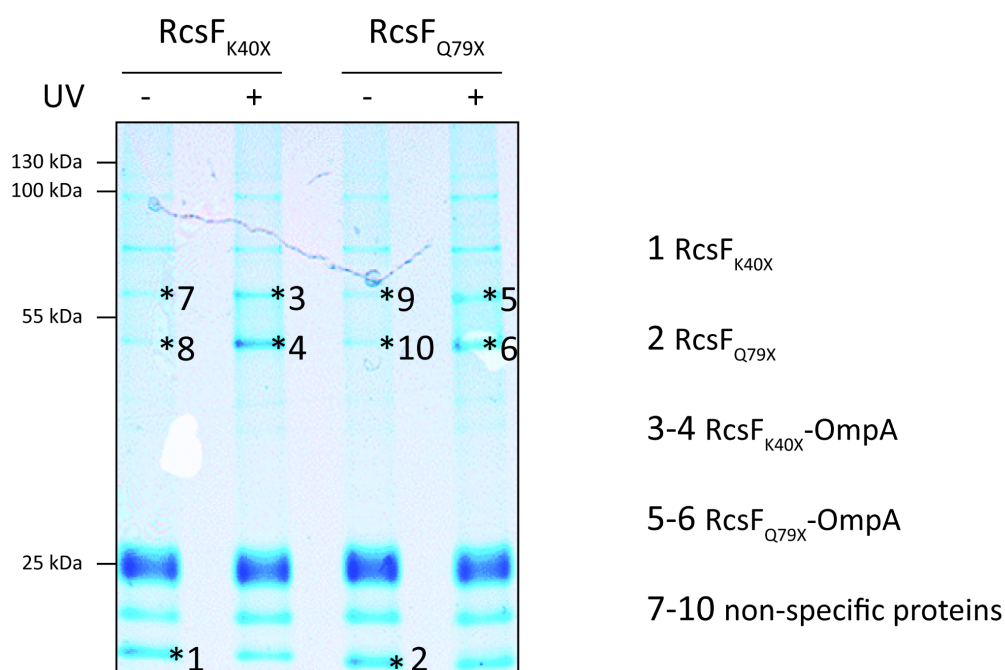


Figure S1. *In vivo* cross-linking of RcsF with OmpA. Cells ($\Delta rcsF$ or $\Delta ompA \Delta rcsF$, as indicated) expressing RcsF mutants containing *pBpa* at the indicated positions were treated (+) or not (-) with UV. Cells were grown in minimal media supplemented with 0.8 mM *pBpa*. The RcsF mutants were expressed from variants of the pSC202 plasmid. After precipitation with TCA, protein samples were analyzed by SDS-PAGE and immunoblotted with an anti-RcsF antibody. *pBpa* was inserted into the RcsF mutants using the system described in **Figure 1**.

A

1	40	
MRALPICLVALMLSGCSMLSRSPVEPVQSTAPQPKAEP	K	
PKAPRATPVRIYTNAEELV	GKPFRDLGEVSGDSCQASN	QD
SPPSIPTARKRMQINASKMKANAVLLHSCEVTSGTPGCYR		
QAVCIGSALNITAK	GSDYKDDDDKHHHHHH	

B



C

	Photo-cross-linked peptide (see the MS2 spectra in Figure S3)	Mass	RcsF peptide (pBpa residues are abbreviated as X)	OmpA peptide (Cross-linked residues are in red)
RcsF _{K40X} X OmpA (from bands 3 and 4)	XL1	2213.1959	AEPAXPKAPR	GIPADKISAR
	XL2	1889.0048	AEPAXPK	GIPADKISAR
	XL3	1785.94	AEPAXPKAPR	GIPADK
RcsF _{Q79X} X OmpA (from bands 5 and 6)	XL1	3804.7902	DLGEVSGDSCQASN ^X DSPPSIPTAR	GIPADKISAR

Figure S2. Identification of the OmpA peptide photo-crosslinked to the pBpa-containing RcsF mutants. (A) The amino acid sequence of RcsF. The amino acid residues (K40, G60, and Q79) that were replaced by pBpa are shown in red. The expressed mutant proteins were fused to a Flag-tag and a His-tag at their C-terminus (the additional residues are shown in green). (B) Pull-down of OmpA photo-crosslinked with RcsF_{K40X} and

RcsF_{Q79X}. Lysates prepared from cells expressing the RcsF variants were irradiated (+) or not (-) with UV and then purified using Ni-affinity resins. After Coomassie staining, the bands numbered 1 to 10 were cut from the gel and analyzed by MS. Note that the Ni-affinity purified RcsF-OmpA complex migrates as two bands as observed previously {Cho, 2014 #899}. **(C)** The sequence of the OmpA and *pBpa*-containing RcsF peptides were found to be photo-crosslinked.

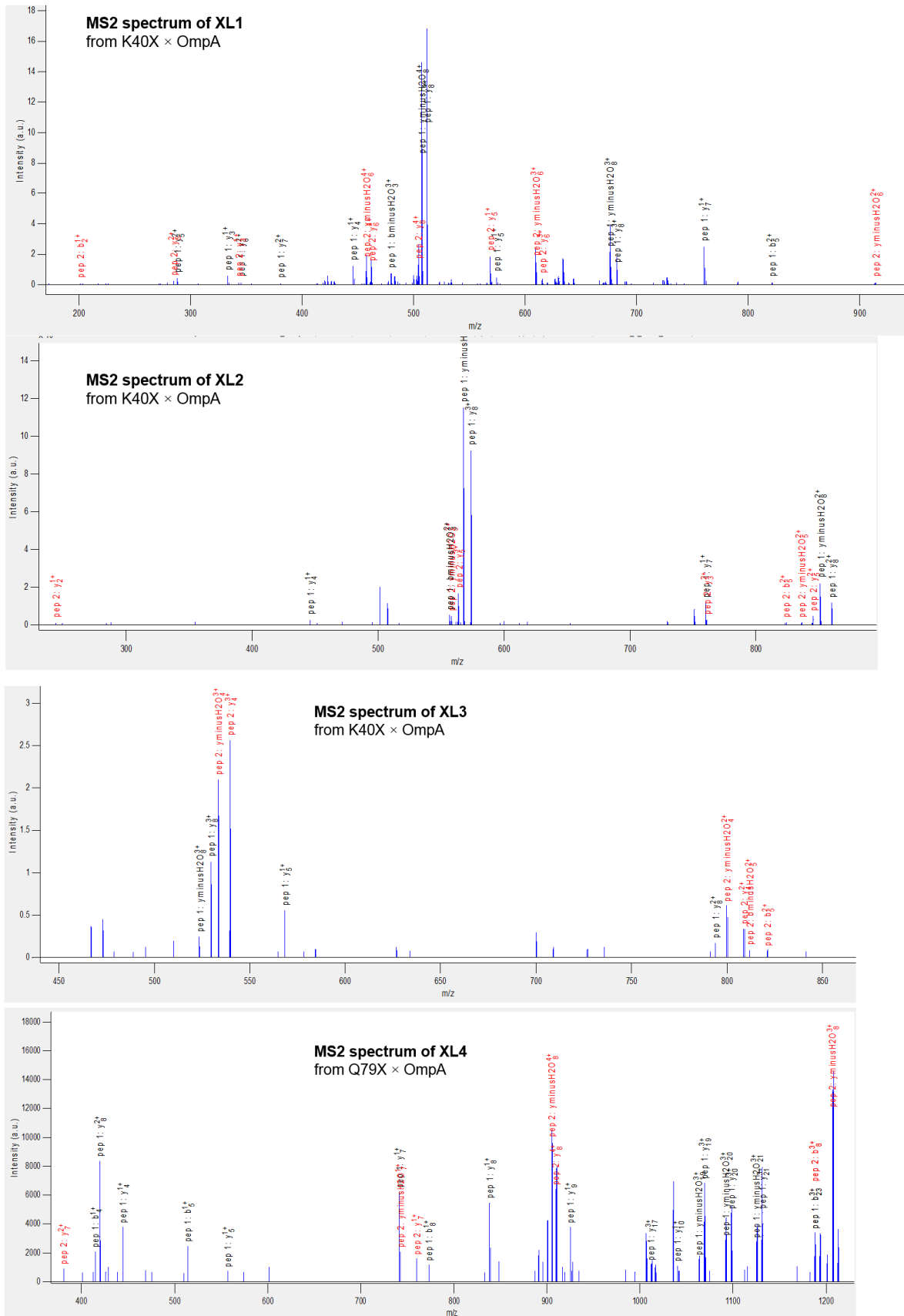
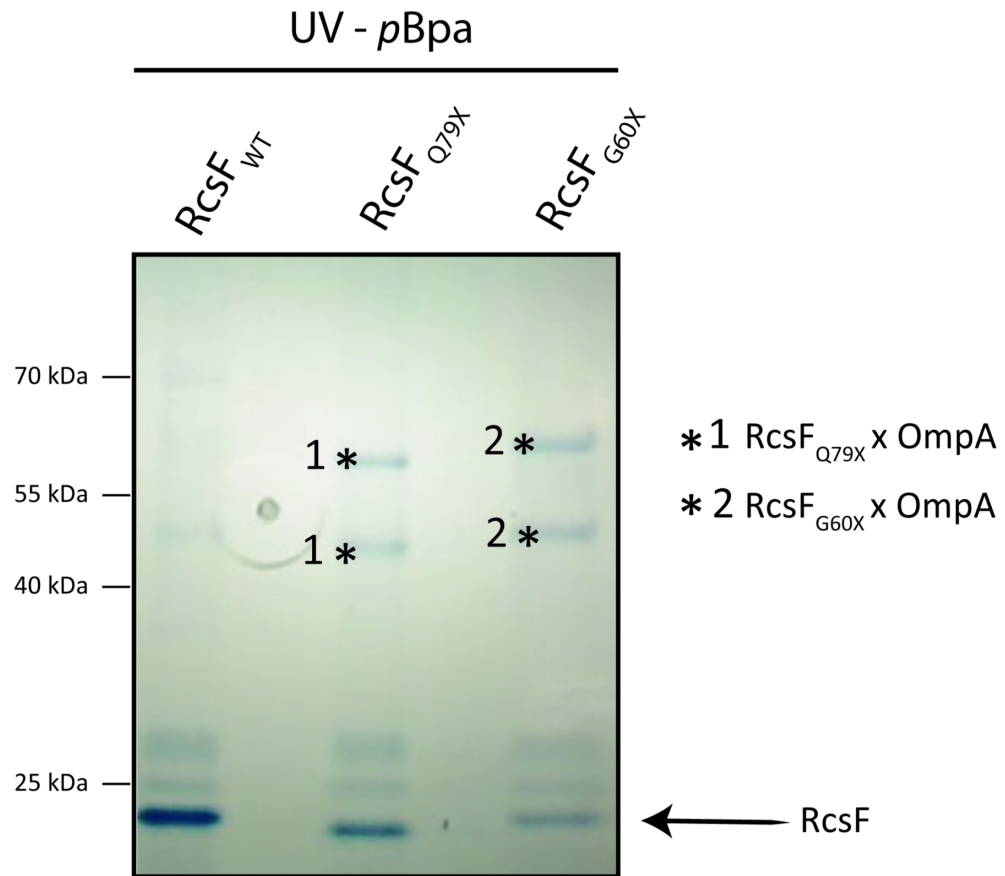


Figure S3. MS2 spectra of the photo-crosslinked peptides between OmpA and either RcsF_{K40X} (XL1-3) or RcsF_{Q79X} (XL4).

A



B

	Photo-cross-linked peptide (see the MS2 spectra in Figure S5)	Mass	<i>RcsF</i> peptide (<i>pBpa</i> is indicated by X)	<i>OmpA</i> peptide (Crosslinked residues are in red)
<i>RcsF</i> _{G60X} × <i>OmpA</i> (from bands 2)	XL1	2856.5176	IYTNAEELV X KPFR	GIPADKISAR
	XL2	2429.2633	IYTNAEELV X KPFR	GIPADK
<i>RcsF</i> _{Q79X} × <i>OmpA</i> (from band 1)	XL3	3804.7903	DLGEVSGDSCQASN X DSPPSIPTAR	GIPADKISAR

Figure S4. Identification of the *OmpA* peptides photo-crosslinked to the *pBpa*-containing *RcsF*_{G60X} mutant. (A) Pull-down of *OmpA* photo-crosslinked with *RcsF*_{Q79X} and *RcsF*_{G60X}. Lysates prepared from cells expressing the *RcsF* variants were irradiated with UV and then purified using Ni-affinity resins. After Coomassie staining, the bands numbered 1 and 2 were cut from the gel and analyzed by MS. Note that the Ni-affinity purified *RcsF*-*OmpA* complex migrates as two bands as observed previously {Cho, 2014

#899}. **(B)** The sequence of the OmpA and *p*Bpa-containing RcsF peptides that were found to be photo-crosslinked.

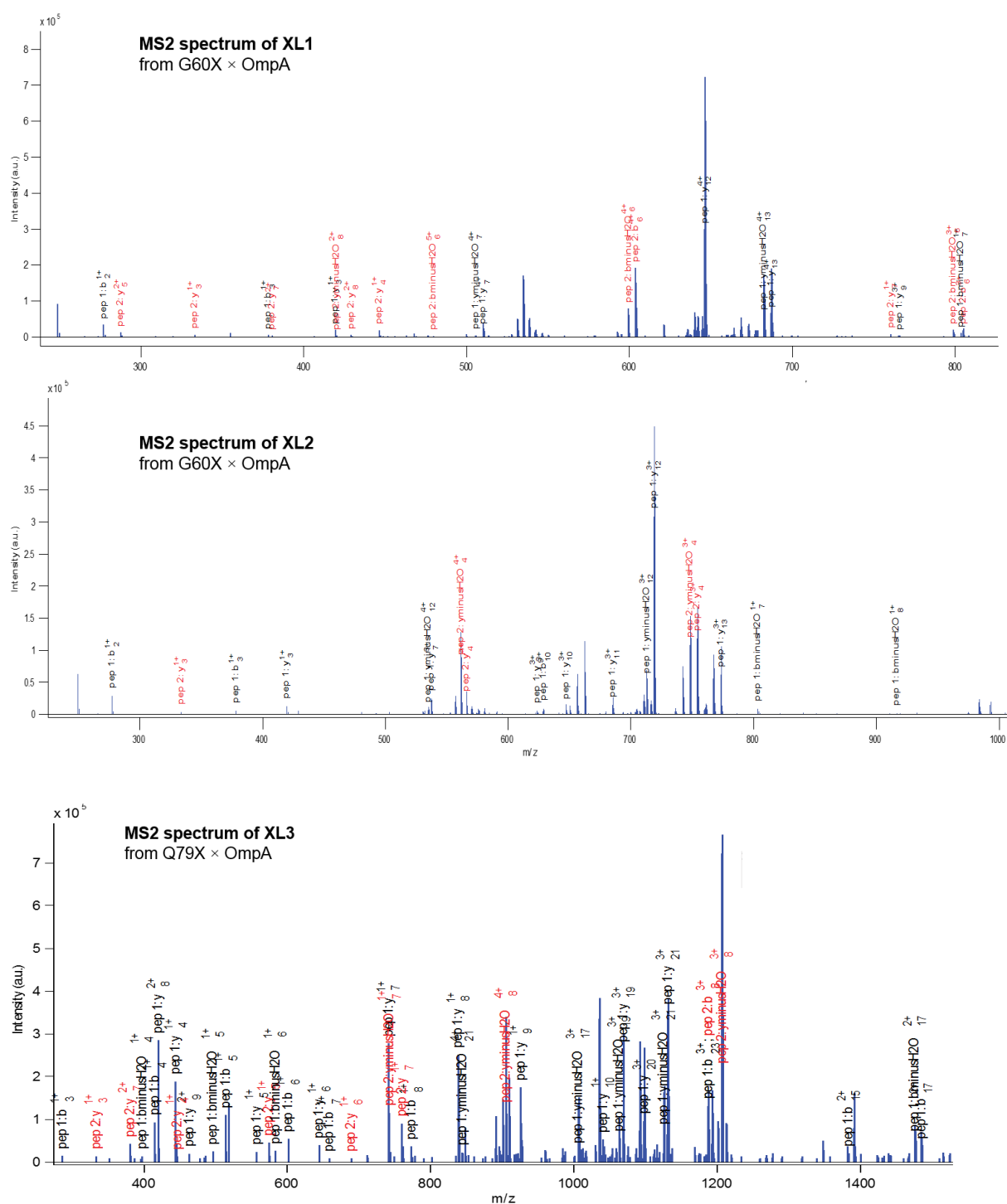
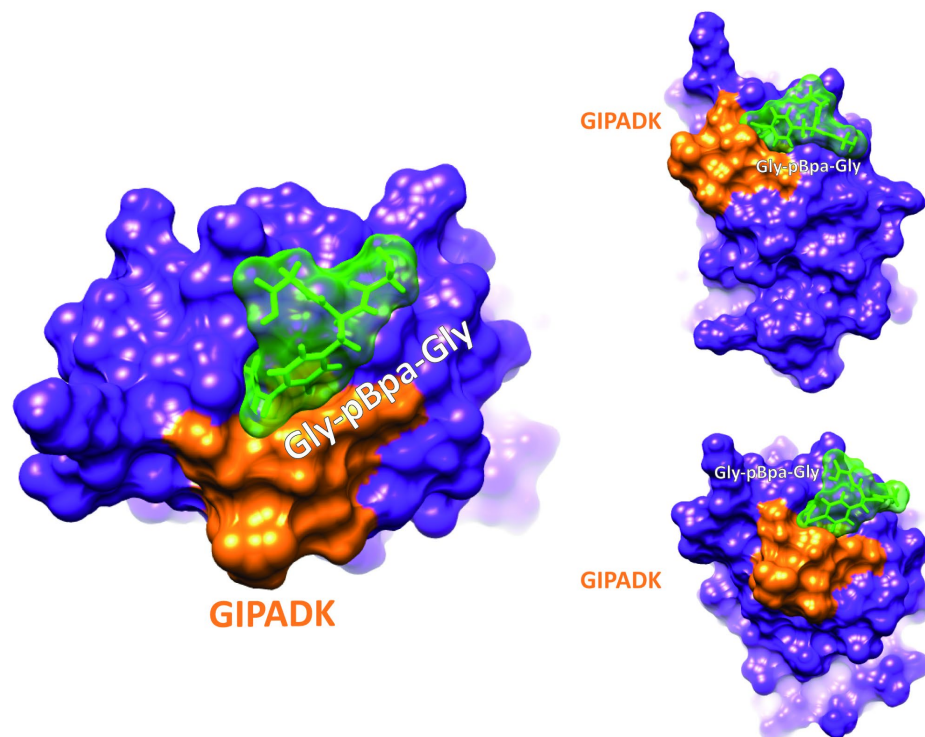


Figure S5. MS2 spectra of the photo-cross-linked peptides between OmpA and either RcsF_{G60X} (XL1 and 2) or RcsF_{Q79X} (XL3).

