The AAA+ ATPase RavA and its binding partner ViaA modulate *E. coli* aminoglycoside sensitivity through interaction with the inner membrane

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Abstract

Enteric bacteria have to adapt to environmental stresses in the human gastrointestinal tract such as acid and nutrient stress, oxygen limitation and exposure to antibiotics. Membrane lipid composition has recently emerged as a key factor for stress adaptation. The *E. coli ravA-viaA* operon is essential for aminoglycoside bactericidal activity under anaerobiosis but its mechanism of action is unclear. Here we characterise the VWA domain-protein ViaA and its interaction with the AAA+ ATPase RavA, and find that both proteins localise at the inner cell membrane. We demonstrate that RavA and ViaA target specific phospholipids and subsequently identify their lipid-binding sites. We further show that mutations abolishing interaction with lipids restore induced changes in cell membrane morphology and lipid composition. Finally we reveal that these mutations render *E. coli* gentamicin-resistant under fumarate respiration conditions. Our work thus uncovers a *ravA-viaA*-based pathway which is mobilised in response to antibiotics under anaerobiosis and has a major impact on cell membrane regulation.

Introduction

Although antibiotics are powerful antibacterial weapons, their widespread and frequently uncontrolled use has led to the emergence of numerous multi-resistant bacteria. Aminoglycosides (AGs) are highly efficient antibiotics against Gram-negative pathogens, yet they cause toxic side effects ¹. Understanding what makes bacteria sensitive to AGs and why AG efficiency is notoriously reduced under anaerobic conditions, often encountered by intestinal pathogens, is essential since administration of smaller AG doses reduces their toxicity towards the host. In two paradigmatic

enterobacteria, *Escherichia coli* and *Vibrio cholerae*, the expression of the *ravA-viaA* operon is enhanced by anaerobiosis and sensitises the bacteria to AGs, whereas a deletion of *ravA-viaA* enhances their AG resistance and reduces the AG-mediated toxic stress²⁻⁵. The extensively studied ATPase RavA⁶⁻⁸ belongs to the versatile AAA+ superfamily^{9,10}, whereas the largely uncharacterised ViaA carries a von Willebrand factor A (VWA) domain¹¹ and stimulates RavA ATPase activity^{6,12}. *E. coli* RavA-ViaA is suggested to act as a chaperone for the maturation of two respiratory complexes - Complex I (Nuo)³ and fumarate reductase (Frd)¹². Increased AG uptake may therefore result from facilitated Nuo assembly that contributes to proton motive force generation. However, *E. coli* and *V. cholerae* respiratory pathways are very different, and *V. cholerae* lacks the Nuo complex¹³. In addition, the documented RavA-ViaA-dependent inhibition of Frd activity¹² contradicts the hypothesis that *ravA-viaA* enhances respiration. Furthermore, *in vitro* RavA is sequestered by the acid stress-inducible lysine decarboxylase LdcI in the form of a huge LdcI-RavA cage proposed to function in the acid stress response upon oxygen and nutrient limitation^{6,8,14}. Thus, the molecular mechanism of RavA-ViaA action in bacterial physiology in general, and in AGs sensitisation in particular, appears enigmatic. Our recent study has unexpectedly revealed that LdcI forms supramolecular assemblies under the *E. coli* membrane, seemingly at lipid microdomains¹⁵. Here we biochemically and biophysically characterise the ViaA protein and its interaction with RavA, and show that both proteins localise to the *E. coli* inner membrane, affect membrane morphology and modify cellular lipid homeostasis. We further reveal that both RavA and ViaA interact with specific lipids, identify the lipid-binding sites and demonstrate that their mutations abolish lipid binding *in vitro* and strongly attenuates the effect on lipid homeostasis *in vivo*. Moreover, we introduce these mutations into the *E. coli* chromosome and show that they abrogate the effect of the *ravA-viaA* operon on the AG sensitisation. Altogether, this work reveals a *ravA-viaA*-based pathway that is mobilised in the response of *E. coli* to AGs under anaerobic conditions and has a major impact on the regulation of the bacterial cell membrane. Building upon these results, we propose a possible scenario for the *ravA-viaA* function and open up further research perspectives.

Results

ViaA is a dimeric, soluble two-domain protein

The protein sequence of ViaA reveals that it has an N-terminal part (N-Terminal domain of ViaA, NTV) with a predominantly α -helical character that does not show sequence similarity to any other known protein (Figure 1A, residues 1 - 319). The C-terminal part (C-Terminal domain of ViaA, CTV), starting from residue 320, is predicted to be a VWA domain and contains the characteristic MIDAS motif (Figure 1A). While studies of the potential role of *E. coli* ViaA in the maturation of Nuo and Frd have been initiated^{3,12}, no protocols for the preparative purification of ViaA are available, its biochemical/biophysical characterization remains scarce, and binding to its potential interaction partners has only been shown indirectly^{3,6,12}. Addition of an N-terminal AviTag to the C-terminally His-tagged ViaA (hereafter named AviTag-ViaA-His) stabilised the protein and enabled its high yield purification for characterisation by multi-angle laser light scattering (MALLS) and small-angle X-ray scattering (SAXS) (Figure 1B-D, Materials and Methods). MALLS experiments with purified AviTag-ViaA-His sample showed a monodisperse peak and yielded a molecular weight (MW) of 123 kDa, corresponding to roughly two times the theoretical MW of the AviTag-ViaA-His construct (60.4 kDa). SAXS studies confirmed the dimeric state of ViaA in solution and resulted in predicted MWs of 130 and 117 kDa using SAXSMoW^{16,17} and ScÅtter¹⁸ respectively. Moreover, the distance distribution function calculated from the SAXS data suggests an elongated particle, while the Kratky plot reveals a degree of flexibility, and corresponds to a Kratky plot expected for a multidomain protein with flexible linker (Figure 1C, 1D). We note that the elongated character and apparent flexibility of ViaA might be accentuated by the presence of non-cleaved tags.

The strength and kinetics of the RavA-ViaA interaction are nucleotide-dependent

While a previous observation that ViaA or NTV slightly enhance the ATPase activity of RavA suggested a physical interaction between the two proteins via the N-terminal part of ViaA^{6,12}, direct measurements of the RavA-ViaA interaction are unavailable. Pull-down assays using SPA-tagged RavA did not bring down ViaA, and vice versa, pointing to a rather weak or transient interaction¹². The advantage of AviTag-containing constructs is that they can be biotinylated *in vitro* and coupled to Streptavidin (SA)-biosensors for binding studies using Bio-Layer Interferometry (BLI). To address potential effects of the protein orientation on the biosensor, either N- or C-terminally tagged constructs (Materials and Methods) were immobilised and transferred to wells containing a concentration series of purified RavA in the absence or presence of ADP or ATP (Figure 2A, 2B). The affinity of the apo-RavA to immobilised His-ViaA-AviTag and AviTag-ViaA-His could be described by dissociation constants (K_D) of 11.0 μ M and 18.9 μ M respectively, showing that the location of the AviTag does not have a major influence and validating the weak nature of the RavA-ViaA interaction. ADP binding to RavA increased the strength of the RavA-ViaA interaction to ~ 2.2 μ M, whereas the presence of ATP altered the shape of the BLI curves that could only be reliably fitted using a 2:1 heterogeneous ligand binding model, resulting in K_{D1}/K_{D2} values of 30.3/1.0 μ M and 37.5/0.5 μ M for His-ViaA-AviTag and AviTag-ViaA-His respectively. Taken together, these BLI binding studies revealed that ViaA directly interacts with RavA, and that the strength of this interaction is dependent on the nucleotide-bound state of RavA. Complementary BLI studies using NTV-AviTag demonstrated similar RavA binding kinetics as the full-length ViaA despite the monomericity of the NTV construct (Supplementary Figure 1), confirming that NTV is sufficient for RavA binding¹². In addition, the absence of the LARA domain of RavA responsible for the LdcI-RavA cage formation^{7,14,19} did not affect the binding of RavA to ViaA (Supplementary Figure 1), indicating that the LARA domain is not involved in the RavA-ViaA interaction.

Previous observation that upon cell fractionation RavA was mostly present in the cytoplasmic fraction whereas the majority of ViaA partitioned into the inner membrane fraction³, led the authors to the detection of ViaA interaction with specific subunits of Nuo³ and Frd¹² by pull down assays with SPA-tagged baits. Thus, using BLI, we interrogated the interaction between ViaA and its proposed respiratory complexes' partners: the soluble catalytic NADH dehydrogenase domain of *E. coli* NADH:Ubiquinone Oxidoreductase I (NuoEFG) and the flavin-containing subunit of the *E. coli* Fumarate Reductase (FrdA)^{3,12}. Expression and purification of NuoEFG and FrdA were performed following previously published protocols²⁰⁻²² (Materials and Methods), and resulted in purified fractions with a red/brown and a yellow color for NuoEFG and FrdA respectively, corresponding to the presence of Riboflavin/Iron-Sulfur clusters for NuoEFG and FAD for FrdA. However, using concentrations as high as 5 μ M of NuoEFG and FrdA did not result in any discernible interaction with either His-ViaA-AviTag or AviTag-ViaA-His (Figure 2C).

Both RavA and ViaA show membrane localisation in *E. coli* cells

Surprised by the absence of *in vitro* interactions between ViaA and its purified proposed binding partners, we wondered what might then be the reason for its described partitioning into the inner membrane fraction³, and therefore sought to localise ViaA and RavA *in cellulo* by single molecule localisation microscopy imaging. Considering the low amount of natively expressed RavA and ViaA³, we opted for overexpression (OE) of these proteins with particular tags (collectively called RavA-OE and ViaA-OE for overexpressed RavA and ViaA constructs respectively). The N- or the C-terminus of RavA was tagged with a 12 amino acid BC2 peptide and the resulting RavA-OE was immunolabelled with an anti-BC2 nanobody²³ coupled to the Alexa Fluor 647 fluorescent dye for stochastic optical reconstruction microscopy (STORM) imaging. ViaA-OE constructs were produced by fusion of the PAmCherry fluorescent protein²⁴ to either the N- or the C-terminus of ViaA, and imaged by photo-activated localisation microscopy (PALM). In addition, PAmCherry was fused to the C-terminus of the NTV and to the N-terminus of the CTV to make NTV-OE and CTV-OE respectively.