A

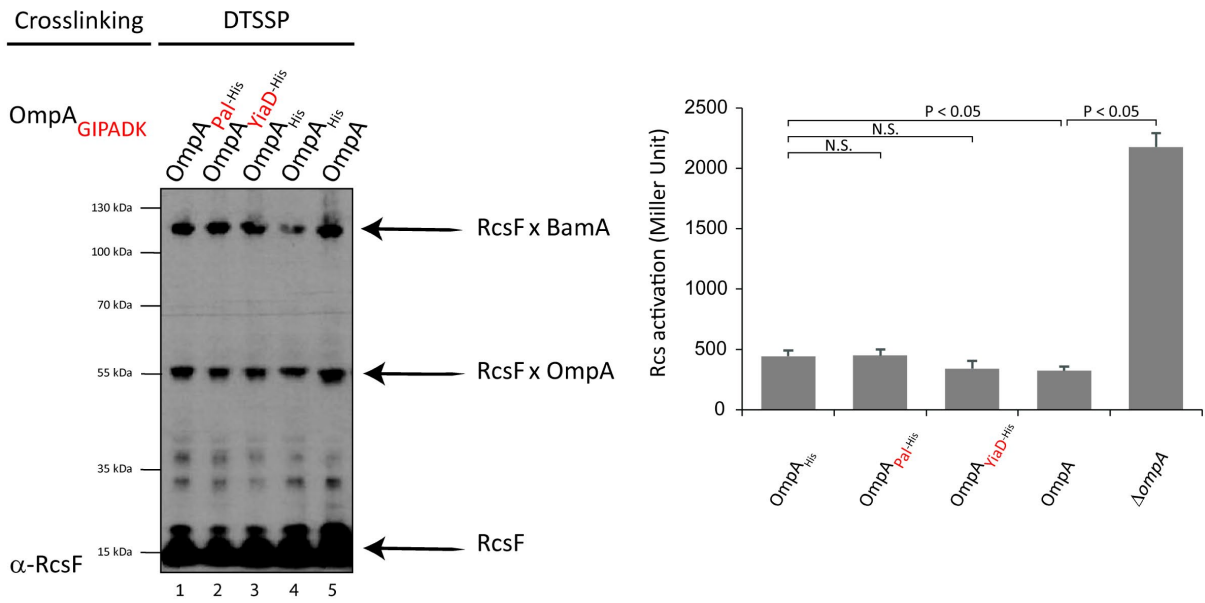


B

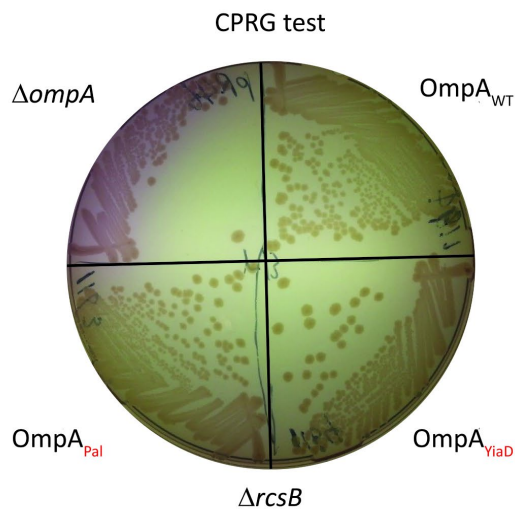
OmpA_m	APKDNITMYTGAKLGHSQYHDTGFIINNGPTHENQLGAGAFGGYQVNPVVGFEFGYDNLGR	60
Pal_m	-----	0
YiaD_m	-----	0
OmpA_m	MPYKGSVENGAYKAQGVQLTAKLGYPITDDLDIYTRLGGVWRADTKSNVYGNHDTGV	120
Pal_m	-----CSSNKN-----	6
YiaD_m	-----CTNPTGEREAGKS	15
OmpA_m	PVFAGGVEYAITPEIATRLEYQWTHNIGDAHT-IGTRPONGHLSLGVSYRFGQGEAA---	176
Pal_m	-----ASNDGSEGM LGAGTGMDANGNGAPSS	33
YiaD_m	ATGAG-LGSLVG-----AGIGALSSSKKDRGKALIGAAAGALGGGVGYMD	62
OmpA_m	----PWAP----APAPAPEVQTKHFTLKSQVLFNFNKATLKPEGQAALDQLYSQSLND	228
Pal_m	EEQARLQMQ-----QLQQNIVVFDLDKYDIRSDFAQMLDAHANFLRSN-	77
YiaD_m	VQEAKLRDKMRGTGVSVTRSGDNIILNMPIMVTFDSSSATLKPGAGANTLTGVAVVLKEY-	121
OmpA_m	PKDGSVAVLGYTIRISDAYIQGSESRRAQSVDYITSGKTPADKISARGNGESHPVTGN	288
Pal_m	-PSYKVTVEGHAEIRITPEYIISGERMANAWMYLQGGVVSADQISIVSYGKEKPAVLG	136
YiaD_m	-PKTAVINIGYTDSTGHDLNRLSQQRADSASALITQGVQASRLRTQGLGPANPIASN	180
OmpA_m	TCDNVQRAALIDCLAPDRRVEIEVKGTKDVTPQQA	325
Pal_m	HDEAAY-----SKNRRRAVLVY-----	152
YiaD_m	STAEGK-----AQNRREITLSPL-----	199

Figure S6. Structural features of the GIPADK motif found in the periplasmic domain of OmpA. (A) Top and side view of the docking complex between the periplasmic domain of OmpA (colored in purple with the GIPADK motif in orange, PDB: 2MQE) and the Gly-pBpa-Gly tripeptide (colored in green). (B) Multiple sequence alignment of OmpA, Pal and YiaD. The signal sequences of the proteins are not shown. The identical residues are squared in yellow, while the GIPADK motif and the corresponding sequence in Pal and YiaD are squared in blue.

A



B



C

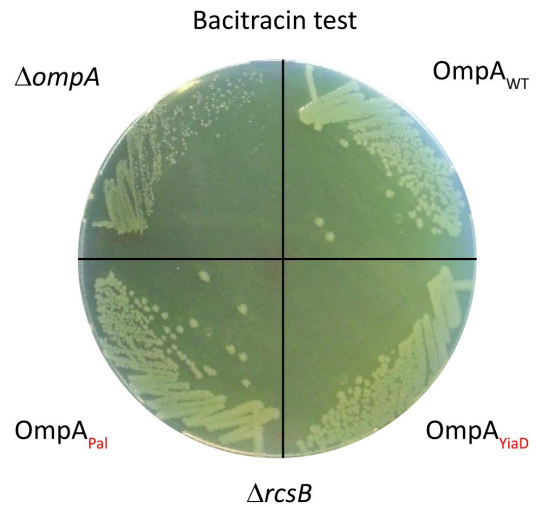


Figure S7. OmpA_{Pal-His} and OmpA_{YiaD-His} are functional. (A) Left panel: Cells expressing OmpA_{Pal-His} (lane 1), OmpA_{YiaD-His} (lane 2) or OmpA_{His} (lanes 3 and 4) were treated with DTSSP for chemical cross-linking. All tested OmpA constructs remain able to form a complex with RcsF. The previously reported complex {Cho, 2014 #899} between RcsF and BamA was also observed in all samples. The complexes were detected by immunoblotting with anti-RcsF antibodies. Right panel: The Rcs system is induced in a $\Delta ompA$ mutant compared to the wild type. OmpA_{His}, OmpA_{Pal-His} and OmpA_{YiaD-His} fully complement the deletion of *ompA* when expressed from the chromosome. The activity of

the Rcs system was measured from a chromosomal *rprA::lacZ* fusion integrated onto the chromosome. Mean (n=3) and standard deviation are shown. P values were evaluated with Student's *t* test (N.S., not significant). **(B)** PR209, SEN1220, SEN1221, and SEN1222 cells were streaked on LB containing chlorophenyl red- β -D-galactopyranoside (CPRG; 20 μ g/ml) and grown at 30 °C to evaluate envelope integrity. CPRG cannot cross the outer membrane of wild-type cells. Outer membrane defects caused by *ompA* deletion allow penetration of CPRG into the cell, where it is hydrolyzed by cytoplasmic β -galactosidase, producing a red color {Paradis-Bleau, 2014 #2334}. Expression of OmpA_{Pal-His} and OmpA_{YiaD-His} restores wild-type outer membrane permeability. In these experiments, we used cells in which *rscB*, which is required for Rcs induction, is deleted. In $\Delta rscB$ cells, basal LacZ activity is measured (not shown). **(C)** PR209, SEN1220, SEN1221 and SEN1222 cells were streaked on LB containing bacitracin (625 μ g/ml) and grown at 37 °C. Deletion of *ompA* impairs outer membrane integrity compared to the wild type, making $\Delta ompA$ cells more sensitive to bacitracin. Expression of OmpA_{YiaD-His} and OmpA_{Pal-His} rescues bacitracin sensitivity.

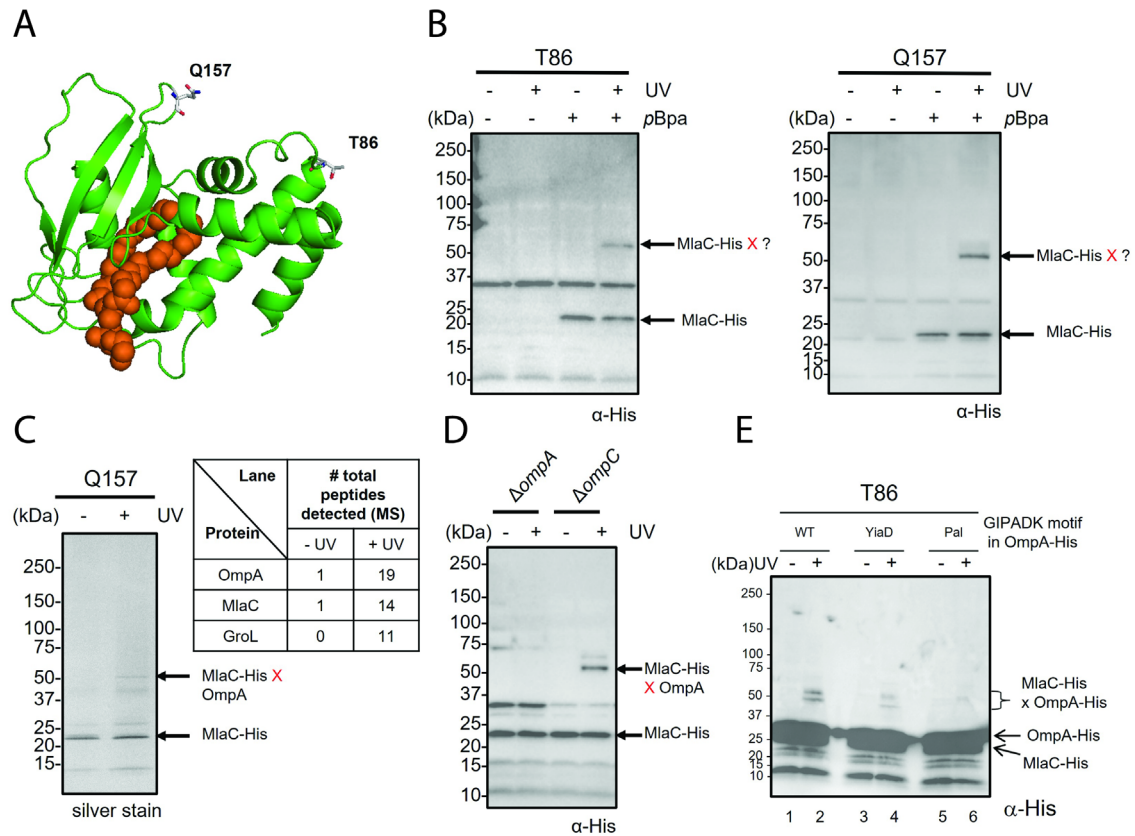


Figure S8. *In vivo* photo-crosslinking of MlaC with OmpA variants containing GIPADK mutations. (A) The structure of *E. coli* MlaC (green) bound to a phospholipid (orange) was modeled based on the crystal structure of its ortholog in *Ralstonia solanacearum* (2QGU) using the SWISS homology modeling software. The two residues on MlaC, Q157, and T86, are indicated as ribbons when replaced with *pBpa* cross-link with OmpA. (B) Whole-cell lysate photo-crosslinking in MC4100 $\Delta mlaC$ pSup-BpaRS-6TRN cells expressing MlaC_{Q157X-His} and MlaC_{T86X-His} at low levels from pET23/42. Samples were subjected to SDS-PAGE and visualized by immunoblot analysis using an anti-His antibody. A crosslinked band at ~60kDa can be detected for both variants in the UV/*pBpa* positive lanes. (C) Co-TALON affinity purification of MC4100 $\Delta mlaC$ pSup-BpaRS-6TRN cells expressing MlaC_{Q157X-His}. Samples were subjected to SDS-PAGE and visualized by silver staining. The cross-linked band in the “+ UV” lane and the corresponding position in the “– UV” control lane were excised and sent for tandem-MS sequencing. The top three hits detected for the cross-linked band at ~60 kDa are shown. Since OmpA had the most number of peptides detected, OmpA was identified as the most probable cross-linking

partner of MlaC. **(D)** Whole-cell lysate photo-crosslinking in MC4100 pSup-BpaRS-6TRN cells expressing MlaC_{Q157X-His} but lacking OmpA or OmpC. Samples were subjected to SDS-PAGE and visualized by immunoblot analysis using an anti-His antibody. The absence of the cross-linked band at ~60kDa in the $\Delta ompA$ strain validates that OmpA is the cross-linking partner of MlaC. **(E)** MlaC_{T86X-His} was expressed from pET23/42-*mlaC-His* containing the corresponding amber codon on *mlaC*. Expressed from the chromosome as in C. were OmpA_{His} (lane 1 and 2), OmpA_{YiaD-His} (lane 3 and 4), and OmpA_{Pal-His} (lane 5 and 6). Anti-His antibody was used to detect the proteins on Western blot. In the odd lanes, no UV was illuminated.

Supplementary Tables

Table S1. Strains used in this study

Strains	Relevant Genotype or Features	Source or notes
XL1-Blue	<i>endA1 gyrA96(nal^R) thi-1 recA1 relA1 lac glnV44</i> F'[::Tn10 <i>proAB</i> ⁺ <i>lacI</i> ^q $\Delta(lacZ)$ M15] <i>hsdR17</i> (r _K ⁻ m _K ⁺)	Stratagene
DH300	<i>rprA-lacZ</i> MG1655 (<i>argF-lac</i>) <i>U169</i>	{Majdalani, 2002 #1116}
MC4100	<i>F- araD139 $\Delta(argF-lac)$ U169 rpsL150 relA1 flbB5301</i> <i>ptsF25 deoC1 ptsF25 thi</i>	{Casadaban, 1976 #321}
MG1655	(<i>argF-lac</i>) <i>U169</i>	
Keio collection single mutants	$\Delta rcsF::kan$, $\Delta ompA::kan$, $\Delta rcsB::kan$, $\Delta mlaC::kan$, $\Delta dolP::kan$ $\Delta yedD::kan$	{Baba, 2006 #322}
PL358	DH300 $\Delta rcsF$	{Cho, 2014 #899}
SEN588	DH300 $\Delta ompA::kan$	{Cho, 2014 #899}
PR46	DH300 $\Delta ompA$	{Dekoninck, 2020 #2324}
SEN589	PL358 $\Delta ompA::kan$	{Cho, 2014 #899}
SEN860	DH300 $\Delta ompA::cat$, <i>sacB</i>	this study
SEN861	PL358 $\Delta ompA::cat$, <i>sacB</i>	this study
SEN1194	SEN860 <i>ompA-His</i>	this study

SEN905	SEN861 <i>ompA-His</i>	this study
SEN1163	SEN860 <i>ompA(GIPADK/GVSADQ_{Pal})-His</i>	this study
SEN1166	SEN860 <i>ompA(GIPADK/GVSADQ_{Pal})-His</i> <i>ΔrcsF::kan</i>	this study
SEN1164	SEN860 <i>ompA(GIPADK/GVDASR_{YiaD})-His</i>	this study
SEN1167	SEN860 <i>ompA(GIPADK/GVDASR_{YiaD})-His</i> <i>ΔrcsF::kan</i>	this study
SEN1220	SEN1163 <i>ΔrcsB::kan</i>	this study
SEN1221	SEN1164 <i>ΔrcsB::kan</i>	this study
SEN1222	SEN1194 <i>ΔrcsB::kan</i>	this study
PR209	PR46 <i>ΔrcsB::kan</i>	this study
sKiD328	DH300 <i>dolP::kan</i>	this study
sKiD329	SEN1163 <i>dolP::kan</i>	this study
sKiD330	SEN1164 <i>dolP::kan</i>	this study
sKiD331	SEN1194 <i>dolP::kan</i>	this study
AG297	MG1655 <i>yedD::kan</i>	
AG320	MG1655 <i>yedD-</i>	this study
AG745	MG1655 <i>yedD- ompA::cat,sacB</i>	this study
AG762	MG1655 <i>yedD- ompA(GIPADK/GVSADQ_{Pal})</i>	this study
	MC4100 <i>ΔmlaC::kan</i>	this study
MLA000	MC4100 <i>ΔompA::kan</i>	{Chong, 2015 #2328}

CZS015	MC4100 $\Delta ompC::kan$	{Chong, 2015 #2328}
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Table S2. Plasmids used in this study

Plasmids	Features	Source or notes
pAM238	IPTG-regulated P_{lac} , pSC101-based, spectinomycin	{Gil, 1991 #1056}
pBAD18	Arabinose regulation, ampicillin	{Guzman, 1995 #323}
pET23/42	pT7 inducible expression vector contains multiple cloning site of pET42a(+) in pET23a(+) backbone, ampicillin	{Wu, 2006 #161}
pEVOL	$aaRS$, $tRNA_{CUA}^{opt}$, p15A origin, chloramphenicol	{Young, 2010 #2329}
pSup-Mb-DiZPK-RS	$PylRS$, $tRNA_{CUA}^{Pyl, opt}$, p15A origin, chloramphenicol	{Zhang, 2011 #806}
pSup-BpaRS-6TRN	$aaRS$, $tRNA_{CUA}^{opt}$, p15A origin, chloramphenicol	{Chin, 2002 #1016}
pSIM5-tet	pSC101-based, $repA^{ts}$, tetracycline	{Koskiniemi, 2011 #2330}
pCP20	FLP^+ , λ cI857 ⁺ , λ_{PR} Rep ^{ts} , ampicillin, chloramphenicol	{Cherepanov, 1995 #1015}

pSC202	pAM238 with RcsF	{Cho, 2014 #899}
pJS11	pAM238 with RcsF-Flag-His	{Cho, 2014 #899}
pSC252	pBAD18 with RcsF-Flag-His	this study
pPR11	pDSW204 with OmpA-His	{Dekoninck, 2020 #2324}
pPR11 _{Pal}	pDSW204 with OmpA(GIPADK/GVSADQ _{Pal})- His	this study
pPR11 _{YiaD}	pDSW204 with OmpA(GIPADK/GVDASR _{YiaD})- His	this study
pKiD41	pBAD18 with DolP-triple Flag	this study
pAG310	pAM238 with YedD	this study
pET23/42- <i>mIaC-His</i>	pET23/42 with MlaC-His	this study

Table S3. Primers used in this study

Name	Sequence (5' -> 3')
rcsB -100	acatctgattcgtgagaagg
rcsB +100	taatgggaatcgtaggccgg
pal_GVSADQ_F	tacctgatctccaaaGGTgTCtCtGCAGACcAGatctccgca cgtggta
pal_GVSADQ_R	taccacgtgcggagatCTgGTCTGCaGaGAcACCtttggag atcaggta
yaiD_GVDASR_ F	tacctgatctccaaaGGtGTGGACGCCAGCCGCatctccg cacgtggtatg
yaiD_GVDASR _R	cataccacgtgcggagatGCGGCTGGCGTCCACaCCtttg gagatcaggta
ompA_delCmSB F	atattcatggcgtattttggatgataacgaggcgcaaaaaAAAATG AGACGTTGATCGGCACG
ompA_delCmSB R	aaaaccccgagcagcgggggttttctaccagacgagaacATCAAA GGGAAAACGTGTCCA
cmSB to OmpA_F	atattcatggcgtattttggatgataacgaggcgcaaaaaATGAAA AAGACAGCTATCGCG
cmSB to OmpAhis_R	aaaaccccgagcagcgggggttttctaccagacgagaacTTAGTG GTGGTGGTGGTGGTGAGCCTG
ompA -100	cgccaggggtgctcggcataagccg
ompA +100	tcttctctgaagcaggatctgcaggc

OmpA(NcoI) F	gaga <u>CCATGG</u> gaaaaaagacagc
OmpA-his (XbaI) R	gaga <u>TCTAGA</u> tcaagtgggtgggtgggtgggtgagcctgcggctgagt tac
dolP -88	TCAGGAAATGCATATGCTGACGGT
dolP +100	ATGTCGGATGCGGCGTAAACG
RcsF1-K40X_F	GAAAGCGGAGCCTGCATAGCCGAAAGCGCC GCGCG
RcsF2-K40X_R	CGCGCGGCGCTTTCGGCTATGCAGGCTCCGC TTTC
RcsF3-A43X_F	cctgcaaaaccgaaaTAGccgcgcgccacgccg
RcsF4-A43X_R	cggcgtggcgcgcggCTAttcggttttcagg
RcsF5-K61X_F	GAAGAATTAGTCGGCTAGCCGTTCCGCGATC TC
RcsF6-K61X_R	GAGATCGCGGAACGGCTAGCCGACTAATTCT TC
RcsF7-R64X_F	GTCGGCAAACCGTTCTAGGATCTCGGTGAAG TC
RcsF8-R64X_R	GACTTCACCGAGATCCTAGAACGGTTTGCCG AC
RcsF9-N78X_F	tcttgccaggcctctTAGcaggactctccgccg
RcsF10-N78X_R	cggcggagagtcctgCTAagaggcctggcaaga

RcsF11-Q79X_F	TGCCAGGCCTCTAATTAGGACTCTCCGCCGA GC
RcsF12-Q79X_R	GCTCGGCGGAGAGTCCTAATTAGAGGCCTG GCA
RcsF13-D80X_F	caggcctctaatacagTAGtctccgccgagcatt
RcsF14-D80X_R	aatgctcggcggagaCTActgattagaggcctg
RcsF15-S81X_F	gcctctaatacaggacTAGccgccgagcattcca
RcsF16-S81X_R	tggaatgctcggcggCTAgtcctgattagaggc
RcsF17-K100X_F	CAACGCCTCTAAAATGTAGGCCAATGCTGTA TTAC
RcsF18-K100X_R	GTAATACAGCATTGGCCTACATTTTAGAGGC GTTG
RcsF19-E110X_F	TTACTGCATAGCTGCTAGGTCACCAGCGGTA CG
RcsF20-E110X_R	CGTACCGCTGGTGACCTAGCAGCTATGCAGT AA
RcsF21-T115X_F	gaagtcaccagcggTAgccagctgctatcgt
RcsF-T115X_R	acgatagcagcctggCTAaccgctggtgacttc
RcsF22-P116X_F	GTCACCAGCGGTACGTAGGGCTGCTATCGTC AG
RcsF23-P116X_R	CTGACGATAGCAGCCCTACGTACCGCTGGTG AC

RcsF24-G117X_F	accagcggtacgccaTAGtgctatcgtcaggct
RcsF25-G117X_R	agcctgacgatagcaCTAtggcgtaccgctggt
RcsF26-K134X_F	CTTAACATTACGGCGtagTGA CTGCAGGCATG C
RcsF27-K134X_R	GCATGCCTGCAGTCActaCGCCGTAATGTTAA G
KiDo233_Gibs__	
pBAD18	CTGCAGGCATGCAAGCTTG
_for_DolP_2	
KiDo234_Gibs_R	
_pBAD18	GAATTCGCTAGCCCAAAAAACG
_for_DolP_2	
KiDo231_Gibs_F	tttttgggctagcgaattcGAGCTCTCACCAGGATGAT
_pBAD18DolP_2	TAAG
KiDo232_Gibs_R	ccaagcttgcctgcagGTCGACTATTTGAGGATC
_pBAD18DolP_2	CC
KiDo223_F_Muta	
Dir_DolP-V41X	CCCACGCAGTtagGGCACCCAGG
KiDo224_R_Mut	
aDir_DolP-V41X	GGGTGCCCTAACTGCGTGGGTCA
AG293_F_YedD-	ttccgggctgacctacgcacgttgcggc
L47X	

AG294_R_YedD- gccgcaacgtgcgtaggtcagcccgga

L47X

AG295_F_YedD- gctgcctatcgctacgggctgaccagcg

E51X

AG296_R_YedD- cgctggtcagccgtaggcgataggcagc

E51X

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Section III: General discussion, conclusion and perspectives

IL ÉTAIT UN FOIS DANS LA BOÎTE DE PÉTRI:



Chapter V: General discussion, conclusion and perspectives

Like a theater play, my thesis work would be nothing without its two key actors: OmpA and RcsF. On the one hand, OmpA, a β -barrel protein, plays a role in adhesion, toxicity, invasiveness, and biofilm formation in Gram-negative bacteria (Guan, Bhowmick et al. 2021). On the other hand, we have RcsF, the stress-sensor lipoprotein of Rcs, a complex envelope stress response system in *E. coli* and *Enterobacteriaceae* (Meng, Young et al. 2021). This thesis study aimed to better understand the interaction between these two proteins and the importance of the underlying mechanism for bacterial cell physiology.

The journey began in 2014 when our group and others identified the stress sensor RcsF as a surface-exposed lipoprotein (SLP). This discovery was significant in many ways. First, it was the first time that a lipoprotein was detected on the cell surface of *E. coli* by different groups and using different methods. While displaying lipoproteins on the cell surface was well described in certain bacterial phyla such as in *Borrelia spp.*, it was unsuspected in *E. coli* (Schulze and Zuckert 2006, Cho, Szewczyk et al. 2014, Konovalova, Perlman et al. 2014). Second, identifying RcsF on the surface was key to advancing our understanding of the Rcs system: indeed, although Rcs was known to sense OM and LPS defects, no mechanistic explanation was available (Majdalani and Gottesman 2005). Here, on the basis of this discovery, potential stress sensing mechanisms could be proposed, either depending on the interaction between RcsF and BamA (Cho, Szewczyk et al. 2014, Rodriguez-Alonso, Letoquart et al. 2020) or on the interaction between the N-terminal linker of RcsF and LPS molecules (Konovalova, Perlman et al. 2014,

Konovalova, Mitchell et al. 2016). However, almost 10 years later, further research is still required to fully elucidate how RcsF senses OM defects.

Another crucial question that was raised by the detection of RcsF on the cell surface was about the export mechanism. Here again, BamA was identified as the potential export machinery, funneling the lipoprotein to β -barrel proteins. OmpA, OmpC and OmpF were identified as possible vehicles for RcsF. When I joined the lab, several members of the group were investigating questions related to the export mechanism. Most of their work was focused on exploring the BAM-RcsF complex. This work resulted in the X-ray crystal structure of BamA-RcsF, in which we showed that the globular domain of RcsF is present in the lumen of BamA (see *Figure 1*). In addition, the results of this study further supported the idea that BamA-RcsF is an intermediate in the delivery of RcsF to unfolded OM β -barrels (Rodriguez-Alonso, Letoquart et al. 2020).

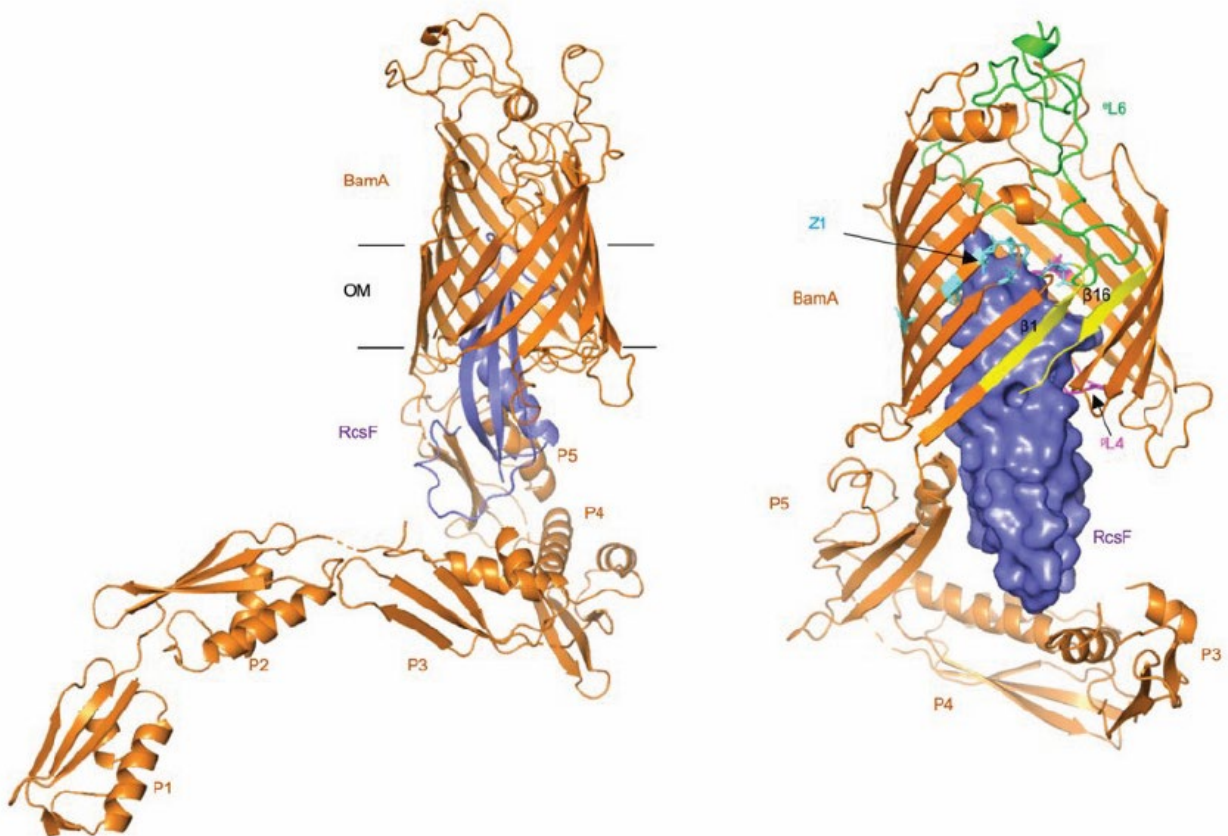


Figure 1. BamA interacts with RcsF suggesting a mechanism for the surface exposure of *E. coli* lipoproteins. Ribbon diagram of the BamA-RcsF complex (PDB:6T1W) in side view. RcsF is colored in blue and BamA in orange. The globular domain of RcsF is presented at the lumen of BamA. This complex is believed to be an intermediate state for RcsF as it is then distributed to OMPs partners. Strand BamA_{b1} and BamA_{b16} are colored in yellow. The interaction zone between BamA and RcsF, called Z1, is colored in cyan. The periplasmic turn L4 is colored in magenta and the extracellular loop L6 is colored in green.(Rodriguez-Alonso, Letoquart et al. 2020).

OmpA buffers the Rcs activity by interacting with RcsF in the periplasm.

My specific goal in the “Rcs project” of the lab was to obtain further structural and molecular insights on the complexes between RcsF and the β -barrels OmpA and OmpC. As OmpA is the main RcsF partner, I first aimed to elucidate the topology of OmpA-RcsF and its role in the Rcs system (Cho, Szewczyk et al. 2014, Konovalova, Perlman et al. 2014, Konovalova, Mitchell et al. 2016). Using a set of biochemical tools, such as *in vivo* site-specific photo-crosslinking, targeted proteolysis, nuclear magnetic resonance (NMR) titration, and bioinformatic modelization; I, together with my colleagues, gained insights into the OmpA-RcsF complex structure. One interesting finding was that we identified the protein-protein interaction interface (PPI) of the complex and showed that RcsF interacts with the C-terminal periplasmic domain of OmpA. This mode of interaction between OmpA and RcsF does not allow OmpA to be the vehicle for the surface exposure of RcsF. Yet, we unraveled that OmpA plays the role of a periplasmic buffer for the Rcs system. The periplasmic domain of OmpA, when interacting with RcsF, prevents the interaction of RcsF with IgaA, the Rcs repressor. Thus, whereas at the start of my research, the working hypothesis was that OmpA would be involved in the surface exposure of RcsF, my results led us to conclude that it was not the case. They also revealed a new level of complexity for the Rcs regulation. Indeed, so far, the regulation of the Rcs was conceptualized as: In non-inducing conditions, RcsF forms complexes with OMPs and is occluded from IgaA, preventing Rcs activation. Upon specific stress, RcsF is no longer funneled to OMPs, accumulates in the periplasm, and is free to bind IgaA, leading to Rcs activation (Cho, Szewczyk et al. 2014). However, our work allows us to hypothesize that the interaction of RcsF with OmpA, occurring in the periplasm, could decrease

the Rcs response: To interact with IgaA, a pool of the RcsF will first have to get rid of its interaction with OmpA. Supporting this hypothesis, we also observe that a $\Delta ompA$ strain background leads to a higher basal Rcs activity highlighting the OmpA buffer role in the Rcs stress response.

The structure of OmpA, with its two domains, is different from that of OmpC and OmpF, which form large β -barrels. It is therefore unlikely that OmpC/F-RcsF complexes are similar to the OmpA-RcsF complex. Further research will be undertaken to investigate the structure of the OmpC/F-RcsF complexes and identify if RcsF is exposed at the surface thanks to OmpC/F. Note that, based on our findings, OmpA-RcsF cannot serve to monitor the state of the lipopolysaccharide leaflet via direct interactions with LPS molecules, which challenges the previously proposed model (Konovalova, Mitchell et al. 2016).

Two major alternative models have been proposed to explain how RcsF senses OM defects (Konovalova, Perlman et al. 2014, Konovalova, Mitchell et al. 2016, Tata and Konovalova 2019, Dekoninck, Letoquart et al. 2020, Rodriguez-Alonso, Letoquart et al. 2020, Tata, Kumar et al. 2021). In the **first model**, the interaction of RcsF with its OM partners (BamA, OmpA, OmpC, and OmpF) plays a significant role in Rcs signaling. Under non-inducing conditions, BamA continuously assembles OMPs and delivers RcsF to them. In complex with OMPs, RcsF is occluded from IgaA, preventing Rcs activation. Under inducing conditions, BamA fails to bind RcsF, the OmpA buffering effect is overcome, and RcsF interacts with IgaA, which releases the inhibition on the Rcs system. Thus, in this first model, it is the BamA-RcsF interaction that is critical: RcsF would sense OM defects by monitoring BAM activity. In the **second model**, which is challenged by my results, RcsF does not monitor BAM activity but somehow transmits stress

signals when in complex with OMPs (Konovalova, Perlman et al. 2014, Konovalova, Mitchell et al. 2016, Saha, Lach et al. 2021) and when interactions between its potentially surface-exposed linker and LPS molecules are perturbed. The two alternative models are shown in *Figure 2*.

We suggest that by gaining more insight into OmpC/F-RcsF complexes, the understanding of the Rcs system will improve. Studying regulation pathways such as Rcs will be important to fight the emergence of resistance in bacteria. Indeed, the cell envelope of Gram-negative bacteria represents a barrier almost impenetrable for most antibiotics. However, by understanding ESR, which maintains envelope integrity and homeostasis, we could identify targets for the development of drugs that would perturb the cell envelope.

The diagram illustrates the Rcs Regulation System in E. coli. It shows the Outer Membrane (OM), Periplasmic Space (PG), and Inner Membrane (IM). In the OM, OmpA is shown. A red starburst labeled 'STRESS' points to the BAM complex (A, B, C, D) and RcsF (green). RcsF is also shown in the PG. A red arrow points from the RcsF in the PG to the Rcs Regulation System (blue box) in the cytoplasm. The Rcs Regulation System is labeled 'Rcs Regulation System'. The diagram also shows OmpC/F (purple) and RcsF (green) in the OM, with a red 'X' over the RcsF in the OM. The diagram is labeled 'periplasm' and 'cytoplasm'.

The diagram illustrates the Rcs Regulation System in *E. coli*. It shows the outer membrane (OM) and periplasmic space (PG) above the inner membrane (IM) and cytoplasm. The BAM complex (A, B, C, D) is embedded in the OM. RcsF (green) is a periplasmic protein that binds to the BAM complex. Unfolded OMP (orange) is shown in the periplasm. RcsF binds to OmpC/F (purple) and OmpA (purple) in the OM. A red starburst labeled "STRESS" indicates the trigger for the system. Red arrows point from the RcsF-OmpC/F and RcsF-OmpA complexes to the Rcs Regulation System (blue box) in the cytoplasm.

Figure 2. Two proposed models for the envelope stress sensing mechanism via RcsF. *Left panel:* The Bam machinery mediates the threading of RcsF into OmpC/F. Upon stress, the interaction of RcsF with the lumen of BamA is compromised so as the formation of OmpC/F-RcsF complexes. RcsF accumulates into the periplasm and activates the Rcs system. RcsF interacts with the periplasmic domain of OmpA, which is a manner to buffer the Rcs system. *Right panel:* RcsF forms complexes with OmpA/C/F by being threaded through their lumens. OM and LPS stresses can impact the complex formation and allow RcsF to activate the Rcs regulation system.

A flaw in the method: Highlighting an artifact using site-specific photo-crosslinking.