Unexpectedly, although RavA is presumed to be cytoplasmic, RavA-OE showed a propensity to localise at the cell periphery rather than being distributed homogeneously through the volume (Figure

3A). Furthermore, both ViaA-OE constructs, as well as CTV-OE, also exhibited a distinct peripheral localisation pattern around the entire circumference of the cell, with a tendency to accumulate at the polar regions (Figure 3A). The NTV-OE, in contrast, formed cytosolic bodies (Supplementary Figure 2). Wide-field images of His-RavA and His-ViaA overexpressing *E. coli* cells labelled with specific anti-RavA and anti-ViaA nanobodies coupled to Alexa Fluor dyes (Materials and Methods) further confirmed the inner membrane distribution of RavA and ViaA (Supplementary Figure 2). Altogether, these experiments indicate that *in cellulo* ViaA is targeted to the bacterial inner membrane despite the absence of ViaA binding to purified NuoEFG and FrdA *in vitro*, with the CTV being required for this targeting.

RavA accumulates under the inner membrane whereas ViaA modifies membrane morphology of *E. coli* cells

In parallel to imaging the RavA-OE and ViaA-OE bacteria by epifluorescence and super-resolution microscopy, these bacteria were subjected to high pressure freezing and freeze substitution followed by ultrathin cell sectioning, and their ultrastructure examined by transmission electron microscopy (EM). A dark layer of matter, supposedly corresponding to the RavA protein, accumulated under the bacterial inner membrane, in line with the fluorescence microscopy observations (Figure 3B). Most strikingly, arrays of ectopic intracellular membrane tubes were discovered to underlie the inner membrane of the ViaA-OE bacteria (Figure 3B). Interestingly, overexpression of few membrane proteins with particular topologies has previously been noticed to induce formation of tubular membrane networks of reminiscent hexagonal phase morphologies^{25,26}. Such cardiolipin (CL)-enriched neo-membranes are proposed to form in order to accommodate the highly overproduced membrane protein, thereby relieving the inner cellular membrane from the associated stress. Thus, on the one side we have biochemically and biophysically characterised a soluble form of ViaA *in vitro*, and on the other side our morphological cellular EM observations agree with and corroborate our optical imaging data that show localisation of ViaA and RavA at the inner membrane of the bacterial cell. This raises two immediate questions: (i) are ViaA and RavA capable of directly binding to lipids, and (ii) do they modulate cellular lipid homeostasis, and in particular, does ViaA overexpression increase the cellular amounts of CL? In this respect, it is interesting to note that the conical shape of CL is known to favor its clustering in negative curvature regions such as cell poles and the division septum^{27,28}, which would agree with the localization of the overexpressed ViaA that we observed by PALM.

Both RavA and ViaA bind specific anionic phospholipids *in vitro*

The inner membranes of *E. coli* consist of three major anionic phospholipids: 70-80% phosphatidylethanolamine (PE), 20-25% phosphatidylglycerol (PG) and ~5% cardiolipin (CL), the exact content of the latter being culture condition and growth phase-dependent²⁹⁻³¹. The universal precursor of these phospholipids is phosphatidic acid (PA). Our dot-blot assays (Figure 4A, Supplementary Figure 3) revealed that purified RavA specifically binds PG, whereas ViaA specifically binds PA and to a lesser extent CL. RavA interactions with ViaA and PG are mutually exclusive; likewise, inside the LdcI-RavA complex, RavA is no longer able to interact with PG. In contrast, lipid-bound ViaA is still capable of interacting with RavA (Figure 4A, Supplementary Figure 3). Surprisingly, RavA_{ΔLARA} loses the PG binding capacity (Supplementary Figure 3), which shows that the LARA domain is implicated not only in the interaction with LdcI but also in the interaction with lipids. In this light, and since the LARA domain is involved in lipid binding but not in ViaA binding (Supplementary Figure 1), the observation that RavA cannot simultaneously interact with ViaA and PG suggests that binding to PG may induce a conformational change in the RavA molecule, making it inapt for ViaA binding.

The observation that ViaA can simultaneously interact with RavA and with lipids is consistent with ViaA being a two-domain protein, with the NTV binding to RavA and the CTV binding to lipids. Since the 3D structure of ViaA is unknown, we examined its primary sequence and the recent 3D structure prediction of the CTV provided by AlphaFold^{32,33} and RoseTTAFold³⁴ (Figure 5) for hints to

possible positions of a lipid binding site. The C-terminal end of ViaA is highly positively charged, with eight arginines, six of which are situated in the predicted α -helix at the C-terminal extremity (Figure 5A). Compelled by the amphipathic nature of this helix (Figure 5B) and its high conservation among enterobacterial ViaA proteins (Figure 5C), we reasoned that the C-terminus of ViaA might be involved in electrostatic interactions with the negatively charged head groups of PA and CL. In particular, R476 and R479 are predicted to be situated at the same side of the α -helix and seem to form a positive patch (Figure 5B). To evaluate this hypothesis, we designed the three following mutants: ViaA₁₋₄₇₂, devoid of the entire C-terminus, ViaA_{R476A/R479A} and ViaA_{R476E/R479E}. As shown in Supplementary Figure 3, mutations of R476 and R479 into alanines notably attenuate interaction with lipids, whereas the removal of the C-terminus and the R-to-E double mutation result in the abrogation of the lipid-binding propensity. Altogether, combined with the membrane distribution and effects on membrane morphology, ViaA unexpectedly joins the list of peripheral membrane proteins tethered to the inner *E. coli* membrane through an amphipathic helix displaying affinity for anionic lipids and in particular CL, which also includes proteins such as the cell division site selection ATPase MinD^{35,36} and the cell shape-determining actin homolog MreB³⁷. Strikingly, although ViaA is a peripheral membrane protein, its membrane-targeting C-terminal sequence contains all the key features of a high-affinity CL-binding site on bacterial integral membrane proteins, recently identified by molecular dynamics simulations³⁸: two or three adjacent basic residues, at least one polar, a neighbouring aromatics and a glycine proposed to confer a higher affinity for the CL head group.

Finally, to further validate the proposed lipid binding sites of RavA and ViaA, we examined the morphology of RavA Δ LARA-OE, ViaA_{R476E/R479E}-OE and ViaA₁₋₄₇₂-OE cells by EM and compared it to the observations presented in Figure 3B. Remarkably, these mutants formed soluble cytosolic bodies inside the bacterial cells (Supplementary Figure 4), although the overexpression levels remained very similar to those of the lipid binding-competent counterparts (Supplementary Figure 5). Thus, the inner membrane localisation and the morphological changes observed with RavA-OE and ViaA-OE are unequivocally related to their propensity to target specific phospholipids.

220 **ViaA modifies cellular lipid homeostasis *in vivo***

We then investigated the effect of RavA or ViaA overexpression on the membrane lipid composition (Figure 4B, Supplementary Figure 6). To this end, lipids were extracted from different cellular samples, and analysed by gas chromatography, thin layer chromatography and mass spectrometry (see Methods). As shown in Supplementary Figure 7, RavA-OE, RavA Δ LARA-OE, ViaA_{R476E/R479E}-OE and ViaA₁₋₄₇₂-OE bacteria contained slightly but statistically significantly fewer lipids, quantified by their fatty acids, than the wild type (WT) MG1655 cells, whereas the total fatty acid content in ViaA-OE remained roughly similar to the WT. A similar behaviour was observed for the relative proportion of PG among the total lipids, whereas the relative proportion of PE remained unchanged. As expected from the electron microscopy observations, the most spectacular changes were observed with CL, the proportion of which was 6 to 10-fold increased in the ViaA-OE cells, whereas overexpression of the other constructs led to a much milder (but still statistically significant) CL increase in comparison to the WT bacteria (Figure 4B). The low native expression level of ViaA precludes conclusive assessments of the potential subtle difference in the lipid metabolism between the WT and the MG1655- Δ ravAviaA strains under anaerobic conditions favoring expression of the *ravA-viaA* operon. One should however keep in mind that as observed for some rare membrane proteins, an inner membrane stress due to its saturation resulting from the ViaA overexpression may be relieved by formation of the CL-enriched neo-membranes that enclose the excess of ViaA.

238 **Lipid binding-deficient chromosomal mutants of RavA and ViaA impair RavA and ViaA function in gentamicin sensitisation**

Having recently shown that *ravA-viaA* sensitise *E. coli* to gentamicin (Gm) in fumarate respiratory condition⁵, we decided to check if lipid binding-deficient chromosomal MG1655 mutants ViaA₁₋₄₇₂, ViaA_{R476E/R479E} and RavA Δ LARA are still capable of Gm sensitisation. To this end, we performed a time-dependent killing experiment, using a Gm concentration equivalent to twice the minimum inhibitory

concentration. As shown in Figure 6, all mutants exhibited increased resistance to Gm compared to the WT strain. This result demonstrates that RavA and ViaA mutants, designed to be unable to bind lipids, prevent Gm toxicity under fumarate respiratory conditions.

An attractive hypothesis could be that ViaA, eventually with the help of RavA, would sensitise *E. coli* to Gm by directly binding to the membrane and modifying its permeability. To address this possibility, we performed planar lipid bilayer experiments (Supplementary Figure 7) that confirmed binding of single molecules of RavA and ViaA to membranes *in vitro* but did not detect formation of membrane pores that would facilitate AG transport. Therefore, we concluded that RavA and ViaA target phospholipids without perforating the lipid bilayer such as to directly enable Gm uptake into the bacterial cell.

Discussion

Since AGs are notoriously inefficient under the anaerobic and acidic conditions of the human gastrointestinal tract, insights into the mechanism of the *ravA-viaA* function in *E. coli* sensitisation to AGs should improve our understanding and fight against the global threat of antibiotic resistance. The *E. coli* MoxR AAA+ ATPase RavA has long been known to tightly bind one of the main enterobacterial acid stress response proteins, the acid stress inducible lysine decarboxylase LdcI, thereby preventing its inhibition by the nutrient stress response alarmone ppGpp^{6,7}. However, a characteristic of the MoxR AAA+ ATPases is also the close co-occurrence of their genes with genes encoding VWA domain-containing proteins, with which they are functionally linked⁶. The *ravA-viaA* operon, shown to be controlled by the anaerobic transcriptional regulator Fnr¹², sensitises *E. coli* to AGs in respiratory conditions when fumarate is used as electron acceptor instead of O₂^{5,39}. Thus, we decided to focus this work on a thorough characterisation of ViaA and the RavA-ViaA interaction, and investigated their molecular functions beyond the LdcI-RavA interaction.

We now provide extensive *in vitro* and *in vivo* biochemical, biophysical, optical imaging and morphological evidence that RavA and ViaA do not only interact in a nucleotide-dependent fashion, but also localise at the bacterial inner membrane, bind specific phospholipids and modulate membrane lipid composition. In particular, we demonstrate specific RavA-PG and ViaA-PA/CL interaction, and reveal a prominent effect of ViaA on the CL-enrichment of the *E. coli* inner membrane. A possible explanation of the effect of ViaA on CL can be proposed based on our finding that ViaA interacts strongly with PA (Figure 4A). Indeed, PA, PG and CL are tightly interconnected: PA, only present in tiny amounts in *E. coli* cells, is a metabolic hub for PG synthesis, whereas two PG molecules are necessary to produce CL⁴⁰. The synthesis of CL in bacteria requires that a first PG receives a phosphatidyl group from a second PG by a transesterification reaction catalysed by cardiolipin synthase (Cls). *E. coli* contains three Cls isoforms⁴¹, with ClsA being the major contributor to CL synthesis during exponential growth. Interestingly, PA was shown to strongly inhibit ClsA activity⁴². Consequently, the association of ViaA with PA at the inner membrane level could trap PA, thereby preventing Cls inhibition and eventually favouring CL synthesis, leading to a modification in the lipid homeostasis. Independently of the concrete mechanism, one may anticipate that by binding to PA and CL, natively expressed ViaA would influence some local membrane properties, for example by acting on the local membrane curvature, affecting membrane permeability or coming into close proximity to proteins present in CL-enriched lipid microdomains. Excitingly, such microdomains form CL-based proton sinks⁴³ that compartmentalise oxidative phosphorylation complexes^{44,45} such as Nuo and F₁F₀ and are proposed to also attract LdcI¹⁵.

Importantly, we show that the RavA and ViaA lipid-binding propensity is directly linked to their effect on the AG bactericidal activity under anaerobiosis, because mutations abolishing interaction with lipids preclude Gm toxicity under conditions of fumarate respiration. Our finding that RavA and ViaA do not permeabilise the lipid bilayer, and the independent observations by us and by others that anaerobic RavA-ViaA-mediated AG sensitivity is dependent on the proton motive force (pmf)^{3,5,39}, suggest that they may positively act on F₁F₀ or other respiratory complexes, thereby enhancing the pmf required for efficient AG uptake. Considering the absence of a direct interaction with purified F₁F₀

and NuoEFG in a BLI setup as described above, as well as the lipid binding and membrane remodelling activity of RavA and ViaA revealed in the present study, we propose that RavA and ViaA chaperone certain respiratory complexes by acting on lipid microdomains in which these complexes are inserted. This hypothesis aligns with our recent observation of a patchy peripheral distribution of LdcI, which we attributed to an attraction of this proton-consuming acid stress response enzyme to anionic phospholipids domains forming proton sinks that compartmentalise oxidative phosphorylation complexes¹⁵. Thus, this work sets the stage for future investigations of an unprecedented molecular network that links the LdcI-RavA-ViaA triad with bacterial stress adaptation, membrane homeostasis, respiratory complexes and aminoglycoside bactericidal activity.

Materials and Methods

Bacterial strains and plasmids construction

All plasmids, primers, and cloning strategies are summarized in Supplementary Table 1. The *ravA* and *viaA* genes were PCR amplified from *Escherichia coli* K-12 MG1655 genomic DNA using Phusion polymerase (Biolabs). All PCR products were purified by DNA cleanup kit (Qiagen). Gibson assemblies were performed using 0.4 U T5 exonuclease, 2.5 U Phusion polymerase, and 400 U Taq ligase (New England Biolabs) in 1× ISO buffer consisting of 100 mM Tris·HCl pH 7.5, 10 mM MgCl₂, 0.8 mM dNTP mix, 10 mM dithiothreitol (DTT), 50 mg polyethylene glycol (PEG)-8000, 1 mM nicotinamide adenine dinucleotide (NAD). A total of 7.5 μL of the Gibson Master Mix was mixed with 2.5 μL DNA, containing ~100 ng of vector. The mix was incubated for 60 min at 50 °C. Transformations were performed in Top10 competent bacteria (One Shot TOP10 chemically competent *E. coli*, Invitrogen) and selected using 100 μg/mL ampicillin or/and 34 μg/mL chloramphenicol (Euromedex). Agarose gel purification and DNA plasmid extractions were performed using a QIAquick Gel extraction kit (QIAGEN). Restriction enzyme digests were performed according to manufacturer recommendations (Biolabs). Bacteria were made electrocompetent by growing cells to an OD₆₀₀ of around 0.7. Cells were then placed on ice and washed three times with ice-cold, sterile water. Electroporation using the MicroPulser (BioRad) was followed by 1 h growth in fresh LB medium.

Mutant strains of K-12 substrain MG1655 (ATCC 47076) were generated by recombineering following the protocols of earlier publications^{46,47}. Briefly, the pKD46 plasmid was used to express the three proteins Gam, Exo, and Beta, that mediate homologous recombination (HR) and that are originally derived from the bacteriophage lambda. On pKD46, expression of those genes is controlled by the *araBAD* promoter. pKD46 has an ampicillin resistance gene and a temperature sensitive origin of replication (ORI), hence cells with pKD46 were grown at 30°C with ampicillin. Recombineering requires DNA substrates with regions homologous to the target genome. Those substrates were produced by PCR using specifically designed pKD3-plasmids serving as PCR-template. pKD3 encodes the gene *CAT* allowing selection of engineered bacteria using chloramphenicol. The *CAT* cassette is flanked by FRT sites and can be removed using FLP recombinase. FLP is expressed from the pCP20 plasmid. pCP20 has a temperature-sensitive ORI and encodes the ampicillin resistance gene *ampR*, hence cells were grown at 30°C with ampicillin to keep the plasmid. To induce expression of FLP and to lose the plasmid, cells were grown at 43°C.

To engineer the here described mutant strains MG1655 Δ*ravA*/*viaA*(1-472)::cat, MG1655 Δ*ravA*/*viaA*-R476E/R479E::cat and MG1655 *ravA*Δ*LARA*/Δ*viaA*::cat by HR, we targeted the following genomic sequence flanking the *ravA*/*viaA* operon: (5'-GTATGGCCAGCTGCTGTTTCGCGAGAGCGTCCCTTCTCTGCTGTAAGCCATGGTCCATATGAATATCC-3') and (5'-CTCGCAATTTACGCAGAACTTTTGACGAAAGGACGCCACTTCATTATGGCTCACCTCATTATTAGC-3'). To obtain PCR products for HR that encode mutant *viaA* and lack *ravA*, the corresponding mutant *viaA* genes were cloned into pKD3 (pKD3_ViaA(1-472), pKD3_ViaA(R476E/R479E)). Likewise, to get PCR products for HR that encode mutant *ravA* and lack *viaA*, the corresponding mutant *ravA* gene was cloned into pKD3 (pKD3_RavA-Δ*LARA*).

For recombineering, the pKD46 plasmid was electroporated into MG1655ΔravA/ΔviaA target cells and grown on ampicillin plates over night at 30°C. On the second day, single colonies of MG1655ΔravA/ΔviaA:pKD46 were re-streaked on ampicillin plates and grown overnight at 30°C. In the afternoon of the third day, a 20 ml pre-culture of MG1655:pKD46 cells was prepared and grown over night at 30°C. On the fourth day, in order to induce expression of *gam*, *exo* and *beta*, about 3 ml of pre-culture was diluted into 100 ml fresh medium containing ampicillin and 0.2% arabinose (final concentration) and grown to an optical density of about 0.7. Cells were immediately made electrocompetent and electroporated with the specific PCR product that served as template for homologous recombination. All cells from the electroporation cuvette were plated on chloramphenicol plates and grown overnight at 37°C. On the fifth day, (all) single clones were re-streaked on fresh chloramphenicol plates for overnight growth at 37°C. The next day, single colonies were tested by colony PCR and re-streaked on chloramphenicol plates. Growth on test-plates that contain either ampicillin or no antibiotics served as control for expected loss of the pKD46 plasmid and to make glycerol stocks of bacteria respectively. PCR products with the expected size were sent for sequencing (Eurofins). In order to move the recombineered part of the genome into fresh cells, P1 phage transfer was conducted⁴⁸.