Nowadays, most of our antibiotics kill bacteria by targeting the cell wall, protein, and nucleic acid synthesis. Novel approaches are urgently required to develop new types of drugs and tackle the public health threat caused by the emergence of multiresistant bacterial strains. In this context, recent years have seen an increasing amount of research aiming to identify new targets for drug development. Among others, RcsF and its OMPs partners, given the role they play in maintaining the cell envelope integrity, have been designated the enemy to kill (Cho, Szewczyk et al. 2014, Konovalova, Perlman et al. 2014, Konovalova, Mitchell et al. 2016, Konovalova, Kahne et al. 2017, Tata and Konovalova 2019, Guan, Bhowmick et al. 2021). However, it is one thing to identify a target to shoot; it is another to know how to do it. In particular, this requires a full understanding of biological pathways in which the target plays a part. A way to contribute to this goal is by unraveling the target interaction networks which can be achieved using various methods such as site-specific photo-crosslinking. It is an increasingly popular technique in which a photoactivable amino acid, such as *p*-benzoyl-L-

phenylalanine (*p*Bpa), is genetically inserted in a protein of interest to trap its interacting partners.

We intensively used site-specific photo-crosslinking during our research on the Rcs system. To that end, we used two different crosslinkers: The First is the *p*Bpa which is based on benzophenone's ability, under UV exposure, to give rise to biradical species. The second, is the DiZPK which is based on the ability, under UV, of the diazirine group to give rise to a carbene reactive group. While these two crosslinkers do not share chemical and reactive similarities, they are both efficiently used in site-specific photo-crosslinking experiments. Note that diazirine required a shorter UV exposure time compared to benzophenone. While optimizing the method to study the OmpA-RcsF complex, we discovered a flaw in the method that can lead and already led to wrong identification (Ranava, Yang et al. 2021) of protein-protein interaction interfaces.

We decided to investigate this issue to warn people about the potential misinterpretation of results obtained by site-specific photo-crosslinking. We demonstrated that all *p*Bpa-containing RcsF variants bind a hydrophobic pocket in OmpA, regardless of the location of *p*Bpa in RcsF. The ability of this pocket to bind *p*Bpa was reduced by changing its chemical properties. Moreover, when inserted in other bacterial cell envelope proteins, *p*Bpa was also found to bind the OmpA's hydrophobic pocket. Overall, our findings suggest that OmpA's hydrophobic pocket acts like a molecular magnet, or as we like to call it, a black hole. Indeed similar to black hole drawing lights, the hydrophobic pocket of OmpA will attract *p*Bpa regardless of its location in partner proteins, making photo-crosslinking with *p*Bpa a counterproductive method for identifying interaction interfaces on OmpA.

In this work, we elucidated an unexpected flaw of the site-specific photocrosslinking method. We believe it is important to report this kind of artifactual effects of method; for instance, in a recent work by *Ranava et al.* (Ranava, Yang et al. 2021), site-specific photo-crosslinking were wrongly interpreted. In their study, these authors investigated the interaction of the lipoprotein DolP with OM partners, and described OmpA and its hydrophobic pocket as the interaction interface for DolP. By doing so, they probably have drawn an erroneous conclusion from their work. Because similar pockets with an affinity for *p*Bpa or other photoactivable amino acids are likely to exist in proteins other than OmpA, great care should be taken when mapping the interaction zone between two proteins using methods that we often imagine as foolproof.

Conclusion and Perspectives.

In the context of this thesis, we had a particular interest in the OM of *E. coli*. By studying the Rcs stress response system, we explored questions related to the function of OMPs in stress response systems. The results presented in this manuscript explore the complex's topology and function between RcsF and OmpA. We bring evidence that OmpA is unlikely the vehicle allowing RcsF to be surface-exposed and that OmpA is involved in fine-tuning Rcs activity. During my work on OmpA-RcsF, I came upon discovering an unsuspected flaw in the widely used site-specific photocrosslinking method.

Yet, my work is just a beginning as much more remains to be done. What we discovered about OmpA-RcsF actually generates more questions that need to be addressed than answers. Among all of these unsolved questions, the most crucial is determining which RcsF partners in the OM are the vehicles for its surface exposure. The answer will likely reveal how RcsF senses LPS and OM defects and luckily will lead to identifying new targets for developing antimicrobial compounds inhibiting crucial steps in the OM biogenesis of *E. coli*.

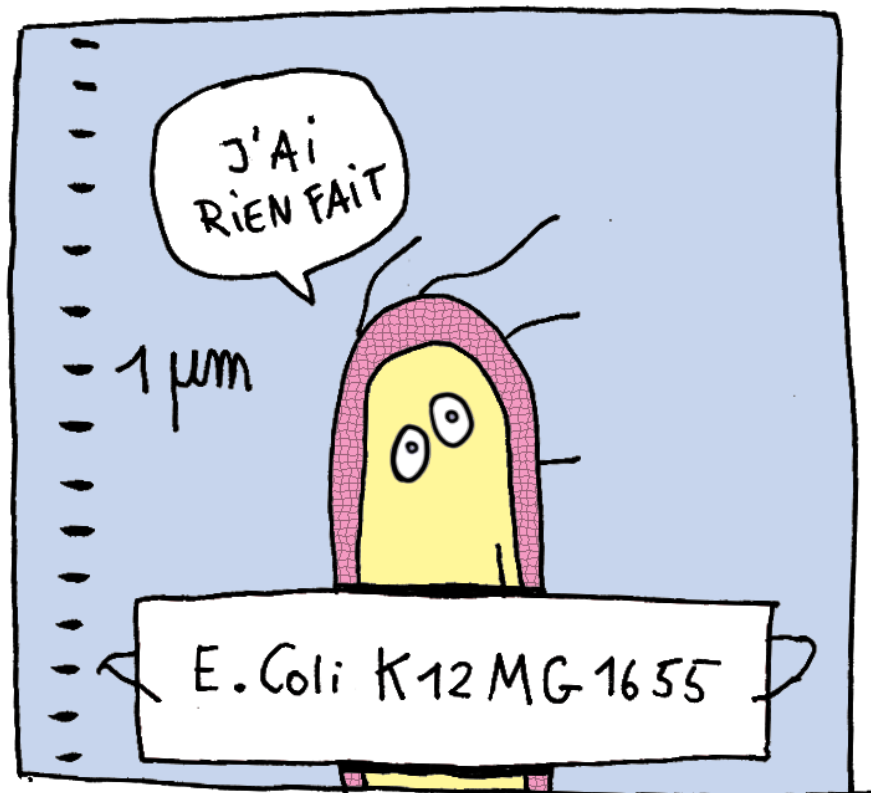


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Section IV: Annex



I. Defining the function of OmpA in the Rcs stress response

Defining the function of OmpA in the Rcs stress response

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Abstract OmpA, a protein commonly found in the outer membrane of Gram-negative bacteria, has served as a paradigm for the study of β -barrel proteins for several decades. In *Escherichia coli*, OmpA was previously reported to form complexes with RcsF, a surface-exposed lipoprotein that triggers the Rcs stress response when damage occurs in the outer membrane and the peptidoglycan. How OmpA interacts with RcsF and whether this interaction allows RcsF to reach the surface has remained unclear. Here, we integrated in vivo and in vitro approaches to establish that RcsF interacts with the C-terminal, periplasmic domain of OmpA, not with the N-terminal β -barrel, thus implying that RcsF does not reach the bacterial surface via OmpA. Our results suggest a novel function for OmpA in the cell envelope: OmpA competes with the inner membrane protein IgaA, the downstream Rcs component, for RcsF binding across the periplasm, thereby regulating the Rcs response.

Introduction

The cell envelope is the morphological hallmark of Gram-negative bacteria. It consists of an inner membrane (IM) surrounding the cytoplasm as well as an outer membrane (OM), an asymmetric bilayer with phospholipids in the inner leaflet and lipopolysaccharides in the outer leaflet (Silhavy et al., 2010). The two membranes are separated by the periplasm, a compartment in which lies a thin layer of peptidoglycan. The cell envelope is essential for viability: the OM serves as a permeability barrier against toxic compounds present in the environment while the peptidoglycan provides shape and osmotic protection to cells (Okuda et al., 2016; Typas et al., 2012; Egan et al., 2020).

Given the functional and structural importance of the envelope, bacteria need to respond to breaches in envelope integrity in a fast and adequate manner. Bacteria have therefore evolved sophisticated signaling systems that monitor envelope integrity and respond to perturbations (Ruiz and Silhavy, 2005; MacRitchie et al., 2008; Delhaye et al., 2019). In *Escherichia coli* and the Enterobacteriaceae, the Rcs system detects damage to the OM and the peptidoglycan (Wall et al., 2018; Laubacher and Ades, 2008; Farris et al., 2010). In response, Rcs modulates the expression of dozens of genes, including those involved in the biosynthesis of colanic acid, an exopolysaccharide that accumulates on the cell surface to form a protective capsule (Wall et al., 2018; Laloux and Collet, 2017).

Rcs signal transduction involves a multi-step phosphorelay (Wall et al., 2018). Under stress, the IM histidine kinase RcsC autophosphorylates, transfers the phosphoryl group to the IM protein RcsD and finally to the cytoplasmic response regulator RcsB. Rcs activity is modulated by two proteins that are not part of the phosphorylation cascade: RcsF and IgaA. RcsF is an OM lipoprotein that senses most Rcs-inducing cues, while IgaA is an essential IM protein that down-regulates Rcs (Takeda et al., 2001; Domínguez-Bernal et al., 2004) by interacting with RcsD (Wall et al., 2020). When perturbations occur in the peptidoglycan or in the OM, RcsF, while remaining anchored in the OM, reaches across the periplasm to interact with IgaA, leading this protein to alleviate its inhibition of the phosphorelay, turning on Rcs (Cho et al., 2014; Hussein et al., 2018). In the absence of stress, RcsF is occluded from IgaA by interacting with OM proteins. A complex between RcsF and BamA, the core component of the β -barrel assembly machinery (BAM), was identified (Cho et al., 2014; Konovalova et al., 2014) and its structure solved (Rodríguez-Alonso et al., 2020). This complex forms as an intermediate (Cho et al., 2014; Konovalova et al., 2014): delivery of unfolded OM β -barrels (OMPs) to BAM triggers the release of RcsF from BamA and its transfer to OMP partners (Rodríguez-Alonso et al., 2020). Three abundant OMPs (OmpA, OmpC and OmpF) have been identified as RcsF partners (Cho et al., 2014; Konovalova et al., 2014). Under stress conditions, newly synthesized RcsF molecules fail to interact with BamA (Cho et al., 2014): they remain in the periplasm, free to bind IgaA, triggering Rcs.

Crucially, whereas the general view is that OM lipoproteins are oriented toward the periplasm, previous work concluded that at least a portion of RcsF, a protein which is composed of an N-terminal disordered linker and a C-terminal globular domain required for signaling (Leverrier et al., 2011; Rogov et al., 2011), is exposed on the cell surface; OmpA, OmpC, and OmpF, but not BamA (Cho et al., 2014), were identified as potential vehicles for RcsF surface exposure (Cho et al., 2014; Konovalova et al., 2014; Konovalova et al., 2016). In a topological model of RcsF surface exposure (Konovalova et al., 2014), the lipid moiety of RcsF is anchored in the outer leaflet of the OM and the N-terminal disordered linker is exposed on the cell surface before being threaded through the lumen of the OMP partners. However, definitive evidence for this model is still lacking.

In addition, because OmpC, OmpF, and OmpA belong to two distinct structural groups, it is unclear whether RcsF interacts with its three OMP partners in a similar way. Indeed, OmpC and OmpF form 16-stranded β -barrels that associate into trimers in the OM. Because they form large β -barrels, they display a central pore (Baslé et al., 2006; Yamashita et al., 2008; Radhakrishnan et al., 2010; Housden et al., 2013) that is large enough to accommodate a disordered peptide such as the RcsF linker. The situation is less clear for OmpA: although this protein, the most abundant OMP in *Escherichia coli*, has been studied for more than four decades, how it folds remains controversial. While some studies indicate that OmpA can also fold into a 16-stranded β -barrel with a large central pore (Singh et al., 2003; Stathopoulos, 1996), the predominant view is that OmpA adopts a two-domain structure with an N-terminal eight-stranded β -barrel inserted in the OM (Pautsch and Schulz, 1998) and a C-terminal, globular domain in the periplasm (Mot and Vanderleyden, 1994; Park et al., 2012). In this conformation, the β -barrel of OmpA is too small to accommodate a polypeptide. Thus, despite the tremendous work that has been done on OmpA, we do not know whether this protein adopts the large β -barrel structure when in complex with RcsF, or whether it folds into the predominant two-domain conformation. If the latter, where does RcsF bind OmpA?

To resolve these outstanding structural and mechanistic questions, here we dissected the OmpA-RcsF complex. By combining in vivo site-specific photo-crosslinking, targeted proteolysis, and nuclear magnetic resonance (NMR) titration, we established that OmpA adopts its two-domain structure when in complex with RcsF and that it is the C-terminal, periplasmic domain—not the β -barrel—that interacts with the lipoprotein. In addition, we identified residues in RcsF and in OmpA that are involved in the interaction, thus providing information about the binding interface. Taken together, our results indicate that the topology of OmpA-RcsF is different from that of OmpC/F-RcsF; they also imply that RcsF does not use OmpA to reach the cell surface. This has important implications for how RcsF senses OM stress: if the linker of RcsF is not on the surface in the OmpA-RcsF complex, then OmpA-RcsF cannot serve to monitor the state of the lipopolysaccharide leaflet via direct interactions with lipopolysaccharide molecules, as previously proposed (Konovalova et al., 2016). Finally, we determined the equilibrium dissociation constants of both the C-terminal domain of OmpA and the periplasmic domain of IgaA for RcsF and provide evidence suggesting that OmpA

and IgA compete for RcsF binding across the periplasm. Our results support a model in which OmpA serves as a buffer for RcsF, titrating it from IgA, thereby fine-tuning Rcs activity.

Results

RcsF interacts with the C-terminal region of OmpA in vivo

The stress sensor lipoprotein RcsF was previously shown to be surface-exposed (Cho *et al.*, 2014; Kononova *et al.*, 2014), and OmpA was described as a possible vehicle for its surface exposure (Cho *et al.*, 2014; Kononova *et al.*, 2014). However, how OmpA folds when in complex with RcsF and whether this interaction allows RcsF to become surface-exposed remain to be determined.

To close this gap, we characterized the OmpA-RcsF interaction. In a previous study, we identified six RcsF residues (in the N-terminal disordered linker and at the tip of the signaling domain) as being part of the interaction interface between RcsF and OmpA (Cho *et al.*, 2014). These residues were identified using a site-specific photo-crosslinking strategy in which a photoreactive, crosslinkable amino acid is inserted at specific positions in the protein of interest, with the help of an exogenous orthogonal tRNA/aminoacyl-tRNA synthetase pair (Chin *et al.*, 2002). We first sought to confirm and extend these results to more clearly define the binding interface in RcsF. Instead of using the hydrophobic crosslinker *p*-benzoyl-L-phenylalanine like before (Cho *et al.*, 2014), we used *N*^ε-((3-(3-methyl-3*H*-diazirin-3-yl)propyl)carbamoyl)-L-lysine (DiZPK), a lysine analog with substantially higher photo-crosslinking efficiency than *p*-benzoyl-L-phenylalanine (Zhang *et al.*, 2011). We selected 11 positions distributed along the RcsF sequence, including four (R21, Q28, Q33, R45) in the disordered linker and seven on the surface of the signaling domain (N54, Q79, R89, K98, E110, P116, Q121) (Figure 1). After UV illumination, a ~ 55 kDa band, the size of the OmpA (40 kDa)-RcsF (14 kDa) complex, formed and was detected with an anti-RcsF antibody (Figure 1) for the following variants (in decreasing intensity): RcsF_{R89X}, RcsF_{P116X}, RcsF_{Q79X}, RcsF_{R45X}, RcsF_{K98X}, RcsF_{Q121X}, RcsF_{N54X}, and RcsF_{E110X}. The identity of the ~55 kDa band as OmpA-RcsF was further verified by showing that it did not form in Δ ompA cells (Figure 1—figure supplements 1 and 2). Thus, as in our previous study (Cho *et al.*, 2014), complex formation was observed when the photoactivatable amino acid was inserted in the linker (R45) or at the tip of the signaling domain (Q79 and P116). The complex also formed when DiZPK was incorporated in other regions of the signaling domain, such as α -helix 1 (N54), α -helix 2 (R89 and K98), β -strand 2 (E110), and β -strand 3 (Q121) (Figure 1). Noteworthy, residues Q79, R89 and P116 are part of the binding interface between RcsF and the luminal wall of the BamA β -barrel in the recently published structure of the BamA-RcsF complex (Rodríguez-Alonso *et al.*, 2020). Taken together, these observations substantially enlarge the region of RcsF known to interact with OmpA. Of note, we observed that the RcsF_{R45X}, RcsF_{R89X} and RcsF_{K98X} variants formed a UV-dependent band migrating slightly higher than the OmpA-RcsF complex (Figure 1). Focusing on RcsF_{R89X}, we identified this band as a complex between RcsF and OmpC/F because it did not form when *ompR*, a transcription factor required for the production of OmpC and OmpF (Hall and Silhavy, 1979; Chubiz and Rao, 2011), was deleted (Figure 1—figure supplement 2).

We next sought to identify where RcsF binds OmpA. The general view is that OmpA consists of two domains, an eight-stranded β -barrel anchoring the protein in the OM and a soluble C-terminal domain located in the periplasm, where it binds the peptidoglycan (Mot and Vanderleyden, 1994; Park *et al.*, 2012; Figure 2A). However, an alternative conformation has been proposed in which OmpA folds into a large, 16-stranded β -barrel (Singh *et al.*, 2003; Stathopoulos, 1996; Figure 2A). To gain insight into where RcsF binds OmpA and to characterize OmpA's conformation when in complex with RcsF, we first engineered an OmpA variant with a thrombin cleavage site inserted after residue V189 (OmpA_{TH_189}), in the middle of the OmpA sequence (Figure 2B). Taking the two-domain structure as a reference, the cleavage site was inserted between the N- and C-terminal domains. We then selected three DiZPK-containing RcsF mutants (RcsF_{R45X}, RcsF_{Q79X}, RcsF_{R89X}) that formed a covalent complex with OmpA at high levels when exposed to UV light (Figure 1). These variants display DiZPK in three regions of RcsF: in RcsF_{R45X}, DiZPK is present at the end of the disordered linker, while RcsF_{Q79X} displays DiZPK in the large loop at the tip of the signaling domain and RcsF_{R89X} displays it on the central α -helix 2 (Figure 1). These variants were expressed in *E. coli* cells also producing OmpA_{TH_189}, and complex formation was induced with UV light (Figure 2C). For all

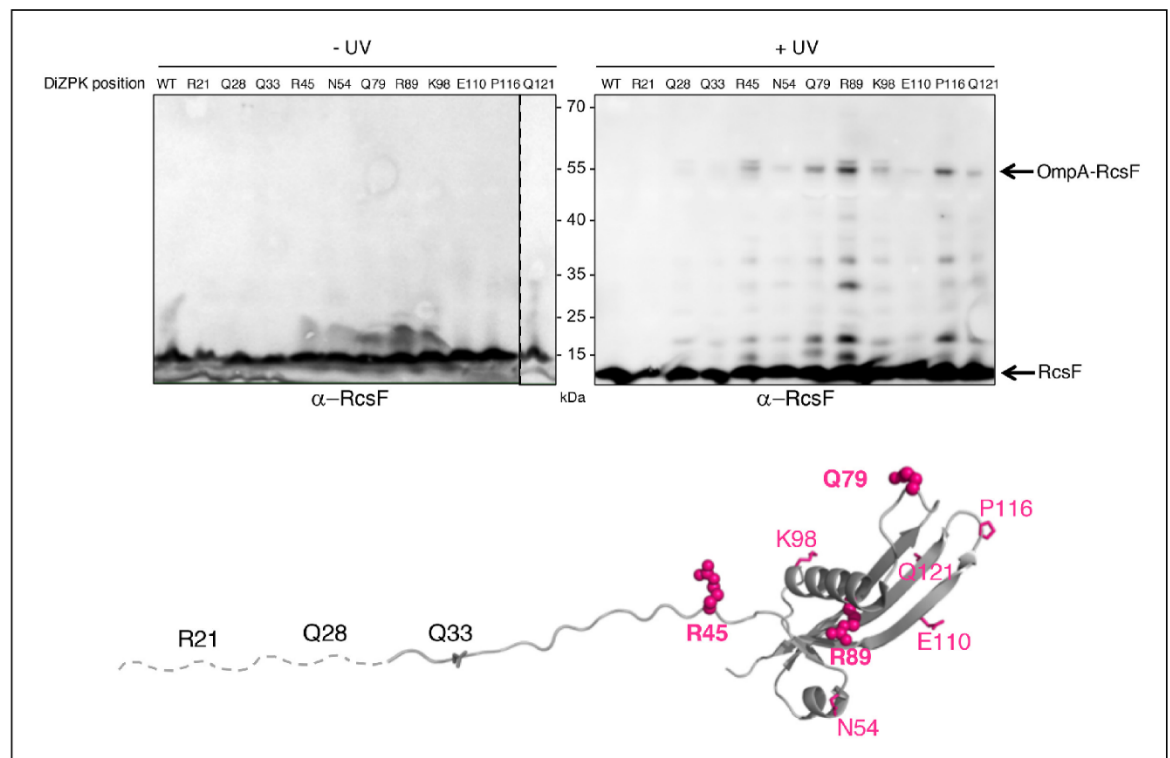


Figure 1. Defining the binding interface of RcsF on OmpA using in vivo site-specific photo-crosslinking. Upper panel: Δ rscF cells expressing wild-type (WT) RcsF or DiZPK-containing RcsF variants (from pSC253) were irradiated with UV light (+) or not (-), and protein samples were immunoblotted with an anti-RcsF antibody. A 55 kDa band, corresponding to the size of the OmpA-RcsF complex, was observed for eight of the mutants (R45, N54, Q79, R89, K98, E110, P116, Q121). Lower panel: residues of RcsF were replaced by DiZPK to map the zone of interaction with OmpA. In this cartoon of the NMR structure of RcsF (PDB: 2L8Y), the truncated N-terminal portion of the protein is shown as a dashed line and the residues that were found to interact with OmpA appear in magenta. The side chains of the residues that were selected for further experiments are shown as spheres, and other side chains are represented as sticks.

The online version of this article includes the following figure supplement(s) for figure 1:

Figure supplement 1. The UV-dependent 55 kDa band is the OmpA-RcsF complex.

Figure supplement 2. The band migrating above the OmpA-RcsF complex is the OmpC/F-RcsF complex.

three RcsF variants, digestion of the 55 kDa OmpA_{TH189}-RcsF complex with thrombin yielded a ~35 kDa band that was recognized by both anti-RcsF and anti-His antibodies (Figure 2C,D). Given the presence of a His-tag in the C-terminus of OmpA (Materials and Methods), we concluded that RcsF (14 kDa) interacts with the C-terminal region of OmpA (~16 kDa) in vivo.

To further delineate the region of OmpA that is important for complex formation, a second OmpA mutant with a thrombin cleavage site inserted after residue I243 (OmpA_{TH243}) was generated (Figure 2B). Like OmpA_{TH189}, OmpA_{TH243} (~10 kDa) could be photo-crosslinked to the three DiZPK-containing RcsF variants described above (Figure 2C,D). In all three cases, thrombin digestion of the OmpA_{TH243}-RcsF complexes generated a smaller band (~30 kDa) that was recognized by anti-RcsF antibodies (Figure 2C,D). In contrast to OmpA_{TH189}-RcsF, which underwent complete digestion, OmpA_{TH243}-RcsF only underwent partial cleavage (Figure 2C,D), which probably reflected decreased accessibility of the cleavage site to the protease. This ~30 kDa band was also detected by anti-His antibodies (Figure 2D), indicating that the three tested residues of RcsF bind the region of OmpA between residue I243 and the C-terminus.

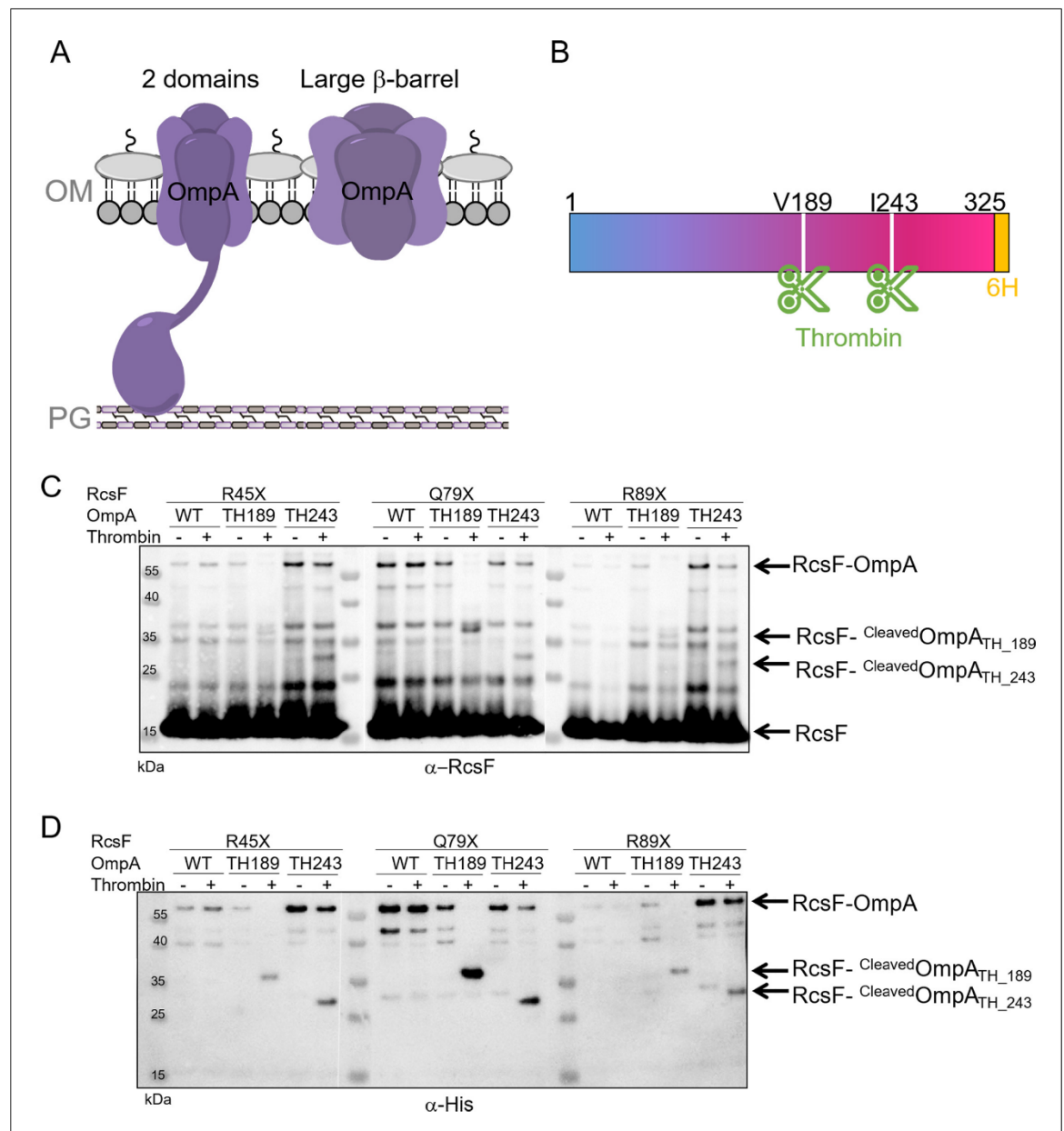


Figure 2. RcsF interacts with the C-terminal portion of OmpA in vivo. (A) Schematic of the two conformations of OmpA. Left: the predominant view is that OmpA adopts a two-domain structure, with an N-terminal β -barrel embedded in the OM and a C-terminal periplasmic domain binding the peptidoglycan. Right: an alternative conformation in which OmpA folds into a large β -barrel has also been proposed (Singh et al., 2003; Stathopoulos, 1996). (B) Schematic of OmpA variants containing a thrombin-specific cleavage site (scissors at positions V189 and I243) and a 6x-histidine tag (orange) at the C-terminus. (C–D) In vivo site-specific photo-crosslinking of RcsF. Δ rscF cells co-expressing one of the DiZPK-containing RcsF variant (R45X, Q79X, or R89X) together with OmpA (wild-type, or with a thrombin site inserted at V189 or I243) were UV-irradiated. The RcsF

Figure 2 continued on next page

Figure 2 continued

variants were expressed from pSC253; OmpA, OmpA_{TH_189}, and OmpA_{TH_243} were expressed from the chromosome. After immunoprecipitation with anti-RcsF, protein samples were incubated (+) or not (-) with thrombin and immunoblotted with an anti-RcsF (C) or an anti-His-tag antibody (D). At least partial digestion of the ~55 kDa complex corresponding to OmpA-RcsF occurred with all three DiZPK-containing RcsF variants, yielding a band (RcsF^{-cleaved}OmpA_{TH_189} or RcsF^{-cleaved}OmpA_{TH_243}) migrating at lower molecular weights that was detected by both antibodies.

The C-terminal region of OmpA is necessary and sufficient for binding RcsF

The results above indicated that RcsF interacts with the C-terminal region of OmpA, raising the question of whether the N-terminal region also participates in this interaction. To probe this potential interaction directly, we generated an OmpA variant lacking the C-terminal moiety (OmpA₁₋₁₇₀) and tested whether it could be crosslinked to RcsF (Figure 3A). Chemical crosslinking was carried out with 3,3'-dithio-bis[sulfosuccinimidylpropionate] (DTSSP) (Cho et al., 2014). Although complexes formed in cells expressing wild-type OmpA, no complex was detected in cells producing OmpA₁₋₁₇₀ (Figure 3A). In addition, expression of OmpA₁₋₁₇₀ did not suppress the activation of the Rcs system (Figure 3A, see Figure 3—source data 1 for statistics) that occurs in cells lacking OmpA and that results from the inability of RcsF to interact with this β -barrel (Cho et al., 2014).

To test whether the N-terminal domain of OmpA was indirectly required for the OmpA-RcsF complex to form, we generated a hybrid protein (OmpA_X) in which the C-terminal region of OmpA (OmpA₁₇₁₋₃₂₅), corresponding to the periplasmic domain in the two-domain structure, was fused to OmpX (Figure 3B). OmpX is a small eight-stranded β -barrel that constitutes a structural homolog of the N-terminal region of OmpA when it folds as a small β -barrel (the two β -barrels can be superimposed with a root mean square deviation (RMSD) of 2.49 Å; Figure 3—figure supplement 1A). OmpX does not share sequence homology with the N-terminal region of OmpA (~25%) and can thus be considered as an OM anchor for the C-terminal region when fused to the latter as in OmpA_X (Figure 3B). We found that OmpA_X could be crosslinked to RcsF (Figure 3B) and that its expression fully suppressed Rcs activation (Figure 3B, see Figure 3—source data 1 for statistics). Because we could not completely exclude the unlikely possibility that OmpA_X could rearrange into a large β -barrel able to bind RcsF, we prepared an additional variant of OmpA (OmpA_{Pal}) in which the C-terminal domain (OmpA₁₇₁₋₃₂₅) was fused to the signal sequence and lipobox (for lipid modification; Szewczyk and Collet, 2016) of the OM lipoprotein Pal, thus converting the C-terminal domain of OmpA into a lipoprotein (Figure 3C). Remarkably, expression of OmpA_{Pal} led to the formation of OmpA_{Pal}-RcsF and substantially decreased Rcs activity (Figure 3C, see Figure 3—source data 1 for statistics). In these experiments, we confirmed that the expression levels of OmpA₁₋₁₇₀, OmpA_X and OmpA_{Pal} were similar to those of the wild type (Figure 3—figure supplement 2). Thus, the C-terminal region of OmpA is necessary and sufficient for the interaction with RcsF, and the N-terminal region is dispensable. Altogether, these results are consistent with the conclusion that OmpA adopts its two-domain structure—and not the large β -barrel conformer—when in complex with RcsF. We therefore conclude that RcsF interacts with the C-terminal, globular domain of OmpA and that this interaction takes place on the periplasmic side of the OM.

RcsF interacts with the periplasmic domain of OmpA in vitro

We next sought to validate our in vivo observations by probing the formation of a complex between RcsF and the soluble, periplasmic domain of OmpA (OmpA₁₈₆₋₃₂₅) in vitro using purified proteins. However, attempts to pull-down OmpA₁₈₆₋₃₂₅ with a soluble, His-tagged version of RcsF failed (data not shown), suggesting that the interaction between these two proteins was weak. Because NMR is a highly effective tool to investigate weak protein-protein interactions (Vaynberg and Qin, 2006), we employed NMR titration experiments of ¹⁵N-labeled OmpA₁₈₆₋₃₂₅ by RcsF. In this approach, the ¹⁵N-¹H 2D NMR spectra of OmpA were recorded upon addition of increasing amounts of RcsF (2 and 10 molar equivalents); when OmpA and RcsF interact, concentration-dependent perturbations in the NMR spectra appear. Upon addition of RcsF, several OmpA residues showed chemical shift variations in ¹⁵N-¹H 2D correlation experiments (Figure 4A,B, see Figure 4—source data 1 for details). Most of the shifted residues (T240, G244, S245, D246, A247, G251, L252, K294 and A297; Figure 4B) were located near the tip of the periplasmic domain (Figure 4C,D), identifying this

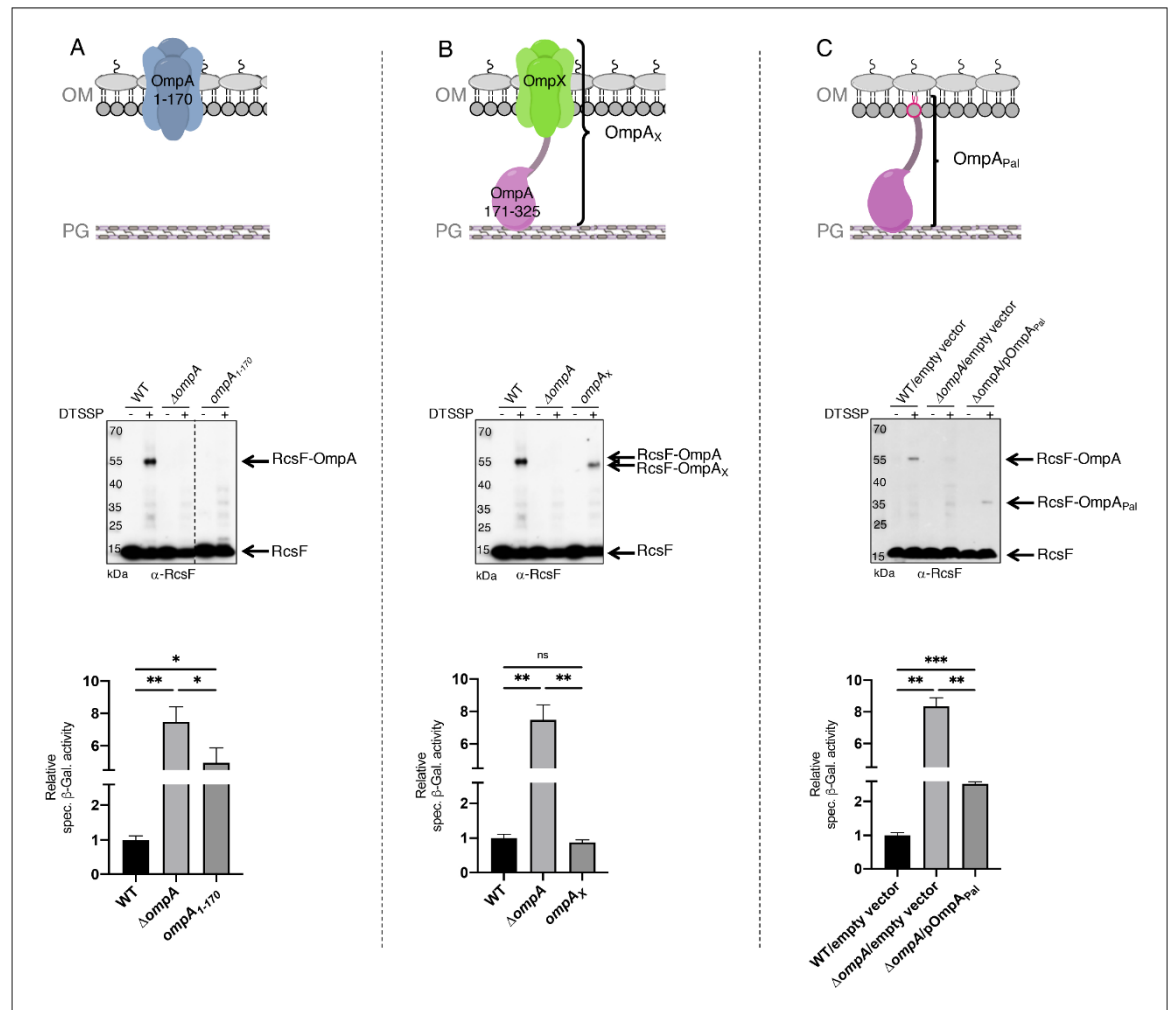


Figure 3. The periplasmic domain of OmpA is necessary and sufficient for the interaction with RcsF in vivo. (A) *Upper panel:* Schematic of the truncated OmpA variant corresponding to the 8-stranded β -barrel domain (OmpA₁₋₁₇₀). *Middle panel:* In vivo chemical crosslinking of RcsF to OmpA and OmpA₁₋₁₇₀. WT, Δ ompA and Δ ompA::ompA₁₋₁₇₀ DH300 cells were incubated with or without 3,3'-dithio-bis[sulfosuccinimidylpropionate] (DTSSP). Proteins were immunoblotted with anti-RcsF (same for the middle panel in B and C). The OmpA-RcsF complex was detected in WT cells but not in cells expressing OmpA₁₋₁₇₀. *Lower panel:* β -galactosidase (β -gal) activity was measured using the transcriptional *rprA-lacZ* fusion on the chromosome (Majdalani et al., 2002) using the same DH300 strains as in the middle panel (same for the lower panel in B and C). Deleting *ompA* induces Rcs, expression of OmpA₁₋₁₇₀ does not restore basal Rcs activity. (B) *Upper panel:* Schematic of the hybrid protein consisting of a fusion between the 8-stranded β -barrel OmpX and the periplasmic domain of OmpA (OmpA₁₇₁₋₃₂₅) (OmpA_X). *Middle panel:* In vivo chemical crosslinking of RcsF to OmpA and OmpA_X. WT, Δ ompA and Δ ompA::ompA_X cells were incubated with or without DTSSP. The RcsF-OmpA complex was detected in WT cells as well as in cells expressing OmpA_X (RcsF-OmpA_X). *Lower panel:* Deleting *ompA* induces Rcs, expression of OmpA_X restores basal Rcs activity. (C) *Upper panel:* Schematic of the C-terminal domain of OmpA (OmpA₁₇₁₋₃₂₅) fused to the signal sequence and lipobox of the OM lipoprotein Pal (OmpA_{Pal}). *Middle panel:* In vivo chemical crosslinking of RcsF to OmpA and OmpA_{Pal}. WT and Δ ompA harboring pDSW204, an empty vector, used as control, and Δ ompA cells harboring pKid22, expressing OmpA_{Pal} from an IPTG-inducible promoter, were incubated with or without DTSSP. The OmpA-RcsF complex was detected in WT cells as well as in cells expressing OmpA_{Pal} (OmpA_{Pal}-RcsF). *Lower panel:* Deleting *ompA* induces Rcs, expression of OmpA_{Pal} substantially decreases Rcs activity. See Figure 3—source data 1 for details and statistics of middle panels. Mean (n = 3) and standard deviation (error bars) are shown. Differences were evaluated with Student's t test (ns, not significant; *p<0.05; ***p<0.001).