***Escherichia coli* ViaA cloning, expression and purification**

E. coli ViaA (Genebank nr.: AAT48203.1, Uniprot ID: P0ADN0) cloned in the p11-Toronto1 vector and containing an N-terminal hexahistidine (6xHis) tag was obtained from Prof. Dr. Walid Houry. Overlap extension PCR was used to add a C-terminal AviTag (GLNDIFEAQKIEWHE) to the His-ViaA construct. Additionally, Gibson assembly was used to clone ViaA in the pET22b vector containing a C-terminal His-tag and an added N-terminal AviTag.

N- and C-terminally AviTagged ViaA constructs, cloned in the pET22b and p11-Toronto1 vectors respectively, were transformed in chemically competent BL21 (DE3) *E. coli* cells. The resulting transformants were grown in a 50 ml preculture supplemented with Ampicillin to an OD of 3.5. Next, 2 l of LB supplemented with Ampicillin was inoculated with 40 ml of the pre-culture and grown at 37 °C until an OD of 0.8 was reached. Cells were subsequently induced with 0.5 mM IPTG and incubated at 18 °C overnight (ON). The next day, the cells were harvested by centrifuging the cultures at 4,000 g for 45 min.

For purification of expressed AviTag-ViaA-His and His-ViaA-AviTag constructs, pellets (4 for a 2 l culture) were resuspended in 30 ml 25 mM HEPES pH 7.5, 300 mM NaCl, 10 mM MgCl₂, 10 % Glycerol, 1 μl benzonase, 1 x Complete tablet, and disrupted by three passages through a Homogeniser at 18,5000 psi. Cell debris was removed by centrifugation for 1 h at 20.00048,384g, and the supernatant was filtered using a 0.2 μl filter and flown over a 5 ml NiNTA Immobilised Metal Affinity Chromatography (IMAC) column (GE Healthcare) equilibrated with buffer A (25 mM HEPES pH 7.5, 300 mM NaCl, 10 mM MgCl₂, 10 % Glycerol, 25 mM Imidazole). AVI-ViaA-HIS was washed with 5 CV of buffer A, and eluted using a gradient of 0 – 100% buffer B (25 mM HEPES pH 7.5, 300 mM NaCl, 10 mM MgCl₂, 10 % Glycerol, 300 mM Imidazole). The top fractions after IMAC were pooled and desalted using a 5 ml HiTrap Desalting column (GE Healthcare) equilibrated with 25 mM HEPES pH 7.5, 300 mM NaCl, 10 mM MgCl₂, 10 % Glycerol. After desalting, the buffer exchanged protein was concentrated and injected on a SD200 10/300 increase column (GE Healthcare) equilibrated with 25 mM HEPES pH 7.5, 300 mM NaCl, 10 mM MgCl₂, 10 % Glycerol, 1 mM DTT. Purified ViaA fractions, eluted at around 14 ml, were flash-frozen and stored at -80 °C for later use.

***Escherichia coli* NuoEF, NuoEFG and FrdA expression and purification**

Expression of soluble NuoEF and NuoEFG was based on Braun *et al.*²⁰ and Bungert *et al.*²¹. Chemically competent *E. coli* BL21 (DE3) cells were transformed with a pET-11a vector containing NuoB-G subunits with NuoF harboring an N-terminal Strep-Tag, kindly provided by Thorsten Friedrich²¹. The resulting transformants were grown for approx. 5 hours in a 2 L culture of LB medium supplemented with 100 mg/L ferric ammonium citrate added in aliquots of 20 mg/L per hour,

2 mg/L cysteine, and 20 mg/L riboflavin. Cells were grown at 37 °C and 0.1 mM IPTG was added when the OD reached 0.8. After three hours, cells were pelleted by centrifugation at 4,000g for 10 min. The cell pellet was resuspended in 50 mM MES, 50 mM NaCl, pH 6.6, 10 µg/ml DNase and 1X Complete tablet and disrupted by one passage through a Homogeniser at 18,000 psi. Cell debris was removed by centrifugation for 1 h at 250,000g. The supernatant was filtered using a 0.2 µl filter and flown over a 5 ml StrepTactin-Sepharose column (GE Healthcare) equilibrated with buffer A (50 mM MES-NaOH, 50 mM NaCl, pH 6.6). The column was washed with buffer A, and the NuoEF and EFG fragments were eluted with buffer A supplemented with 2.5 mM D-desthiobiotin. The eluted NuoEF and NuoEFG mixture appeared reddish/brown, and was further purified using a SD200 10/300 increase column (GE Healthcare) equilibrated with 50 mM MES, 500 mM NaCl, pH 6.6, resulting in two separated peaks containing NuoEFG and NuoEF respectively. Fractions containing NuoEFG and NuoEF, verified by SDS-PAGE, were concentrated separately, flash frozen and stored at -80 °C.

Expression of soluble FrdA was based on the protocol by Léger *et al.*²². Chemically competent *E. coli* BL21 (DE3) cells with a knock-out in FrdB were transformed with N-terminal His-tagged FrdA in the pnEA_His6_3C vector, gratefully obtained by Dr. Christophe Romier, Strasbourg. The resulting transformants were used to inoculate 200 ml of LB + 200 µl Ampicillin in a 1 l flask and the culture was grown at 37 °C with vigorous aeration (250 rpm) ON. The pre-culture was used to inoculate 0.5 of LB medium with 500 ml Ampicillin. The resulting culture was grown at 37 °C until the OD reached 0.6. Gene expression was induced with 0.5 mM IPTG. After +/- 18h of expression at 18 °C, cells were pelleted by centrifugation at 4000 g for 45 min. The cell pellet was resuspended in 30 ml Buffer A (50 mM KH₂PO₄ pH 7, 500 mM NaCl, 1% glycerol) supplemented with 1x Complete tablet and 0.5 µl benzonase. Cells were opened by sonication, and cell debris was removed by centrifugation for 1 h at 20,000 g. The supernatant was filtered using a 0.2 µl filter and flown over a 5 ml NiNTA IMAC column (GE Healthcare) equilibrated with buffer A (50 mM KH₂PO₄ pH 7, 500 mM NaCl, 1% glycerol). Bound FrdA was washed with 5% buffer B (50 mM KH₂PO₄ pH 7, 500 mM NaCl, 1% glycerol, 500 mM Imidazole), followed by elution using a gradient from 5 – 100 % buffer B. Eluted FrdA fractions appeared bright yellow due to the present FAD cofactor. The eluted FrdA fraction was subsequently concentrated and injected onto a SD200 10/300 increase column (GE Healthcare) equilibrated with 20 mM HEPES pH 7.5, 300 mM NaCl, 10% glycerol, 1 mM DTT. Purified FrdA fractions were flash-frozen and stored at -80 °C for later use.

Multi-Angle Laser light Scattering (MALLS)

Prior to conducting MALLS experiments, flash-frozen purified AviTag-ViaA-His and His-NTV-Avi samples were thawed and centrifuged at 20,000g for 30 min. SEC-MALLS experiments were conducted at 4 °C on a high-performance liquid chromatography (HPLC) system (Schimadzu, Kyoto, Japan) consisting of a DGU-20 AD degasser, an LC-20 AD pump, a SIL20-AC_{HT} autosampler, a CBM-20A communication interface, an SPD-M20A UV-Vis detector, a FRC-10A fraction collector, an XL-Therm column oven (WynSep, Sainte Foy d'Aigrefeuille, France) and static light scattering miniDawn Treos, dynamic light scattering DynaPro NANOSTAR and refractive index Optilab rEX detectors (Wyatt, Santa-Barbara, USA).

Purified AviTag-ViaA-His (80 µl at 3 mg/ml) or His-NTV-AviTag (80 µl at 2 mg/ml) were injected on a Superdex 200 increase 10/300 GL column (GE Healthcare), equilibrated at 4°C with a buffer containing 25 mM HEPES pH 7.5, 300 mM NaCl, 10 mM MgCl₂ and 10 % Glycerol, at a flow rate of 0.5 ml/min. Bovine serum albumin (BSA) at 2 mg/ml in phosphate-buffered saline (PBS) buffer was injected as a control. The extinction coefficient and refractive index increments for the proteins were calculated from the amino acid sequences using the SEDFIT software.

Small Angle X-Ray Scattering (SAXS)

SAXS data were collected on the BM-29 BIOSAXS beamline at the ESRF (Grenoble, France)⁴⁹, equipped with a Pilatus3 2M detector operated at a wavelength of 0.9919 Å and using a sample-detector distance 2.867 m, resulting in a scattering momentum transfer range of 0.003 Å⁻¹ to 0.494 Å⁻¹. Purified AviTag-ViaA-His was measured at a concentration of 3 mg/ml in a buffer containing 25

444 mM HEPES pH 7.5, 300 mM NaCl, 10 mM MgCl₂ and 10 % Glycerol. Measurements were
445 performed at 20 °C, and 10 frames with an individual exposure time of 0.5 s were taken per sample.
446 Initial data integration, averaging and background subtraction were performed using PRIMUS-QT
447 from the ATSAS software package ⁵⁰.

448 The forward scattering (I_0) and radius of gyration (R_g) were determined by PRIMUS-QT using
449 Guinier approximation ⁵¹. The Porod volume estimate (V_p) was evaluated using Autoporod ⁵². The
450 maximum particle dimension D_{max} and distance distribution function $P(r)$ were evaluated using
451 ScÅtter ¹⁸. The molecular mass of the His-ViaA-AviTag sample was calculated using the online
452 SAXSMoW 2.0 program ¹⁷ and the ScÅtter software package.