Figure 3 continued on next page

Figure 3 continued

The online version of this article includes the following source data and figure supplement(s) for figure 3:

Source data 1. Raw source data for middle panels of **Figure 3A,B,C**.

Figure supplement 1. The eight-stranded β -barrels of OmpA and OmpX are structurally similar and do not display an open channel through the membrane.

Figure supplement 2. The expression levels of the OmpA variants are similar to those of wild-type OmpA.

region, and in particular a flexible loop between β -strand two and α -helix 3, as part of the binding interface with RcsF. The importance of this loop for the OmpA-RcsF interaction was confirmed using site-specific photo-crosslinking: OmpA was strongly crosslinked to RcsF when DIZPK was introduced at residue D246, while weak complex formation was observed with OmpA_{R242X} and OmpA_{V248X} (**Figure 4E**). These results nicely fit with those obtained using OmpA_{TH_243} (**Figure 2B**) that identified the same region of the C-terminal domain of OmpA as part of the zone of interaction with RcsF. Thus, taken together, our results allow us to conclude that RcsF interacts with the C-terminal domain of OmpA in its globular conformation, not only in vivo but also in vitro.

IgaA and OmpA likely compete for RcsF across the periplasmic space

RcsF turns on the Rcs response by interacting with the periplasmic domain of the IM protein IgaA under stress (*Hussein et al., 2018*). Here, we found that the interaction between RcsF and OmpA takes place in the periplasm (**Figure 5A**), thus suggesting that OmpA and IgaA compete for binding RcsF across this compartment. To investigate this hypothesis, we determined the effect of artificially increasing the IgaA concentration on the formation of the IgaA-RcsF and OmpA-RcsF complexes. In these experiments, a triple Flag-tagged, functional version of IgaA (*Hussein et al., 2018*) was expressed from an inducible plasmid. To monitor complex formation, we carried out chemical cross-linking using bis(sulfosuccinimidyl)suberate (BS3), a bifunctional crosslinker. Increasing the levels of IgaA led to changes in the IgaA-RcsF and OmpA-RcsF complexes that were inversely correlated: whereas overexpressing IgaA led to more IgaA-RcsF, it decreased the levels of the OmpA-RcsF complex (compare lanes 9 and 10 with lanes 7 and 8 in **Figure 5B**). Thus, IgaA and OmpA seem to compete for RcsF across the periplasm. Because IgaA (200 copies per cell; *Li et al., 2014*) is far less abundant than OmpA (200,000 copies), even when overexpressed (we estimate that IgaA levels were increased 8–40 fold over baseline (**Figure 5—figure supplement 1**; Materials and methods) in the experiment above), these results suggested that IgaA has a substantially higher affinity for RcsF than OmpA. To probe this directly, we determined the affinity constants of the periplasmic domains of OmpA and IgaA for RcsF. Note that the periplasmic domain of IgaA interacts with RcsF in vivo and in vitro (*Cho et al., 2014*; *Hussein et al., 2018*). First, from the NMR shift data, we calculated the equilibrium dissociation constant (K_D) of OmpA₁₈₆₋₃₂₅ for RcsF as being $125 \pm 85 \mu\text{M}$ (**Figure 5C**). Second, using biolayer interferometry, we measured that the periplasmic domain of IgaA has a K_D of $1.6 \pm 0.3 \text{ nM}$ for RcsF (**Figure 5D**, see **Figure 5—source data 2** for statistics). Thus, these values confirm that IgaA has substantially more affinity for RcsF than OmpA (see Discussion). Interestingly, we noted that the BamA-RcsF complex was not modified by the increased expression of IgaA (lanes 7–10 in **Figure 5B**), which is consistent with the fact that BamA has a much higher affinity for RcsF ($K_D \sim 400 \text{ nM}$; *Rodríguez-Alonso et al., 2020*) than OmpA.

Discussion

OmpA is unlikely the vehicle allowing RcsF to reach the surface

OmpA was first purified from *E. coli* membranes in 1977 (*Chai and Foulds, 1977*) and has served as a model for OMP assembly since then. Although the predominant view is that OmpA folds into a two-domain conformation, with an N-terminal eight-stranded β -barrel and a C-terminal periplasmic domain, an alternative conformation in which OmpA forms a single, 16-stranded β -barrel, has been proposed to also exist (*Singh et al., 2003*; *Stathopoulos, 1996*). Here, we integrated in vivo (**Figures 2, 3, 4 and 5**) and in vitro (**Figures 4 and 5**) approaches to dissect the interaction between OmpA and the lipoprotein RcsF; altogether, our data establish that OmpA is in the two-domain conformation in the OmpA-RcsF complex and that it is the C-terminal, periplasmic domain of OmpA

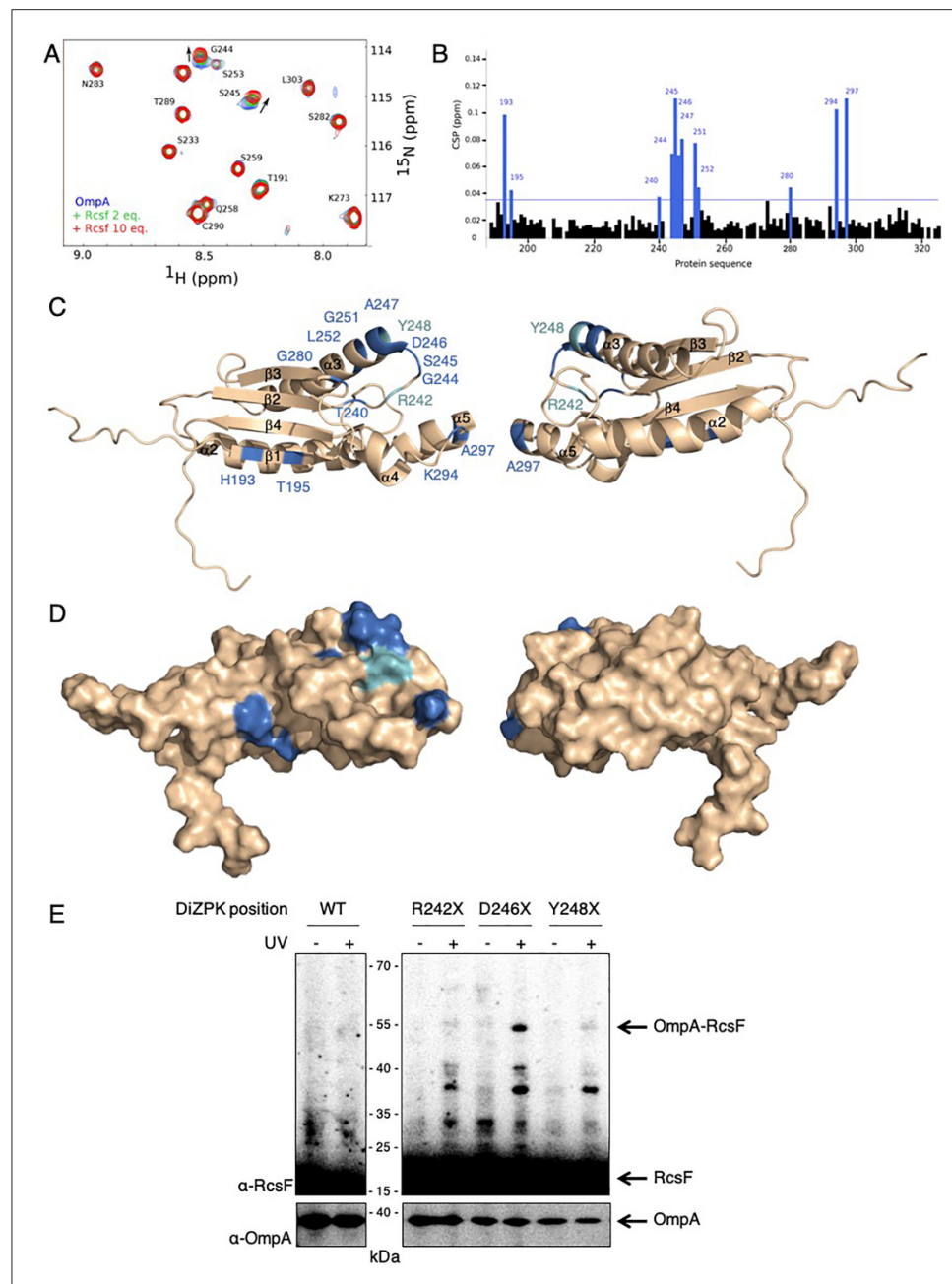


Figure 4. RcsF interacts with the periplasmic domain of OmpA in vitro. (A) An expanded region of the HSQC titration spectra of ^{15}N -labeled OmpA₁₈₆₋₃₂₅ with RcsF. Several residues in the $^1\text{H}/^{15}\text{N}$ BEST-TROSY-HSQC spectrum of OmpA (blue) show chemical shift perturbations (CSP) upon addition of RcsF at molar ratios of RcsF to OmpA of 2 (green) and 10 (red). Arrows indicate the direction of the chemical shift changes upon addition of RcsF to OmpA. (B) CSPs induced by the addition of RcsF to ^{15}N -labeled OmpA₆₆₋₃₂₅. Residues that showed CSP larger than two standard deviation are highlighted. *Figure 4 continued on next page*

Figure 4 continued

colored in blue. Cartoon (C) and surface (D) view of OmpA₁₈₆₋₃₂₅ (PDB: 2MQE): top view (on the left) and bottom view (on the right). The residues in OmpA that undergo a CSP larger than two standard deviation (as in panel B) appear as dark blue and are labeled. Most of these residues are located between β -strand 2 and α -helix 3. The residues R242 and Y248 that do not interact well with RcsF in (E) are colored in light blue. (E) To confirm the importance of the loop between β -strand 2 and α -helix 3 of OmpA for the interaction with RcsF, we used site-specific photo-crosslinking. Δ ompA rcsF⁺ cells expressing wild-type (WT) OmpA or three DiZPK-containing OmpA variants (OmpA_{R242X}, OmpA_{D246X}, and OmpA_{Y248X}) from pPR21 were irradiated with UV light (+) or not (-), and protein samples were immunoblotted with an anti-RcsF antibody. A strong 55 kDa band, corresponding to the size of the OmpA-RcsF complex, was observed with the OmpA_{D246X} variant, confirming the NMR data. Weak complex formation was also observed with OmpA_{R242X} and OmpA_{Y248X}. The expression levels of the OmpA variants were verified by immunoblotting (lower panel). The online version of this article includes the following source data for figure 4:

Source data 1. Raw source data for **Figure 4A,B**: the HSQC titration spectra of ¹⁵N-labeled OmpA₁₈₆₋₃₂₅ with RcsF.

that interacts with RcsF. Using protein-protein docking and molecular dynamics simulations, we built a three-dimensional model taking into accounts the results of the cross-linking experiments; in this model, RcsF and the periplasmic domain of OmpA show a good surface complementarity, with a buried accessible surface of 1087 Å². In this model, all six residues mutated to DiZPK (RcsF_{R45X}, RcsF_{Q79X}, RcsF_{R89X}, OmpA_{R242X}, OmpA_{D246X}, and OmpA_{Y248X}) are situated in close proximity to residues from the binding partner (**Figure 6A**), in good agreement with the crosslinking data. Interestingly, the peptidoglycan-binding region of OmpA (**Park et al., 2012**) remains accessible and is oriented in the direction opposite to the N-terminal end of RcsF. Our conclusions have two important implications.

First, we and others previously showed that portions of RcsF are surface-exposed (**Cho et al., 2014; Konovalova et al., 2014**) and proposed OmpA, OmpC, and OmpF as vehicles for surface exposure (**Cho et al., 2014; Konovalova et al., 2014**). Understanding how RcsF reaches the surface is crucial: lipoprotein surface exposure is an emerging concept in *E. coli* and Enterobacteriaceae (**Szewczyk and Collet, 2016; Konovalova and Silhavy, 2015**) and how lipoproteins cross the OM remains to be clearly established. It was proposed that it is the N-terminal linker of RcsF that is exposed on the surface before being threaded through the lumen of OmpA and other OMPs (**Konovalova et al., 2014**). However, when OmpA adopts the two-domain conformation, as in the OmpA-RcsF complex, its β -barrel domain does not have an open channel (**Figure 3—figure supplement 1B**) to accommodate the RcsF linker. We therefore conclude that RcsF does not reach the surface when in complex with OmpA, and predict that only OmpC and OmpF (which form large β -barrels) serve as vehicles for surface exposure. The fact that only a subset of the DiZPK-containing variants of RcsF that form a complex with OmpA also form a complex with OmpC/F (**Figure 1**) also supports the idea that the topology of OmpA-RcsF is different from that of OmpC/F-RcsF.

Second, a model was proposed in which RcsF, when in complex with OmpA, uses its positively charged, surface-exposed N-terminal linker to sense when interactions between lipopolysaccharide molecules are disturbed (**Konovalova et al., 2016**). However, if the RcsF linker is not surface-exposed in the OmpA-RcsF complex, as we show here, then the function of OmpA-RcsF in Rcs needs to be re-evaluated (see below). Importantly, we found that lipopolysaccharide alterations caused by sub-lethal concentrations of polymyxin B induce Rcs in MG1655 cells lacking *ompA* (**Figure 6—figure supplement 1**, see **Figure 6—figure supplement 1—source data 1** for statistics), in contrast to what was previously reported in another strain background (MC4100) (**Konovalova et al., 2016**), which further questions the potential role of OmpA-RcsF in sensing lipopolysaccharide defects.

It will also be interesting to investigate the role of the BAM machinery in the assembly of OmpA-RcsF. It was reported that the formation of the OmpA-RcsF complex was likely mediated by BAM during the assembly of OmpA (**Cho et al., 2014; Konovalova et al., 2014**). However, our finding that RcsF interacts with the C-terminal, periplasmic domain of OmpA, whose folding is unlikely to depend on BAM (**Noinaj et al., 2017**), questions this conclusion. Further experiments, including pulse-chase experiments, will be carried out to clarify this point. Given that BAM both interacts with RcsF and assembles OmpA in the OM, we anticipate that interpreting the data will be challenging, and therefore these experiments are outside the scope of this publication.