453 **Bio-Layer Interferometry**

454 For BLI binding studies, biotinylated AviTag-ViaA-His, His-ViaA-AviTag and His-NTV-AviTag
455 samples were expressed and purified as described earlier, but with the addition of 50 µM D-biotin to
456 the LB medium during expression overnight at 18 °C. BLI experiments were performed in HBS
457 kinetics buffer, containing 25 mM HEPES pH 7.5, 300 mM NaCl, 10 mM MgCl₂, 10 % Glycerol,
458 0.1% w/v BSA and 0.02% v/v Tween-20), using an Octet RED96 instrument (FortéBio) operated at
459 293 K. Streptavidin-coated biosensors (FortéBio) were functionalised with biotinylated AviTag-ViaA-
460 His, His-ViaA-AviTag or His-NTV-Avi to a maximum signal of 1 nm, subsequently quenched with
461 10 µg ml⁻¹ biocytin, and transferred to wells containing 5 different concentrations of ligand. When
462 RavA or RavAΔLARA was used as a ligand, 1mM ADP was added to the HBS kinetics buffer and
463 RavA or RavAΔLARA samples were incubated with 1 mM ADP for 10 min prior to conducting
464 measurements. Buffer subtraction was performed using a functionalized biosensor measuring running
465 buffer. To check for nonspecific binding during the experiments, non-functionalized biosensors were
466 used to measure the signal from the highest ligand concentration as well as running buffer. All data
467 were fitted with the FortéBio Data Analysis 9.0 software using a 1:1 interaction model. All binding
468 experiments were performed in triplicate (technical triplicates), and calculated K_D , k_d and k_a values
469 represent the averages of these triplicate experiments.

470 **Nanobody production and labelling**

471 The anti-RavA-Nb and anti-ViaA-Nb were obtained from the nanobody generation platform of the
472 AFMB laboratory (Marseille, France) as described ¹⁵. The anti-BC2-Nb ²³ was kindly provided by
473 Ulrich Rothbauer. Nanobodies were labelled as described ¹⁵.

474 **Overexpression of recombinant RavA and ViaA constructs for fluorescence imaging, cellular 475 EM observations, and lipid extraction and analysis**

476 MG1655 cells were transformed with a low-copy auxiliary pT7pol26 (Kan^R) plasmid that codes
477 for T7 RNA polymerase under the control of a lac promoter ⁵³. The resulting MG1655/pT7pol26
478 (Kan^R) strain was then transformed with a plasmid (Amp^R) carrying a gene coding for a desired RavA
479 or ViaA construct under the control of the T7 promoter. The cells were cultured at 37 °C until the
480 OD₆₀₀ reached 0.6, and then RavA-OE or ViaA-OE was induced with 40 µM IPTG for 12 hours, at 18
481 °C, under aerobic conditions.

482 **Fluorescence imaging**

483 For immunofluorescence staining cells were harvested, fixed and permeabilised as described ¹⁵.
484 For epifluorescence imaging of fluorescent protein fusion constructs the procedure was the same
485 except that the fixation was performed with 1% formaldehyde solution in PBS, and that no
486 permeabilisation was necessary.

487 **Wide field imaging**

488 For each sample, 2 µL of cells in suspension were mounted between a glass slide and a 1.5h glass
489 coverslip, and observed using an inverted IX81 microscope, with the UPLFLN 100× oil immersion
490 objective from Olympus (numerical aperture 1.3), using a fibered Xcite™ Metal-Halide excitation

491 lamp in conjunction with the appropriate excitation filters, dichroic mirrors, and emission filters
492 specific for DAPI/Hoechst, AF488, mCherry or AF647 (4X4MB set, Semrock). Acquisitions were
493 performed with Volocity software (Quorum Technologies) using a sCMOS 2048 × 2048 camera
494 (Hamamatsu ORCA Flash 4, 16 bits/pixel) achieving a final magnification of 64 nm per pixel.

495 **STORM and PALM imaging**

496 Super-resolution single molecule localization microscopy was performed using STORM (after
497 nanobody labeling) and PALM (PAmCherry fusion proteins) approaches. For STORM imaging, cells
498 labeled as mentioned above, were transferred to a glucose buffer containing 50 mM NaCl, 150 mM
499 Tris (pH 8.0), 10% glucose, 100 mM MEA (mercaptoethylamine) and 1x Glox solution from a 10x
500 stock containing 1 μM catalase and 2.3 μM glucoseoxidase. For PALM, cells expressing the ViaA
501 fused constructs with PAmCherry, were resuspended in PBS. In each case, 2 μL of the cells in
502 suspension were mounted as specified in Jessop and al.(2021). Mounted samples were imaged on an
503 IX81 microscope (Olympus) by focusing the excitation lasers to the back focal plane of an oil
504 immersion UAPON100X (N.A. 1.49) objective. Acquisitions were performed using an Evolve 512
505 camera with a gain set to 200 (electron- multiplying charge-coupled device EMCCD, 16 bits/pixel,
506 Photometrics) using Metamorph (Molecular Devices). Excitation lasers power was controlled by an
507 Acousto-Optical Tunable Filter (OATF, Quanta Tech). SMLM datasets of about 30,000 frames were
508 generated using 3 kW/cm² of a 643 nm laser in conjunction with up to 1 W/cm² of a 405 nm laser at a
509 framerate of 20 frames per seconds (STORM) or approximately 1 kW/cm² of a 561nm laser after
510 photoactivation of PAmCherry using a few watts/cm² of a 405 nm laser (PALM). Data processing was
511 performed using Thunderstorm plugin in ImageJ ^{54,55}.

512 **Cellular EM observations**

513 WT, RavA-OE and ViaA-OE cells were centrifuged at 4000 g for 5 min. A pellet volume of 1.4 μl
514 was dispensed on the 200 μm side of a type A 3 mm gold platelet (Leica Microsystems), covered with
515 the flat side of a type B 3 mm aluminum platelet (Leica Microsystems), and was vitrified by high-
516 pressure freezing using an HPM100 system (Leica Microsystems). Next, the samples were freeze
517 substituted at -90 °C for 80 h in acetone supplemented with 1% OsO₄ and warmed up slowly
518 (1°C h⁻¹) to -60 °C in an automated freeze substitution device (AFS2; Leica Microsystems). After 8
519 to 12 h, the temperature was raised (1°C h⁻¹) to -30°C, and the samples were kept at this temperature
520 for another 8 to 12 h before a step for 1 h at 0 °C, cooled down to -30 °C and then rinsed four times in
521 pure acetone. The samples were then infiltrated with gradually increasing concentrations of Epoxy
522 Resin (Epoxy Embedding Medium kit, Merck) in acetone (1:2, 1:1, 2:1 [vol/vol] and pure) for 2 to 8 h
523 while raising the temperature to 20°C. Pure epoxy resin was added at room temperature. After
524 polymerization 24 h at 60°C, 60 to 80 nm sections were obtained using an ultra-microtome UC7
525 (Leica Microsystems) and an Ultra 35° diamond knife (DiATOME) and were collected on formvar-
526 carbon-coated 100 mesh copper grids (EMS). The thin sections were post-stained for 10 min with 2%
527 uranyl acetate, rinsed and incubated for 5 min with lead citrate. The samples were observed using a
528 FEI Tecnai12 120kV LaB6 microscope with an Orius SC1000 CCD camera (Gatan).

529 **Lipid extraction**

530 Lipids were extracted from freeze-dried cells. First, cells were harvested by centrifugation and
531 then immediately frozen in liquid nitrogen. Once freeze-dried, the pellet was suspended in 4 mL of
532 boiling ethanol for 5 minutes to prevent lipid degradation and lipids were extracted according to Folch
533 *et al.* ⁵⁶ by addition of 2 mL methanol and 8 mL chloroform. The mixture was saturated with argon
534 and stirred for 1 hour at room temperature. After filtration through glass wool, cell remains were
535 rinsed with 3 mL chloroform/methanol 2:1, v/v and 5 mL of NaCl 1% were added to initiate biphasic
536 formation. The chloroform phase was dried under argon and the lipid extract was stored at -20°C.

537 **TLC and GC-FID/MS phospholipid quantification**

538 Total phospholipids were quantified from their fatty acids. The lipid extract was solubilised in
539 pure chloroform and 5 µg of C21:0 (internal standard) were added in an aliquot fraction. Fatty acids
540 were converted to methyl esters (FAME) by a 1-hour incubation in 3 mL 2.5% H₂SO₄ in pure
541 methanol at 100°C⁵⁷. The reaction was stopped by addition of 3 mL water and 3 mL hexane. The
542 hexane phase was analyzed by gas chromatography (Clarus 80, Perkin Elmer) on a BPX70 (SGE)
543 column. FAMES were identified by comparison of their retention times with those of standards
544 (Sigma) and by their mass fragmentation spectra. They were quantified using C21:0 for calibration.
545 To quantify each class of phospholipid, 300 µg of lipids were separated by two-dimensional thin layer
546 chromatography (TLC) onto glass-backed silica gel plates (Merck)⁵⁸. The first solvent was
547 chloroform:methanol:water (65:25:4, v/v) and the second one chloroform:acetone:methanol:acetic
548 acid:water (50:20:10:10:5, v/v). Lipids were sprayed with 2% 8-anilino-1-naphthalenesulfonic acid in
549 methanol, then visualised under UV light and scraped off the plate. Lipids in the scraped silica were
550 quantified by methanolysis and GC-FID/MS as described above.

551 **Dot blot assay (Protein-lipid overlay assay)**

552 All phospholipids were purchased from Avanti Lipids (Sigma). 2 µl of chloroform solutions (5,
553 0.5 and 0.05 mg/ml) of 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (16:0-18:1 PC), 1-palmitoyl-
554 2-oleoyl-sn-glycero-3-phospho-L-serine (16:0-18:1 PS), 1-palmitoyl-2-oleoyl-sn-glycero-3-
555 phosphoethanolamine (PE 16:0-18:1 PE), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-
556 glycerol) (16:0-18:1 PG), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate (16:0-18:1 PA) and 1',3'-
557 bis[1-palmitoyl-2-oleoyl-sn-glycero-3-phospho]-glycerol (16:0-18:1 Cardiolipin) were spotted using a
558 Hamilton syringe onto a nitrocellulose membrane (Biorad trans-blot turbo RTA Midi 0.2 µm) to yield
559 10, 1 and 0.1 µg of the lipid per spot. The membranes were blocked using a Tris-buffered saline
560 (TBS) solution (pH 7.4) supplemented with 10 % (w/v) non-fat milk powder at room temperature for
561 1 h before incubated with 10 µg/ml of the purified proteins (RavA, ViaA, LdcI) or protein complex
562 (LdcI-RavA) in TBS containing 0.05 % (v/v) Tween-20 (TBST). After overnight incubation at 4 °C,
563 the membranes were washed with TBST (3×15 min) and probed either with anti-ViaA mouse
564 monoclonal antibody or a rabbit polyclonal antibody raised against the purified RavA or LdcI proteins
565 diluted in TBST at room temperature for 1 h. The membranes were washed with TBST (3×15 min)
566 and further incubated with either anti-mouse or anti-rabbit IgG secondary antibody conjugated with
567 horseradish peroxidase (Merk) at room temperature for 1 h. After washing with TBST (3×15 min), the
568 chemiluminescence signal was developed using a Pierce ECL Western blotting substrate (Thermo
569 Fisher Scientific, USA) and recorded using a ChemiDoc XRS+ System (Bio-rad, USA).

570 For RavA bound via ViaA, the milk-blocked membrane was incubated with TBST alone (RavA
571 only) or with 10 µg/ml ViaA overnight at 4 °C, washed three times with TBST buffer and incubated
572 with 10 µg/ml RavA for 2 h at room temperature. The chemiluminescent signal was developed after
573 the incubation of the membranes with anti-RavA serum followed by incubation with the HRP-
574 conjugated secondary antibody. Likewise, for ViaA bound via RavA, the membrane was incubated
575 with 10 µg/ml RavA overnight before incubation with 10 µg/ml ViaA. The LdcI-RavA complex was
576 prepared by mixing the purified LdcI and RavA proteins in molar ratio of 1:2.25 (LdcI:RavA) before
577 incubating for 30 min at room temperature. The milk-blocked membranes were incubated with RavA
578 (10 µg/ml) or LdcI (6.5 µg/ml) alone or with the LdcI-RavA complex (corresponding to the final
579 concentration of LdcI and RavA proteins of 6.5 µg/ml and 10 µg/ml, respectively) diluted in TBST
580 supplemented with 10 mM MgCl₂ and 2 mM ADP. After overnight incubation at 4 °C, the
581 membranes were washed and the chemiluminescent signal was developed after incubation of the
582 membranes with anti-RavA or anti-LdcI sera followed by incubation with the HRP-conjugated
583 secondary antibody (both diluted also in TBST-Mg-ADP buffer).

584 **Time-dependent killing assay**

585 This experiment was performed as previously described⁵. Briefly, overnight cultures were diluted
586 (1/100) and grown anaerobically at 37 °C to an OD₆₀₀ of 0.2 in LB medium supplemented with
587 fumarate. At this point (T0), Gm was added to the cells at 16 µg/mL. After 1.5 and 3 hours, 100 µL of

cells were diluted in sterile phosphate buffered saline solution (PBS buffer), spotted on LB agar and incubated at 37 °C for 48 hours. Cell survival was determined by counting colony-forming units per mL (CFU/mL). The absolute CFU at time-point zero (used as the 100%) was approximately 5x10⁷ CFU/mL. This assay was performed in an anaerobic chamber (Jacomex, France).

Planar lipid bilayer experiments

Planar lipid bilayer measurements were performed at 25 °C using a home-made Teflon cells separated by a diaphragm with a circular hole with the diameter of 0.5 mm. The membrane was formed by a painting method using a mixture of lipids composed of PA:PG:PE (10:45:45) dissolved in *n*-decane and 3 % asolectin dissolved in *n*-decane:butanol (9:1) for ViaA and RavA proteins and the *B. pertussis* pore-forming CyaA toxin, respectively. The membrane current was recorded by Theta Ag/AgCl electrodes after addition of 2 nM ViaA and RavA proteins or 250 pM CyaA toxin⁵⁹ in 10mM Tris-HCl (pH 7.4), 0.15 M KCl and 2 mM CaCl₂ using applied potential of -50 and +50 mV. The signal was amplified by an LCA-200-100G Ultra-Low-Noise Current Amplifier (Femto, Germany) and digitised by a Keithley KPCI-3108 PCI Bus Data Acquisition Boards card. The signal was analysed using QuB (<https://qub.mandelics.com>) and processed using a 10-Hz filter.

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Author contributions

JF, LB, CL, AF, FR, JYEK, KH, BG, CM, JPK, YD, MJ, EK and IG performed experiments, JF, LB, CL, AF, FR, JYEK, JPK, YD, MJ, FB, JJ and IG analysed data. IG designed the overall study, supervised the project and wrote the paper with significant input from JF and contributions from LB, AF, FR, KH, JPK, YD and JJ. JF prepared the figures with contributions from LB, CL, AF, FR, JYEK, JPK, YD and IG. All authors edited the manuscript prior to submission.

References

1. Krause, K. M., Serio, A. W., Kane, T. R. & Connolly, L. E. Aminoglycosides: An overview. *Cold Spring Harb. Perspect. Med.* **6**, (2016).

- 633 2. Girgis, H. S., Hottes, A. K. & Tavazoie, S. Genetic architecture of intrinsic antibiotic
634 susceptibility. *PLoS One* **4**, e5629 (2009).
- 635 3. Wong, K. S. *et al.* The MoxR ATPase RavA and its cofactor ViaA interact with the NADH:
636 Ubiquinone oxidoreductase I in *Escherichia coli*. *PLoS One* **9**, (2014).
- 637 4. Baharoglu, Z., Babosan, A. & Mazel, D. Identification of genes involved in low
638 aminoglycoside-induced SOS response in *Vibrio cholerae*: A role for transcription stalling and
639 Mfd helicase. *Nucleic Acids Res.* **42**, 2366–2379 (2014).
- 640 5. El Khoury, J., Zamarreño Beas, J., Huguenot, A., Py, B. & Barras, F. The AAA+ ATPase RavA-
641 ViaA complex sensitizes *Escherichia coli* to aminoglycosides under anaerobic low energy
642 conservation conditions. *bioRxiv* (2022). doi:10.1101/2021.08.03.455006
- 643 6. Snider, J. *et al.* Formation of a distinctive complex between the inducible bacterial lysine
644 decarboxylase and a novel AAA+ ATPase. *J. Biol. Chem.* **281**, 1532–46 (2006).
- 645 7. El Bakkouri, M. *et al.* Structure of RavA MoxR AAA+ protein reveals the design principles of
646 a molecular cage modulating the inducible lysine decarboxylase activity. *Proc. Natl. Acad. Sci.*
647 *U. S. A.* **107**, 22499–504 (2010).
- 648 8. Jessop, M. *et al.* Structural insights into ATP hydrolysis by the MoxR ATPase RavA and the
649 LdcI-RavA cage-like complex. *Commun. Biol.* **3**, 46 (2020).
- 650 9. Puchades, C., Sandate, C. R. & Lander, G. C. The molecular principles governing the activity
651 and functional diversity of AAA+ proteins. *Nat. Rev. Mol. Cell Biol.* **21**, 43–58 (2020).
- 652 10. Jessop, M., Felix, J. & Gutsche, I. AAA+ ATPases: structural insertions under the magnifying
653 glass. *Curr. Opin. Struct. Biol.* **66**, 119–128 (2021).
- 654 11. Whittaker, C. A. & Hynes, R. O. Distribution and evolution of von Willebrand/integrin A
655 domains: widely dispersed domains with roles in cell adhesion and elsewhere. *Mol. Biol. Cell*
656 **13**, 3369–87 (2002).
- 657 12. Wong, K. S., Bhandari, V., Janga, S. C. & Houry, W. A. The RavA-ViaA Chaperone-Like
658 System Interacts with and Modulates the Activity of the Fumarate Reductase Respiratory
659 Complex. *J. Mol. Biol.* **429**, 324–344 (2017).
- 660 13. Minato, Y., Fassio, S. R., Reddekopp, R. L. & Häse, C. C. Inhibition of the sodium-
661 translocating NADH-ubiquinone oxidoreductase [Na⁺-NQR] decreases cholera toxin
662 production in *Vibrio cholerae* O1 at the late exponential growth phase. *Microb. Pathog.* **66**, 36–
663 39 (2014).
- 664 14. Malet, H. *et al.* Assembly principles of a unique cage formed by hexameric and decameric *E.*
665 *coli* proteins. *Elife* **3**, e03653 (2014).
- 666 15. Jessop, M. *et al.* Supramolecular assembly of the *Escherichia coli* LdcI upon acid stress. *Proc.*
667 *Natl. Acad. Sci. U. S. A.* **118**, (2021).