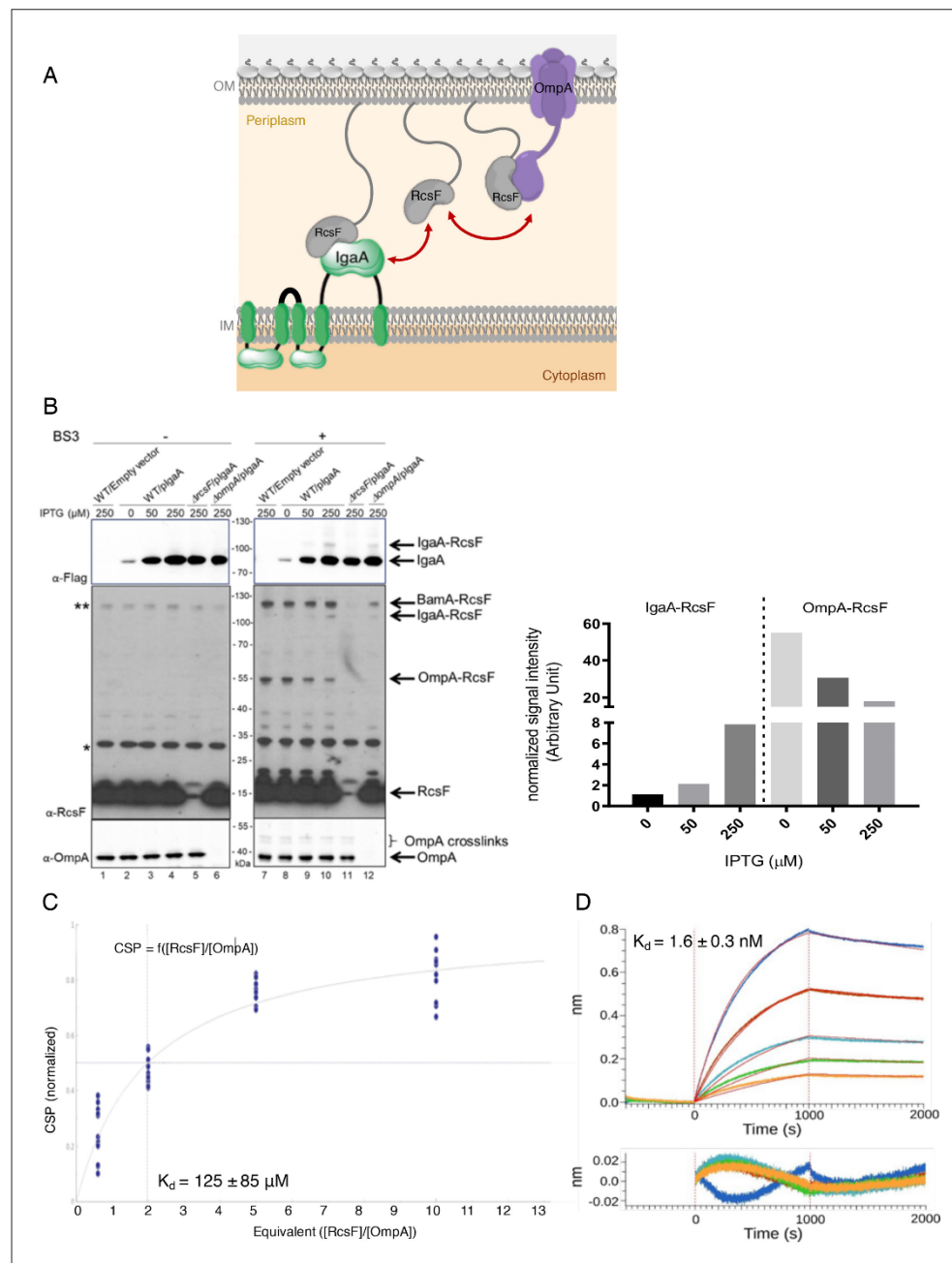


Figure 5. OmpA likely competes with the IM protein IgA for RcsF. (A) Cartoon of the *E. coli* cell envelope, with IgA in the IM and RcsF and OmpA in the OM. RcsF, an OM lipoprotein, interacts with OmpA. When in complex with RcsF, OmpA adopts its two-domain conformation, with an N-terminal β -barrel embedded in the OM and a C-terminal domain soluble in the periplasm. The periplasmic domain of OmpA interacts with RcsF, likely competing with IgA for RcsF binding. Copy numbers are from Li et al., 2014. (B) Impact of over-expressing IgA on the OmpA-RcsF complex (left). Cells

Figure 5 continued on next page

Figure 5 continued

harboring pSC231, an empty vector used as control, or pIgaA (pSC237, expressing IgaA-Flag₃ from an IPTG-inducible promoter) were harvested at mid-log phase and incubated without (lanes 1–6) or with (lanes 7–12) BS3. Protein samples were immunoblotted with α -Flag (upper panel), α -RcsF (middle panel), or α -OmpA₁₇₁₋₃₂₅ (lower panel) antibodies. IgaA-Flag₃ was expressed in WT (lanes 2–4 and 8–10), Δ rscF (lanes 5 and 11), and Δ ompA cells (lanes 6 and 12) with the indicated IPTG concentrations. Quantitation (see **Figure 5—source data 1** for details) of the IgaA-RcsF and OmpA-RcsF complexes detected by the anti-RcsF antibodies is shown (right panel). * and **, non-specific bands detected by the polyclonal α -RcsF antibody. (C) Plot of the chemical shift perturbation (CSP) measured on OmpA resonances as a function of the RcsF:OmpA ratio. Only the residues with significant CSP (colored in blue in **Figure 4B**) are plotted and used to fit the K_D . (D) The interaction between RcsF and the periplasmic domain of IgaA was probed by biolayer interferometry (BLI). Sensortips carrying immobilized RcsF were dipped into increasing concentrations of IgaA (5.9, 8.9, 13.3, 20, 30 nM) from 0 to 1000s then into buffer (1000–2000s). Association and dissociation phases were fitted (red lines) to extract a K_D value. Residuals from the fits are shown at the bottom of the panel (see **Figure 5—source data 2** for statistics).

The online version of this article includes the following source data and figure supplement(s) for figure 5:

Source data 1. Raw source data for **Figure 5B**.

Source data 2. Raw source data for **Figure 5D**.

Figure supplement 1. Estimation of the concentrations of IgaA and OmpA in the cell envelope.

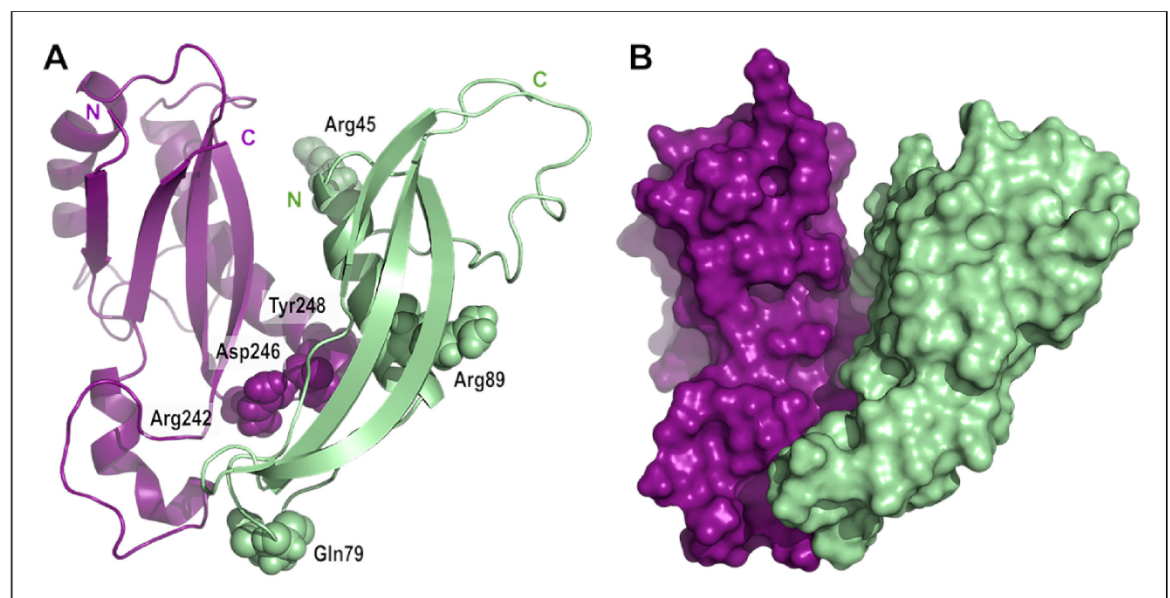


Figure 6. Model of the complex between RcsF and the C-terminal domain of OmpA. (A) Cartoon and (B) surface representation of a complex between RcsF (residues 44–134, colored in light green) and OmpA (residues 190–315, colored in purple). The atoms from the six residues mutated to DiZPK (RcsF_{R45X}, RcsF_{C279X}, RcsF_{R89X}, OmpA_{R242X}, OmpA_{D246X}, and OmpA_{Y248X}) are represented as spheres with the van der Waals radius. This model, which shows a good surface complementarity between the two proteins, was obtained by protein-protein docking with constraints from the crosslinking experiments and further refined by all-atom molecular dynamics (50 ns). The N-terminal residue of RcsF points out in the same direction as the extremities of the periplasmic domain of OmpA, that is toward the OM, and this binding mode is compatible with the interaction of the periplasmic domain of OmpA with the peptidoglycan.

The online version of this article includes the following source data and figure supplement(s) for figure 6:

Figure supplement 1. The Rcs system responds to polymyxin exposure in cells lacking OmpA.

Figure supplement 1—source data 1. Raw source data for **Figure 6—figure supplement 1**.

Figure supplement 2. OmpA functions as a buffer for RcsF.

OmpA functions as a buffer for RcsF

Finally, if OmpA does not allow RcsF to reach the surface, what could be the function of the OmpA-RcsF complex? On the basis of our results, we propose that the role of OmpA in Rcs is to modulate the activity of this system. Consider the equilibrium dissociation constants of the IgaA-RcsF and OmpA-RcsF complexes:

$$K_d^{\text{OmpA-RcsF}} = \frac{[\text{OmpA}][\text{RcsF}]}{[\text{OmpA-RcsF}]} \quad (1)$$

$$K_d^{\text{IgaA-RcsF}} = \frac{[\text{IgaA}][\text{RcsF}]}{[\text{IgaA-RcsF}]} \quad (2)$$

We estimate that OmpA's C-terminal domain is present in the periplasm at ~1 mM, RcsF at ~15 μM , and IgaA at ~1 μM (Materials and methods). If we take into account these estimates, then the following equation can be derived from **Equations 1 and 2** (Materials and methods):

$$[\text{IgaA-RcsF}] = \frac{K_d^{\text{ratio}}[\text{OmpA-RcsF}]}{1000 + K_d^{\text{ratio}}[\text{OmpA-RcsF}]} \quad (3)$$

where $K_d^{\text{ratio}} = \frac{K_d^{\text{OmpA-RcsF}}}{K_d^{\text{IgaA-RcsF}}}$.

Because OmpA is highly abundant, [OmpA] can be considered constant. Thus, according to **Equation 1**, the concentration of the OmpA-RcsF complex increases linearly with the concentration of RcsF in the periplasm (**Figure 6—figure supplement 2**). From **Equation 3**, we conclude that the concentration of IgaA-RcsF will also increase as a function of [RcsF] in the periplasm. However, in this case, the increase is not linear, but is decelerated and follows a hyperbolic curve (**Figure 6—figure supplement 2**), indicating that OmpA functions as a buffer for RcsF, negatively impacting its ability to activate Rcs. The buffering function of OmpA is nicely illustrated by the fact that exposure to low concentrations of polymyxin B causes higher Rcs induction in cells lacking *ompA* than in the wild type (**Figure 6—figure supplement 1**).

We acknowledge that the ~80,000 fold difference in affinity for RcsF that we measured between IgaA and OmpA (**Figure 5**) does not seem to support the function of OmpA as a buffer for RcsF: with such a high affinity for RcsF, IgaA should always outcompete OmpA and formation of the IgaA-RcsF complex should be constitutive, which is not the case (*Hussein et al., 2018*). To explain the apparent discrepancy between the in vivo observations and the in vitro measurements, we propose that intracellular factors such as attachment of the periplasmic domains of these proteins to their respective membrane anchors (we used the soluble periplasmic domains of OmpA, IgaA and RcsF for the in vitro measurements) and binding of OmpA to the peptidoglycan (*Reusch, 2012*) modulate the affinity of OmpA and IgaA for RcsF in vivo, allowing them to actually compete for RcsF. It is remarkable that OmpA, a protein that had mostly been known for its role in stabilizing the OM, is also involved in the network controlling the activity of Rcs, one of the most complex signal transduction systems in bacteria (*Cho et al., 2014; Hussein et al., 2018; Laloux and Collet, 2017*), whose correct functioning is critical for success of commensal Enterobacteriaceae and virulence of pathogens.

Materials and methods

Bacterial strains, primers, and plasmids

The bacterial strains and primers used in this study are listed in **Supplementary files 1 and 2**, respectively. The parental *E. coli* strain DH300 is a MG1655 derivative containing a deletion of the *lac* region; it also carries a chromosomal *rprA::lacZ* fusion at the λ phage attachment site to monitor Rcs activation (*Majdalani et al., 2002*). The *ompA*, *ompR* and *rscF* deletion mutants were obtained by transferring the corresponding alleles from the Keio collection (kan^R) (*Baba et al., 2006*) into DH300 (*Majdalani et al., 2002*) via P1 phage transduction, which was verified by PCR. To excise the kanamycin-resistance cassette, we used pCP20 as previously described (*Datsenko and Wanner, 2000*). To insert *ompA-His* (encoding OmpA with six histidines at the C-terminus), *ompA_{TH189}-His*,

ompA_{TH243-His}, and *ompX-ompA₁₇₁₋₃₂₅* on the chromosome at the *ompA* locus, we performed λ -Red recombineering (Yu *et al.*, 2000) with the pSIM5-Tet plasmid (Koskiniemi *et al.*, 2011). First, a *cat-sacB* cassette encoding chloramphenicol acetyl transferase (*cat*) and *SacB*, a protein conferring sensitivity to sucrose, was amplified from strain CH1990 using primers 'ompA_delCmSB F' and 'ompA_delCmSB R'. The resulting PCR product shared 40 bp of homology to the 5' UTR and 3' UTR of *ompA* at its 5' and 3' ends, respectively, and was used for λ -Red recombineering (Yu *et al.*, 2000). We selected transformants for chloramphenicol resistance and verified that the *cat-sacB* cassette replaced *ompA* by sequencing across the junctions. The *cat-sacB* cassette was subsequently replaced by one of the versions of *ompA* (*ompA-His*, *ompA_{TH189-His}*, *ompA_{TH243-His}*, or *ompX-ompA₁₇₁₋₃₂₅*) using λ -Red recombineering and negative selection on sucrose-containing medium (Thomason *et al.*, 2014; Gennaris *et al.*, 2015). *ompA-His*, *ompA_{TH189-His}*, and *ompA_{TH243-His}* were amplified via PCR using primers 'cmSB to OmpA_F' and 'cmSB to OmpAHis_R' and plasmids pPR11, pPR11_{189thrombin}, and pPR11_{243thrombin} (see below) as templates, respectively. The PCR product of *ompX-ompA₁₇₁₋₃₂₅* was generated using primers 'JLEo20-F-Chrom-OmpX' and 'JLEo22-R-Chrom-OmpACT', and pJLE17-OmpX-OmpACTer as template. Strains were verified through DNA sequencing. To delete *ompA₁₇₁₋₃₂₅*, we performed λ -Red recombineering on the chromosome at the *ompA* locus in a way similar to the description above. Briefly, the kanamycin cassette from the strain SEN588 (Δ *ompA::kan*) was PCR-amplified using primers 'ompAc_delKm F' and 'ompAc_delKm R' to encompass the flanking regions of *ompA₁₇₁₋₃₂₅*. We selected transformants for kanamycin resistance.

The plasmids used in this study are described in **Supplementary file 3**. pSC253, encoding *RcsF*, was constructed via digestion of pSC202 (Cho *et al.*, 2014) with *KpnI* and *HindIII* and insertion of the generated fragment into pBAD18 (Guzman *et al.*, 1995). To generate the *rscF* variants containing an amber codon (TAG) at selected positions, site-directed mutagenesis was performed on pSC253 using primers described in **Supplementary file 2**. To generate pPR11, *ompA-His* was PCR-amplified using primers 'OmpA(NcoI) F' and 'OmpA-his(XbaI) R' and chromosomal *E. coli* DNA as template. The PCR product was inserted into pDSW204 restricted with *NcoI* and *XbaI*, generating pPR11. To insert thrombin cleavage sites after residues 189 and 243 in *OmpA*, site-directed mutagenesis was performed using primer pair '189VPRGS thr_F' and '189VPRGS thr_R' and primer pair '243LVPR thr_F' and '243LVPR thr_R', respectively, on pPR11, yielding pPR11_{189thrombin} [*OmpA*(189-Val-Val-Pro-Arg-Gly-Ser-Gln-190)] and pPR11_{243thrombin} [*OmpA*(243-Ile-Leu-Val-Pro-Arg-Gly-244)], respectively. pJLE17-OmpX-OmpA₁₇₁₋₃₂₅ (encoding *OmpA_X*) was constructed as follows. The two PCR products corresponding to *ompX* and *OmpA₁₇₁₋₃₂₅* were generated using primer pair 'OmpX(NcoI)F' and 'OmpX-OmpAc_R' and primer pair 'OmpX-OmpAc_F' 'JLEo16-R-XbaI-OmpACT', respectively, and chromosomal *E. coli* DNA as template. To join the two PCR fragments, overlapping PCR was performed using primers 'OmpX(NcoI)F' and 'JLEo16-R-XbaI-OmpACT', generating the PCR product encoding *OmpA_X*. Next, the PCR product of *OmpA_X* was digested with *NcoI* and *XbaI* and ligated with pDSW204 pre-digested with *NcoI* and *XbaI*, yielding pJLE17-OmpX-OmpACTer. pKiD22, expressing *OmpA_{Pal}*, was constructed as follows. The sequence encoding the signal sequence and lipobox of the lipoprotein *Pal* was PCR-amplified using primers 'Palss(NcoI)F' and 'Palss-OmpAc_R' and the sequence encoding *OmpA₁₇₁₋₃₂₅* using primers 'Palss-OmpAc_F' and 'OmpA_stop_Flag3(KpnI)R'. Chromosomal *E. coli* DNA was used as template. To join the two PCR fragments, overlapping PCR was performed using primers 'Palss(NcoI)F' and 'OmpA_stop_Flag3(KpnI)R'. The PCR product encoding ssPal-OmpA₁₇₁₋₃₂₅ was digested with *NcoI* and *KpnI* and ligated with pDSW204, pre-digested with *NcoI* and *KpnI*, yielding pKiD22.

To generate pPR21, site-directed mutagenesis was performed on pPR11 using primers 'OA_stopTGA_F' and 'OA_stopTGA_R' to insert a stop codon upstream of the 6xHis tag. To generate the *ompA* variants containing an amber codon (TAG) at selected positions, site directed mutagenesis was performed on pPR21 using primers described in **Supplementary file 2**. To obtain pKiD5, *OmpA₁₈₆₋₃₂₅* with an N-terminal Strep-tag but no signal sequence was PCR-amplified using primers 'KiDo14-F-NdeI-Strep-OmpACTD' and 'KiDo15-R-SacI-Rbs-OmpACTD' and chromosomal *E. coli* DNA as template. The PCR product was digested with *NdeI* and *SacI* and inserted into pET21a. To prepare a version of pAM238 containing *lacI^q*, a *trc* promoter, and a triple Flag tag (Flag₃), we prepared a PCR product using primers 'lacIq Nsil_F' and 'flag3-KpnI_R' and pMER77 as template (Hemmis *et al.*, 2011). This product was digested with *NsiI* and *KpnI* and ligated with pAM238 pre-digested with *PstI* and *KpnI*. To reduce the basal activity of the *trc* promoter, we modified the -10

region (from TATAAT to CATTAT) and the –35 region (from TTTACA to TTGACA), generating pSC231 (Weiss et al., 1999). The coding sequences for OmpA and IgaA were obtained by PCR-amplification using primer pair 'OmpA(NcoI) F' and 'OmpA XbaI flag R' and primer pair 'IgaA(NcoI) F' and 'IgaA XbaI flag3 R', respectively. Each product was digested with NcoI and XbaI and ligated into pSC231 pre-digested with the same enzymes, generating pPR4 and pSC237, respectively.