- 668 16. Fischer, H., de Oliveira Neto, M., Napolitano, H. B., Polikarpov, I. & Craievich, A. F.
669 Determination of the molecular weight of proteins in solution from a single small-angle X-ray
670 scattering measurement on a relative scale. *J. Appl. Crystallogr.* **43**, 101–109 (2010).
- 671 17. Piiadov, V., Ares de Araújo, E., Oliveira Neto, M., Craievich, A. F. & Polikarpov, I.
672 SAXSMoW 2.0: Online calculator of the molecular weight of proteins in dilute solution from
673 experimental SAXS data measured on a relative scale. *Protein Sci.* **28**, 454–463 (2019).
- 674 18. Rambo, R. P. & Tainer, J. A. Accurate assessment of mass, models and resolution by small-
675 angle scattering. *Nature* **496**, 477–81 (2013).
- 676 19. Kandiah, E. *et al.* Structural insights into the Escherichia coli lysine decarboxylases and
677 molecular determinants of interaction with the AAA+ ATPase RavA. *Sci. Rep.* **6**, 24601 (2016).
- 678 20. Braun, M., Bungert, S. & Friedrich, T. Characterization of the Overproduced NADH
679 Dehydrogenase Fragment of the NADH:Ubiquinone Oxidoreductase (Complex I) from
680 Escherichia coli. *Biochemistry* **37**, 1861–1867 (1998).
- 681 21. Bungert, S., Krafft, B., Schlesinger, R. & Friedrich, T. One-step purification of the NADH
682 dehydrogenase fragment of the Escherichia coli complex I by means of Strep -tag affinity
683 chromatography. *FEBS Lett.* **460**, 207–211 (1999).
- 684 22. Léger, C. *et al.* Enzyme electrokinetics: energetics of succinate oxidation by fumarate reductase
685 and succinate dehydrogenase. *Biochemistry* **40**, 11234–45 (2001).
- 686 23. Virant, D. *et al.* A peptide tag-specific nanobody enables high-quality labeling for dSTORM
687 imaging. *Nat. Commun.* **9**, 930 (2018).
- 688 24. Subach, F. V *et al.* Photoactivatable mCherry for high-resolution two-color fluorescence
689 microscopy. *Nat. Methods* **6**, 153–9 (2009).
- 690 25. Carranza, G. *et al.* Cardiolipin plays an essential role in the formation of intracellular
691 membranes in Escherichia coli. *Biochim. Biophys. Acta - Biomembr.* **1859**, 1124–1132 (2017).
- 692 26. Jamin, N., Garrigos, M., Jaxel, C., Frelet-Barrand, A. & Orłowski, S. Ectopic neo-formed
693 intracellular membranes in Escherichia coli: A response to membrane protein-induced stress
694 involving membrane curvature and domains. *Biomolecules* **8**, (2018).
- 695 27. Mileykovskaya, E. & Dowhan, W. Cardiolipin membrane domains in prokaryotes and
696 eukaryotes. *Biochim. Biophys. Acta* **1788**, 2084–91 (2009).
- 697 28. Renner, L. D. & Weibel, D. B. Cardiolipin microdomains localize to negatively curved regions
698 of Escherichia coli membranes. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 6264–6269 (2011).
- 699 29. Shibuya, I. Metabolic regulations and biological functions of phospholipids in Escherichia coli.
700 *Prog. Lipid Res.* **31**, 245–99 (1992).
- 701 30. Hiraoka, S., Matsuzaki, H. & Shibuya, I. Active increase in cardiolipin synthesis in the
702 stationary growth phase and its physiological significance in Escherichia coli. *FEBS Lett.* **336**,
703 221–4 (1993).

- 704 31. Tropp, B. E. Cardiolipin synthase from *Escherichia coli*. *Biochim. Biophys. Acta* **1348**, 192–
705 200 (1997).
- 706 32. Jumper, J. *et al.* Highly accurate protein structure prediction with AlphaFold. *Nature* **596**, 583–
707 589 (2021).
- 708 33. Tunyasuvunakool, K. *et al.* Highly accurate protein structure prediction for the human
709 proteome. *Nature* **596**, 590–596 (2021).
- 710 34. Baek, M. *et al.* Accurate prediction of protein structures and interactions using a three-track
711 neural network. *Science* **373**, 871–876 (2021).
- 712 35. Szeto, T. H., Rowland, S. L., Rothfield, L. I. & King, G. F. Membrane localization of MinD is
713 mediated by a C-terminal motif that is conserved across eubacteria, archaea, and chloroplasts.
714 *Proc. Natl. Acad. Sci. U. S. A.* **99**, 15693–15698 (2002).
- 715 36. Mileykovskaya, E. *et al.* Effects of phospholipid composition on MinD-membrane interactions
716 in vitro and in vivo. *J. Biol. Chem.* **278**, 22193–22198 (2003).
- 717 37. Salje, J., van den Ent, F., de Boer, P. & Löwe, J. Direct Membrane Binding by Bacterial Actin
718 MreB. *Mol. Cell* **43**, 478–487 (2011).
- 719 38. Corey, R. A. *et al.* Identification and assessment of cardiolipin interactions with *E. coli* inner
720 membrane proteins. *Sci. Adv.* **7**, (2021).
- 721 39. Zamarreño Beas, J., Barras F., P. B. Study of molecular mechanisms allowing *E. coli* to resist
722 against antibiotics. (2018).
- 723 40. Schlame, M. Cardiolipin synthesis for the assembly of bacterial and mitochondrial membranes.
724 *J. Lipid Res.* **49**, 1607–20 (2008).
- 725 41. Jeucken, A., Helms, J. B. & Brouwers, J. F. Cardiolipin synthases of *Escherichia coli* have
726 phospholipid class specific phospholipase D activity dependent on endogenous and foreign
727 phospholipids. *Biochim. Biophys. acta. Mol. cell Biol. lipids* **1863**, 1345–1353 (2018).
- 728 42. Ragolia, L. & Tropp, B. E. The effects of phosphoglycerides on *Escherichia coli* cardiolipin
729 synthase. *Biochim. Biophys. Acta* **1214**, 323–32 (1994).
- 730 43. Haines, T. H. & Dencher, N. A. Cardiolipin: A proton trap for oxidative phosphorylation. *FEBS*
731 *Lett.* **528**, 35–39 (2002).
- 732 44. Arias-Cartin, R., Grimaldi, S., Arnoux, P., Guigliarelli, B. & Magalon, A. Cardiolipin binding
733 in bacterial respiratory complexes: structural and functional implications. *Biochim. Biophys.*
734 *Acta* **1817**, 1937–49 (2012).
- 735 45. Magalon, A. & Alberge, F. Distribution and dynamics of OXPHOS complexes in the bacterial
736 cytoplasmic membrane. *Biochim. Biophys. Acta* **1857**, 198–213 (2016).
- 737 46. Datsenko, K. A. & Wanner, B. L. One-step inactivation of chromosomal genes in *Escherichia*
738 *coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 6640–5 (2000).

- 739 47. Miller, J. H. *A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for*
740 *Escherichia coli and Related Bacteria*. (Plainview, NY, 1992).
- 741 48. Sternberg, N. L. & Maurer, R. Bacteriophage-mediated generalized transduction in *Escherichia*
742 *coli* and *Salmonella typhimurium*. *Methods Enzymol.* **204**, 18–43 (1991).
- 743 49. Pernot, P. *et al.* Upgraded ESRF BM29 beamline for SAXS on macromolecules in solution. *J.*
744 *Synchrotron Radiat.* **20**, 660–664 (2013).
- 745 50. Franke, D. *et al.* ATSAS 2.8 : a comprehensive data analysis suite for small-angle scattering
746 from macromolecular solutions. *J. Appl. Crystallogr.* **50**, 1212–1225 (2017).
- 747 51. Guinier, A. La diffraction des rayons X aux très petits angles : application à l'étude de
748 phénomènes ultramicroscopiques. *Ann. Phys. (Paris)*. **11**, 161–237 (1939).
- 749 52. Petoukhov, M. V. *et al.* New developments in the ATSAS program package for small-angle
750 scattering data analysis. *J. Appl. Crystallogr.* **45**, 342–350 (2012).
- 751 53. Mertens, N., Remaut, E. & Fiers, W. Tight transcriptional control mechanism ensures stable
752 high-level expression from T7 promoter-based expression plasmids. *Biotechnology. (N. Y.)*. **13**,
753 175–9 (1995).
- 754 54. Ovesný, M., Křížek, P., Borkovec, J., Svindrych, Z. & Hagen, G. M. ThunderSTORM: a
755 comprehensive ImageJ plug-in for PALM and STORM data analysis and super-resolution
756 imaging. *Bioinformatics* **30**, 2389–90 (2014).
- 757 55. Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image
758 analysis. *Nat. Methods* **9**, 671–5 (2012).
- 759 56. Folch, J., Lees, M. & Sloane Stanley, G. H. A simple method for the isolation and purification
760 of total lipides from animal tissues. *J. Biol. Chem.* **226**, 497–509 (1957).
- 761 57. Jouhet, J., Maréchal, E., Bligny, R., Joyard, J. & Block, M. A. Transient increase of
762 phosphatidylcholine in plant cells in response to phosphate deprivation. *FEBS Lett.* **544**, 63–8
763 (2003).
- 764 58. Jouhet, J. *et al.* LC-MS/MS versus TLC plus GC methods: Consistency of glycerolipid and
765 fatty acid profiles in microalgae and higher plant cells and effect of a nitrogen starvation. *PLoS*
766 *One* **12**, e0182423 (2017).
- 767 59. Knapp, O. & Benz, R. Membrane Activity and Channel Formation of the Adenylate Cyclase
768 Toxin (CyaA) of *Bordetella pertussis* in Lipid Bilayer Membranes. *Toxins (Basel)*. **12**, (2020).
769

770 Figures and Legends

Figure 1: Biochemical and biophysical characterization of ViaA: A) Annotated sequence of *E. coli* ViaA, with predicted α -helices shown as red tubes and predicted β -strands shown as blue arrows. The von Willebrand A (VWA) domain of ViaA is shown in green, with the MIDAS motif highlighted in dark green. B) Molecular mass determination of AviTag-ViaA-His by SEC-MALLS. The differential refractive index (RI) signal is plotted (left axis, blue curve) along with the UV signal (UV, left axis, green curve) and the determined molecular weight (MW, right axis, grey curve). An SDS-PAGE gel of purified AviTag-ViaA-His is shown as an inset on the right-hand side of the plot. The theoretical monomer MW and the MW as determined by MALLS are annotated on the left-hand side of the plot. C) SAXS curve of purified AviTag-ViaA-His. An inset shows a Guinier Plot zooming in on the low- q region of the scattering curve. The theoretical monomer MW as well as the MW determined by SAXSMOW and ScÅtter are shown on the left. D) Pair-wise distance distribution function $P(r)$ and Kratky plot (Top right inset) of the AviTag-ViaA-His SAXS data.