In vivo site-specific photo-crosslinking using DiZPK

We used the site-specific photo-crosslinking method described previously (Cho et al., 2014) with some modifications. To incorporate N^6 -((3-(3-methyl-3H-diazirin-3-yl)propyl)carbamoyl)-L-lysine (DiZPK) into RcsF, we used the pSup-Mb-DiZPK-RS plasmid encoding an evolved *Methanosarcina barkeri* pyrrolysyl-tRNA synthetase (PylRS) and an optimized tRNA^{Pyl}_{CUA} suppressor (Zhang et al., 2011). DH300 Δ rscF (PL358) cells were co-transformed with pSup-Mb-DiZPK-RS and one of the plasmids containing an amber codon in rscF in pSC253. Cells were grown in 3-(N-morpholino)propane-sulfonic acid (MOPS) minimal medium supplemented with 0.2% glucose, 0.2% L-arabinose (MOPS-glucose/arabinose minimal medium), and 0.8 mM DiZPK (no supplement of other amino acids; see the reasons for using MOPS medium below) (Neidhardt, Bloch, and Smith 1974). Cell cultures were grown to an OD₆₀₀ of 1–1.2 and 1 mL of samples was irradiated with UV light at 365 nm or left unirradiated for 10 min. Cells were precipitated with trichloroacetic acid (TCA), and the pellets were washed with acetone and solubilized in 60 μ L of SDS-sample buffer before further analysis. We used a similar method to incorporate DiZPK into OmpA with minor modifications; DH300 Δ ompA (PR46) cells were co-transformed with pSup-Mb-DiZPK-RS and one of the plasmids containing an amber codon in ompA in pPR21. Cells were grown in LB medium supplemented with 0.2% L-arabinose, 200 μ M IPTG and 1 mM DiZPK. Cell cultures were grown to an OD₆₀₀ of 1 and 0.75 mL of samples was irradiated with UV light at 365 nm or left unirradiated for 10 min. Cells were precipitated with TCA, and the pellets were washed with acetone and solubilized in 100 μ L of SDS-sample buffer before further analysis.

In previous experiments incorporating *p*-benzoyl-L-phenylalanine into RcsF, we used LB as the growth medium (Cho et al., 2014). However, we found that the expression levels of the DiZPK-containing RcsF mutant proteins were substantially lower when cells were grown in LB (data not shown); in contrast, the expression levels of RcsF were greatly enhanced in MOPS-glucose/arabinose minimal medium (data not shown). Therefore, we used MOPS-glucose/arabinose minimal medium for all photo-crosslinking experiments involving DiZPK-containing RcsF variants.

Synthesis of DiZPK

DiZPK was synthesized as described previously (Zhang et al., 2011).

Immunoprecipitation of RcsF-containing complexes and thrombin cleavage

Protein samples in SDS-sample buffer (without reducing agent) were denatured for 15 min at 65°C and 15 min at 95°C with vigorous shaking. Next, the samples were diluted in 750 μ L of KI buffer (50 mM Tris-HCl [pH 8], 2% Triton X-100, 150 mM NaCl, 1 mM EDTA) and centrifuged at 4°C for 3 min at 12,000 $\times$ g to harvest the PG. Photo-crosslinked RcsF complexes were immunoprecipitated by adding 1 μ L of undiluted guinea pig anti-RcsF antibody (Cho et al., 2014) and 10 μ L of protein A/G magnetic beads (Pierce); samples were incubated for 1 hr on a wheel at room temperature. After three washes with 500 μ L of KI buffer, RcsF complexes were eluted with 20 μ L of glycine buffer (100 mM glycine [pH 1.5], 0.1% Triton X-100) after 10 min of incubation at room temperature. Proteins samples were neutralized with 2 μ L of 1.5 M Tris-HCl [pH 8.8] and diluted with 18 μ L of KI buffer before SDS-PAGE and immunoblotting or thrombin cleavage. For thrombin cleavage, 20 μ L of the elution samples were incubated for 1 hr at room temperature with 1 μ L of thrombin (thrombin from bovine plasma, Sigma). Samples were analyzed via SDS-PAGE and immunoblotting as indicated in the figure legends.

Immunoblotting and antibodies

Protein bands were transferred from the gels onto nitrocellulose membranes (Millipore) using a semi-dry electroblotting system. The membranes were blocked with 5% skim milk. The rest of the

immunoblot steps were performed using standard protocols. Signal from antibody binding was visualized by detecting chemiluminescence from the reaction of horseradish peroxidase with luminol. Polyclonal RcsF antibodies were purified against the carboxy-terminal domain of RcsF as previously described (Cho *et al.*, 2014) and used at a dilution of 1:20,000 in 1% skim milk in 50 mM Tris-HCl [pH 7.6], 0.15 M NaCl and 0.1% Tween 20 (TBST). Since we found that the anti-OmpA antibody only recognizes the periplasmic domain (data not shown), we used an antibody directed against loop 4 of the β -barrel of OmpA to detect OmpA₁₋₁₇₀. The anti-OmpA antibodies are gifts from the Llobes and Bernstein laboratories (Cascales *et al.*, 2002; Hussain and Bernstein, 2018). These antibodies were used at dilutions of 1:20,000 and 1:10,000, respectively. The anti-His antibody conjugated to horseradish peroxidase (Qiagen) was used at a dilution of 1:5000. The anti-Flag M2 monoclonal antibody (F1804, Sigma) was used at a dilution of 1:20,000.

In vivo 3,3'-dithio-bis[sulfosuccinimidylpropionate] and bis(sulfosuccinimidyl)-suberate (BS3) crosslinking

DTSSP and BS3 (CovaChem) are bifunctional primary amine crosslinkers; DTSSP contains a disulfide bond in its spacer arm. In vivo crosslinking has been performed as described previously (Cho *et al.*, 2014). The media used are MOPS-glucose minimal medium (Neidhardt *et al.*, 1974; Figure 3) and LB (Figure 5).

β -Galactosidase assay

E. coli cells were grown in MOPS-glucose minimal medium (Neidhardt *et al.*, 1974) or LB to mid-log phase ($OD_{600} = 0.4$ – 0.6). β -galactosidase activity was measured as described previously (Zhang and Bremer, 1995).

Expression and purification of RcsF, OmpA₁₈₆₋₃₂₅ and of the periplasmic domain of IgA

Expression and purification of RcsF with a C-terminal His-tag were performed as previously described (Leverrier *et al.*, 2011). *E. coli* BL21(DE3) cells harboring pKid5 expressing N-terminal Strep-tagged OmpA₁₈₆₋₃₂₅ were grown at 37°C in M9-glucose minimal medium containing 1 g/L $^{15}\text{NH}_4\text{Cl}$ (99%, ^{15}N ; Eurisotop) to uniformly label the protein with ^{15}N . The expression was induced by adding 1 mM IPTG at an OD_{600} of 0.5. After a 5 hr induction, cells were harvested by centrifugation and resuspended in 20 mL of lysis buffer (20 mM Tris-HCl, 500 mM NaCl [pH8], containing a protease inhibitor cocktail (Complete, Roche)). Re-suspended cells were stored at -20°C . Frozen cells were thawed on ice and lysed by two passages through a French pressure cell at 1500 psi. The soluble fraction was isolated after centrifugation for 1 hr at $40,000 \times g$ at 4°C . The supernatant was filtered through 0.45 μm filters and loaded onto a 5 mL Strep-Tactin column (IBA, Lifesciences), previously equilibrated in buffer A (50 mM NaH_2PO_4 , 100 mM NaCl [pH7.0]). After a washing step with buffer A, elution was performed with buffer A supplemented with 2.5 mM D-desthiobiotin. The sample was then further purified using size-exclusion chromatography on a HiLoad 16/60 Superdex 75 column (GE Healthcare) using buffer A. To express IgA₃₆₁₋₆₅₄ with a C-terminal His-tag, *E. coli* SHuffle T7 cells harboring pSC211 were grown at 37°C in LB. Expression of the protein was induced by adding 1 mM IPTG at an OD_{600} of 0.5. After a 5 hr induction, cells were harvested by centrifugation and resuspended in 20 mL of lysis buffer. Re-suspended cells were stored at -20°C . Frozen cells were thawed on ice and processed as explained above for Strep-tagged OmpA₁₈₆₋₃₂₅. IgA₃₆₁₋₆₅₄ was purified using Ni-NTA agarose beads (5 mL; IBA Lifescience), previously equilibrated in buffer B (20 mM NaH_2PO_4 , 150 mM NaCl [pH7.5]). After washing the resin with buffer B supplemented with 20 mM imidazole, proteins were eluted with five column volumes of buffer B supplemented with 200 mM imidazole. As a final purification step, a size-exclusion chromatography was performed using a HiLoad 16/60 Superdex 200 column (GE Healthcare) with buffer B. Purity was checked via SDS-PAGE with Coomassie Staining and concentration was performed using Vivaspin Turbo apparatus (Sartorius) with a 5 kDa molecular weight cut-off.

NMR titration experiments of ^{15}N -labelled OmpA₁₈₆₋₃₂₅ with RcsF

50 μM ^{15}N -labelled OmpA₁₈₆₋₃₂₅ in buffer A at 25°C was titrated with increasing concentrations of unlabelled RcsF in buffer A to reach 2 and 10 RcsF/OmpA₁₈₆₋₃₂₅ molar ratios. To follow ^{15}N - ^1H

resonances chemical shifts perturbations, ^{15}N - ^1H BEST-TROSY-HSQC correlation experiments were recorded at 25°C using Bruker AVANCE spectrometer operating at 700 MHz proton frequency equipped with a TCI cryoprobe (Favier and Brutscher, 2011). OmpA₁₈₆₋₃₂₅ assignments were transposed from BMRB entry 25030. Chemical shift perturbations (CSP) were calculated on a per-residue basis for the highest substrate-to-protein ratio as described previously (Egan et al., 2018). Spectra were processed with Topspin 3.57 (Bruker) and analyzed with ccpnmr 3 (<https://www.ccpn.ac.uk>).

Western blot analysis

Quantitation of the intensity of the bands was done by optical densitometry and analyzed using ImageQuant TL processing software (ImageQuant TL v8.1.0.0, GE Healthcare). After background correction, the values were normalized by the intensity of non-specific bands detected by the polyclonal antibody.

Biolayer interferometry

Biolayer Interferometry Experiments (BLI) were recorded on an OctetRED96e (Fortebio) using streptavidin (SA) biosensors (Fortebio). To biotinylate RcsF, RcsF-His (5 mg/mL in 0.1 M MES [pH 5.5]) was incubated with Biotin-LC-Hydrazide (1.25 mM, final concentration; Sigma) and N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide (EDC; 6.5 mM, final concentration) during 2 hr at 22°C under agitation. Biotinylated RcsF was dialysed against buffer C (10 mM Hepes, 150 mM NaCl [pH 7.5]) and immobilized at 5 µg/mL onto SA tips in buffer C supplemented with 0.02% Tween-20 to reach ~3.5 nM of immobilization level. RcsF-loaded biosensors were dipped into different concentrations of the periplasmic domain of IgA (5.9 nM to 30 nM) in buffer C at 23°C. Kinetics were recorded with 1000s association and 1000s dissociation phases, and repeated four times with 10 mM HCl pulses (18 s in total) used for regeneration between cycles. Sensorgrams were subtracted for contribution of buffer alone and binding of non-functionalized biosensors. Kinetic analysis of the data was performed using 1:1 interaction model in the ForteBio data analysis software. K_D obtained from four independent injection series were averaged and produced a K_D of 1.6 nM with a standard deviation of 0.3.

Estimation of the expression levels of IgA

Overexpressed IgA has a triple Flag tag (IgA-Flag₃). To compare the expression levels of IgA to those of OmpA, we generated a triple Flag-tagged version of OmpA (OmpA-Flag₃); this variant was expressed from an IPTG-inducible plasmid in the *ompA* strain. If we compare the intensity of the signal corresponding to OmpA-Flag₃ detected either by the anti-OmpA or the anti-Flag antibodies, we estimate the anti-Flag antibodies to be ~25 times more sensitive than the anti-OmpA antibodies (lanes 1–4 in Figure 5—figure supplement 1). With this value in hand, we can now compare the expression levels of IgA-Flag₃ to those of untagged OmpA produced from the chromosome and detected with the anti-OmpA antibodies (lanes 5–9 in Figure 5—figure supplement 1); because the intensities of OmpA and IgA-Flag₃ in lane eight are similar, we estimate that IgA-Flag₃ is 25 times less abundant than OmpA when IgA is expressed with 250 µM IPTG; likewise, we estimate the levels of IgA-Flag₃ to be ~1/125 (0.8%) of those of OmpA when IgA is expressed with 50 µM IPTG. Because the concentration of OmpA is ~1 mM, we estimate the concentration of overexpressed IgA to be 40 and 8 µM, respectively.

Molecular modelling

HADDOCK 2.4 web server (van Zundert et al., 2016; Wassenaar et al., 2012) was used for protein-protein docking using the structures 2MQE for OmpA-CTD (Ishida et al., 2014) and 2L8Y for RcsF (Rogov et al., 2011). Six residues identified from the crosslinking experiments were considered as active during the docking calculations: 242, 246, and 248 for OmpA and 45, 79, and 89 for RcsF. The resulting clusters were inspected visually, and the one compatible with the interaction between the periplasmic domain of OmpA and the peptidoglycan (Park et al., 2012) was selected for further refinement using molecular dynamics. Molecular dynamics simulations were carried out with GRO-MACS version 2020.1 (Abraham et al., 2015) using the OPLS-AA (Jorgensen et al., 1996) force field. Each system was energy-minimized until convergence using a steepest descents algorithm. Molecular dynamics with position restraints was then performed (50 ps NVT and 50 ps NPT),

followed by the production run of 50 ns. During the position restraints and production runs, the V-rescale and Parrinello-Rahman methods were used for temperature and pressure coupling, respectively. Electrostatics were calculated with the particle mesh Ewald method. The P-LINCS algorithm was used to constrain bond lengths, and a time step of 2 fs was used throughout.

Estimation of the concentrations of OmpA, RcsF and IgaA in the periplasm

According to *Li et al., 2014*, each cell contains ~200,000 molecules of OmpA in rich media, which corresponds to $\sim 3.448 \times 10^{-19}$ mol (200,000/Avogadro constant). If we consider the volume of an *E. coli* cell to be 10^{-18} m^3 (10^{-15} L) and compare it to a cube with 1 μm edges (*E. coli* has 0.5 μm in width and 2 μm in length; EcoliWiki ecoliwiki.net/colipedia/index.php/Escherichia_coli), we calculate that the cellular concentration of OmpA is $\sim 3.448 \times 10^{-4} \text{ M}$. The volume of the envelope being ~30% of the total cell volume (*Stock et al., 1977*), we calculate that the concentration of the C-terminal domain of OmpA in the periplasm is ~1 mM. If we perform the same calculations for RcsF and IgaA (~3000 and ~200 copies/cell, respectively), we find that their periplasmic concentrations are ~15 μM and ~1 μM , respectively.

OmpA functions as a buffer for RcsF

The equilibrium dissociation constants for the OmpA-RcsF and IgaA-RcsF complexes are:

$$K_d^{\text{OmpA-RcsF}} = \frac{[\text{OmpA}][\text{RcsF}]}{[\text{OmpA-RcsF}]} \quad (4)$$

$$K_d^{\text{IgaA-RcsF}} = \frac{[\text{IgaA}][\text{RcsF}]}{[\text{IgaA-RcsF}]} \quad (5)$$

If we divide **Equation 4** with **Equation 5**, we obtain:

$$\frac{K_d^{\text{OmpA-RcsF}}}{K_d^{\text{IgaA-RcsF}}} = \frac{[\text{OmpA}][\text{IgaA-RcsF}]}{[\text{IgaA}][\text{OmpA-RcsF}]} \quad (6)$$

If we replace $\frac{K_d^{\text{OmpA-RcsF}}}{K_d^{\text{IgaA-RcsF}}}$ by K_d^{ratio} , then we can rearrange **Equation 6** to,

$$\frac{[\text{IgaA-RcsF}]}{[\text{IgaA}]} = \frac{K_d^{\text{ratio}}[\text{OmpA-RcsF}]}{[\text{OmpA}]} \quad (7)$$

Under physiological conditions, we estimate the concentration of IgaA to be ~1 μM (see above). Thus, because $[\text{IgaA}] + [\text{IgaA-RcsF}] = 1 \mu\text{M}$, **Equation 7** can be successively rearranged to,

$$\frac{[\text{IgaA-RcsF}]}{1 - [\text{IgaA-RcsF}]} = \frac{K_d^{\text{ratio}}[\text{OmpA-RcsF}]}{[\text{OmpA}]} \quad (8)$$

$$\frac{1 - [\text{IgaA-RcsF}]}{[\text{IgaA-RcsF}]} = \frac{[\text{OmpA}]}{K_d^{\text{ratio}}[\text{OmpA-RcsF}]} \quad (9)$$

$$\frac{1}{[\text{IgaA-RcsF}]} = \frac{[\text{OmpA}]}{K_d^{\text{ratio}}[\text{OmpA-RcsF}]} + 1 \quad (10)$$

$$\frac{1}{[\text{IgaA-RcsF}]} = \frac{[\text{OmpA}] + K_d^{\text{ratio}}[\text{OmpA-RcsF}]}{K_d^{\text{ratio}}[\text{OmpA-RcsF}]} \quad (11)$$

yielding:

$$[\text{IgaA-RcsF}] = \frac{K_d^{\text{ratio}}[\text{OmpA-RcsF}]}{[\text{OmpA}] + K_d^{\text{ratio}}[\text{OmpA-RcsF}]} \quad (12)$$

In **Equation 4**, [OmpA], which is ~1000 μ M, can be considered as a constant. Therefore, **Equations 4 and 14** become:

$$[\text{OmpA} - \text{RcsF}] = \frac{1000 [\text{RcsF}]}{K_d^{\text{OmpA-RcsF}}} \quad (13)$$

$$\begin{aligned} [\text{IgaA} - \text{RcsF}] &= \frac{K_d^{\text{ratio}} [\text{OmpA} - \text{RcsF}]}{1000 + K_d^{\text{ratio}} [\text{OmpA} - \text{RcsF}]} = \frac{[\text{OmpA} - \text{RcsF}]}{1000/K_d^{\text{ratio}} + [\text{OmpA} - \text{RcsF}]} \\ &= \frac{\frac{1000 [\text{RcsF}]}{K_d^{\text{OmpA-RcsF}}}}{1000/K_d^{\text{ratio}} + \frac{1000 [\text{RcsF}]}{K_d^{\text{OmpA-RcsF}}}} \end{aligned} \quad (14)$$

Thus, from **Equation 14**, we conclude that whereas [OmpA-RcsF] increases linearly to [RcsF], [IgaA-RcsF] increases proportionally, but not linearly, to [RcsF]. Thus, OmpA functions as a buffer for RcsF.

Analysis of protein structures

Protein structures were downloaded from the Protein Data Bank (<http://www.rcsb.org>; PDB codes are indicated) and visualized using PyMOL Molecular Graphics System (Version 2.3.4, Schrödinger, LLC). FASTA protein sequences were downloaded from Uniprot (<http://www.uniprot.org/>).

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Additional files

Supplementary files

- Supplementary file 1. Strains used in this study.
- Supplementary file 2. Primers used in this study.
- Supplementary file 3. Plasmids used in this study.
- Transparent reporting form

Data availability

All data generated or analyzed during this study are included in the manuscript and supporting files. Source data files have been provided for Figures 3, 4A, 4B, 5B, 5D and 6 supplement 1.

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