783 **Figure 2: Characterization of the ViaA-RavA interaction by Bio-Layer Interferometry (BLI)**
784 **binding studies.** A & B) BLI measurements of His-ViaA-AviTag (A) or AviTag-ViaA-His (B)
785 coupled on BLI biosensors and RavA, with or without added ADP/ATP. The blue curves correspond
786 to the measured signal while the red curves correspond to the calculated fit using a 1:1 (no nucleotide,
787 ADP) or 2:1 heterogeneous ligand binding (ATP) interaction model. C) BLI measurements of
788 AviTag-ViaA-His coupled on BLI biosensors and either NuoEF (blue curve), NuoEFG (green curve)
789 or FrdA (red curve). SDS-PAGE gels of purified NuoEF, NuoEFG and FrdA are shown on the right.

790 **Figure 3: Imaging of overexpressed RavA and ViaA by super-resolution microscopy and cellular**
791 **TEM.** A) Single molecule localisation microscopy imaging of *E. coli* cells overexpressing
792 fluorescently labeled RavA and ViaA by STORM (BC2-RavA, RavA-BC2) and PALM (PAmCherry-
793 ViaA, ViaA-PAmCherry and PAmCherry-CTV). B) Imaging of high pressure frozen, freeze-
794 substituted and sectioned wild-type (WT) *E. coli* cells and *E. coli* cells overexpressing RavA (RavA-
795 OE) or ViaA (ViaA-OE) by TEM.

Figure 4: RavA and ViaA binding to specific lipids, and quantification of CL in different RavA and ViaA overexpressing strains. A) Dot-blot assays using purified ViaA and RavA, visualized using anti-ViaA and anti-RavA antibodies and a secondary HRP-antibody conjugate (PC: phosphatidylcholine, PS: phosphatidylserine, PE: phosphatidylethanolamine, PG: phosphatidylglycerol, PA: phosphatidic acid, CL: cardiolipin). For 'ViaA bound via RavA' or 'RavA bound via ViaA': Lipid binding was first allowed for RavA and subsequently ViaA was added, or vice versa. For 'LdcI-RavA-complex', proteins were mixed before incubation on the membrane (see also Materials and Methods). B) Quantification of cardiolipin (CL) levels by TLC and GC-FID/MS in wild-type (WT) MG1655 *E. coli* cells and MG1655 *E. coli* cells overexpressing RavA, RavA_{ΔLARA}, ViaA, ViaA_{R476E/R479E} and ViaA₁₋₄₇₂, visualized by a Tukey representation (showing all points from min. to max.). Each point represents a biological repeat. The overexpressing (OE) series were compared with the control WT MG1655 series using an unpaired nonparametric Mann-Whitney test. A significant difference with the control is shown by * (p value < 0.05) or ** (p value < 0.01). The # indicates a significant difference (p value < 0.05) with ViaA OE.

Figure 5: A) Cartoon presentation of AlphaFold (left, light grey) and RoseTTAFold (right, light grey) structural predictions for the ViaA VWA domain. The MIDAS motif (green) as well as Arginine residues in the C-terminal helix (dark blue) are annotated and shown as sticks. The AlphaFold and RoseTTAFold models are shown superimposed on the right in dark and light grey respectively. B) Helical wheel diagram of the C-terminal helix of ViaA (residues 471 – 483), with annotated and color-coded residues. C) Multiple sequence alignment of the C-terminal helix of different enterobacterial ViaA orthologues. Residues are color-coded and level of conservation is annotated with ‘*’ (perfect alignment), ‘:’ (strong similarity) and ‘.’ (weak similarity).

818 **Figure 6: Lipid binding-deficient chromosomal mutants of RavA and ViaA impair RavA and**
819 **ViaA function in gentamicin sensitisation**
820 Survival of wild type MG1655, *ViaA*₁₋₄₇₂, *ViaA*_{R476E/R479E} and *RavA*_{ΔLARA} *E. coli* strains after Gm
821 treatment. Cells were grown to an OD₆₀₀ of 0.2 in LB supplemented with 10 mM fumarate, after
822 which 16 μg/mL Gm was added. The survival values after 1.5 and 3 hours of treatment are
823 represented. Full and empty symbols are for untreated and Gm-treated bacteria, respectively. Survival,
824 measured by colony-forming units (CFU) per mL, was normalized relative to time zero at which Gm
825 was added (early log phase cells; ~5x10⁷ CFU/mL) and plotted as Log₁₀ of % survival. Values are
826 expressed as a mean value of 3 biological replicates and error bars depict standard deviations. A one-
827 way ANOVA test followed by a Dunnett's multiple comparisons test was performed to compare at
828 each time point (1.5 & 3 hours) the treated WT to each of the treated mutant strains (***) adjusted
829 *p*Value = 0.0002 & **** adjusted *p*Value < 0.0001).

830 **Supplementary Figure Legends**

831 **Supplementary Figure 1:** A) BLI measurements of His-NTV-AviTag coupled on BLI biosensors and
 832 RavA, with or without added ADP/ATP. B) BLI measurements of AviTag-ViaA-His coupled on BLI
 833 biosensors and RavA Δ LARA, with or without added ADP/ATP. For A) and B), the blue curves
 834 correspond to the measured signal while the red curves correspond to the calculated fit using a 1:1 (no
 835 nucleotide, ADP) or 2:1 heterogeneous ligand binding (ATP) interaction model. C) Molecular mass
 836 determination of His-NTV-AviTag by SEC-MALLS. The differential refractive index (RI) signal is
 837 plotted (left axis, blue curve) along with the UV signal (UV, left axis, green curve) and the determined
 838 molecular weight (MW, right axis, grey curve). The theoretical monomer MW and the MW as
 839 determined by MALLS are annotated on the left-hand side of the plot.

840 **Supplementary Figure 2:** A) Single molecule localisation microscopy imaging of *E. coli* cells
 841 overexpressing the N-terminal domain of ViaA (NTV) fused to PAmCherry by PALM at 37° C (left),
 842 30° C (middle) and 18° C (right). B & C) Wide field imaging of *E. coli* cells overexpressing RavA (B)
 843 or ViaA (C) using phase contrast, DNA (Hoechst) staining or anti-RavA (VHH2-AF488) or anti-ViaA
 844 (VHH1-AF647) nanobodies coupled to Alexa Fluor dyes.

845 **Supplementary Figure 3:** Dot-blot assays using purified ViaA constructs (A: ViaA, ViaA₁₋₄₇₂,
 846 ViaA_{R476E/R479E}), purified RavA constructs (B: RavA, RavA Δ LARA), or purified LdcI-RavA complex
 847 (C) visualized using anti-ViaA (A) and anti-RavA (B & C) or anti-LdcI (C) antibodies and a
 848 secondary HRP-antibody conjugate (PC: phosphatidylcholine, PS: phosphatidylserine, PE:
 849 phosphatidyl-ethanolamine, PG: phosphatidylglycerol, PA: phosphatidic acid, CL: cardiolipin).

850 **Supplementary Figure 4:** TEM imaging of high pressure frozen, freeze-substituted and sectioned *E.*
 851 *coli* cells overexpressing different RavA (blue square: RavA, RavA Δ LARA) or ViaA (purple square:
 852 ViaA, ViaA₁₋₄₇₂ or ViaA_{R476E/R479E}.) constructs.

853 **Supplementary Figure 5:** A) Bar chart showing the determination of protein quantity of
 854 overexpressed RavA, RavA Δ LARA, ViaA, ViaA₁₋₄₇₂ and ViaA_{R476E/R479E}, based on SDS-PAGE gels
 855 shown in B (RavA), C (RavA Δ LARA), D (ViaA), E (ViaA₁₋₄₇₂) and F (ViaA_{R476E/R479E}).

856 **Supplementary Figure 6:** A & B) Quantification of fatty acid levels (total,
 857 phosphatidylethanolamine, phosphatidylglycerol or cardiolipin) by TLC and GC-FID/MS in wild-type
 858 (WT) MG1655 *E. coli* cells and MG1655 *E. coli* cells overexpressing RavA, RavA Δ LARA, ViaA,
 859 ViaA_{R476E/R479E} and ViaA₁₋₄₇₂, visualized by bar charts (A) and Tukey representations (B, showing all
 860 points from min. to max.). Bars are the average of 3 to 7 measurements (5 for MG1655, 7 for ViaA-
 861 OE, 3 for the other mutants), plus or minus SD. The overexpressing (OE) series were compared with
 862 the control WT MG1655 series using an unpaired Student t test. In the Tukey representations, each
 863 point represents a biological repeat. The overexpressing (OE) series were compared with the control
 864 WT MG1655 series using an unpaired nonparametric Mann-Whitney test. For both representations,
 865 significant difference with the control is shown by * (p value < 0.05) or ** (p value < 0.01). The #
 866 indicates a significant difference (p value < 0.05) with ViaA-OE.

867 **Supplementary Figure 7:** Overall membrane activities of ViaA, RavA and the *B. pertussis* pore-
 868 forming CyaA toxin as measured by planar lipid bilayer technique. The ViaA and RavA proteins were
 869 diluted to a final concentration of 2 nM and exposed to a lipid membrane containing PA, PG and PE
 870 lipids in molar ratio of 10:45:45. The CyaA toxin, used as a positive control for membrane pore
 871 formation, was diluted to a final concentration of 250 pM and exposed to an asolectin membrane.
 872 The aqueous phase contained 10 mM Tris-HCl (pH 7.4), 150 mM KCl and 2 mM CaCl₂; the applied
 873 voltage was 50 mV; the temperature was 25 °C. The membrane current recordings were processed
 874 using a 10 Hz filter.