Anionic lipids and the cytoskeletal proteins MreB and RodZ define the spatio-temporal distribution and function of membrane stress controller PspA in *Escherichia coli*

Goran Jovanovic,1 Parul Mehta,1 Liming Ying2 and Martin Buck1

1Department of Life Sciences, Imperial College London, London SW7 2AZ, UK
2National Heart and Lung Institute, Imperial College London, London SW7 2AZ, UK

All cell types must maintain the integrity of their membranes. The conserved bacterial membrane-associated protein PspA is a major effector acting upon extracytoplasmic stress and is implicated in protection of the inner membrane of pathogens, formation of biofilms and multi-drug-resistant persister cells. PspA and its homologues in Gram-positive bacteria and archaea protect the cell envelope whilst also supporting thylakoid biogenesis in cyanobacteria and higher plants.

In enterobacteria, PspA is a dual function protein negatively regulating the Psp system in the absence of stress and acting as an effector of membrane integrity upon stress. We show that in *Escherichia coli* the low-order oligomeric PspA regulatory complex associates with cardiolipin-rich, curved polar inner membrane regions. There, cardiolipin and the flotillin 1 homologue YqiK support the PspBC sensors in transducing a membrane stress signal to the PspA-PspF inhibitory complex. After stress perception, PspA high-order oligomeric effector complexes initially assemble in polar membrane regions. Subsequently, the discrete spatial distribution and dynamics of PspA effector(s) in lateral membrane regions depend on the actin homologue MreB and the peptidoglycan machinery protein RodZ. The consequences of loss of cytoplasmic membrane anionic lipids, MreB, RodZ and/or YqiK suggest that the mode of action of the PspA effector is closely associated with cell envelope organization.

INTRODUCTION

Maintaining membrane integrity is fundamental to all cell types and of key importance to energy production, signalling, adaptation to the environment and cellular compartmentalization. Many Gram-negative bacteria mount a major adaptive response to extracytoplasmic stress by inducing the phage shock protein (Psp) system (reviewed by Model et al., 1997; Darwin, 2005; Joly et al., 2010). Related stress control systems are found in Gram-positive bacteria, cyanobacteria, archaea and higher plants (Joly et al., 2010).

The Psp system of Gram-negative bacteria is induced by a variety of membrane stress stimuli such as protein translocation defects (Joly et al., 2010; Wang et al., 2010; Wickström et al., 2011) and production of secretins, e.g. phage f1 protein IV (pIV), PulD, YscC, OutD, which are components of the types II, III and IV secretion systems (reviewed by Joly et al., 2010; Yamaguchi & Darwin, 2012). The Psp response of bacterial pathogens protects the cell envelope during infection and is important for biofilm formation and virulence whilst also being implicated in antibiotic resistance and formation of persister cells (reviewed by Joly et al., 2010; Darwin, 2013; see also Dhamdhere & Zgurskaya, 2010; Vega et al., 2013; Wallrodt et al., 2014). Agents inducing psp impair the plasma membrane and dissipate the proton motive force (pmf). A drop in pmf may not be sufficient to induce the Psp response and multiple signals appear to be integrated to induce the response in enterobacteria (Wang et al., 2010; Engl et al., 2011). The highly conserved bacterial peripheral membrane protein PspA acts as a major effector that, through a yet unknown mechanism, repairs the membrane and so preserves the pmf (Joly et al., 2010). Homologues of PspA in *Mycobacterium* and other Gram-positive bacteria (e.g. LiaH in *Bacillus*) have been postulated to maintain cell wall homeostasis upon extracytoplasmic stress (Joly et al., 2010; White et al., 2011; Darwin, 2013), to confer resistance to cell wall/peptidoglycan (PGL) and membrane integrity-targeting antibiotics (reviewed by Jordan et al., 2008; Joly et al., 2010). In cyanobacteria and plants, the PspA homologue VIPP1 is required for photosynthesis through support of thylakoid
membrane biogenesis and protection of the cell envelope (Westphal et al., 2001; Aseeva et al., 2004, 2007; Vothknecht et al., 2012; Zhang et al., 2012; Zhang & Sakamoto, 2013). PspF, PspA, PspB and PspC are conserved in enterobacteria and constitute the core proteins of the Psp response (Huve et al., 2011). Under non-stress conditions in enterobacteria, σ^{54}-RNA polymerase-dependent psp expression is negatively regulated by PspA via its direct ~1:1 binding to the surface-exposed hydrophobic 'W56 loop' of the hexameric bacterial enhancer-binding protein PspF (Joly et al., 2009; Zhang et al., 2013). Under inner membrane (IM) stress, induction of psp involves the IM-bound PspB and PspC proteins sensing stress and recruiting the PspA-PspF inhibitory complex to the IM (Jovanovic et al., 2010; Yamaguchi et al., 2010). Relieving the inhibition of PspF imposed by PspA involves changing PspA’s interacting partner from PspF to PspBC, resulting in strong induction of psp genes and formation of PspA effector complexes at the IM (Yamaguchi et al., 2013; Mehta et al., 2013). Upon stress, σ^{70}-controlled expression of PspF remains unchanged (Lloyd et al., 2004).

Single molecule sensitivity imaging (SMI) studies of chromosome-expressed fluorescent fusion Venus–PspF (V–PspF) in live Escherichia coli cells established that PspF is predominantly hexameric and that the PspA–V–PspF nucleoid-bound inhibitory complex under non-stress conditions communicates with the IM in a PspA- and PspBC-dependent manner (Mehta et al., 2013). Under IM stress, PspF is stably bound to the nucleoid and involved in transcription while V–PspA [or eGFP (enhanced green fluorescent protein)–PspA] is found within static polar and dynamic lateral IM complexes. Static polar IM complexes correlate with a PspA regulatory function within the signalling complex PspA–PspBC, while the dynamic lateral IM complexes correlate with PspA effector function (Engl et al., 2009; Jovanovic et al., 2014). When the dynamics of lateral membrane eGFP–PspA complexes are abolished or when PspA is mutated so it cannot interact with PspF and PspBC but cannot bind to the IM and form the lateral membrane complexes, it is able to form the polar IM complexes and respond to PspBC-dependent IM stress but is unable, for example, to conserve the pmf. The major oligomerization state of eGFP–PspA found in static polar foci is a 6-mer with a minority of additional high-order oligomers up to ~36-mer (Lenn et al., 2011). Relief of negative control results in PspA binding to the IM, switch in oligomeric state from low-order to high-order oligomers and the appearance of lateral IM PspA effector complexes (Jovanovic et al., 2014). These observations suggest that a switching mechanism linked to the stress signalling pathway in cellular polar membrane regions exists to convert PspA between its negative regulator (potentially 6-mer) and effector (36-mer) forms. Apparently, PspBC, as opposed to PspA, has a direct secretin-damage effector function in Yersinia enterocolitica (PspC has extra amino acids at its N terminus compared with the E. coli PspC) (Horstman & Darwin, 2012). Moreover, Yamaguchi et al. (2013) did not observe lateral IM PspA effector complexes upon stress, suggesting that the action of PspA effectors in stressed Y. enterocolitica cells might be orchestrated differently from that in E. coli. Nevertheless, at the same time it was established that the static polar PspBC sensor co-localizes with PspA at the onset of stress, consistent with the polar PspA–PspBC complex functioning in a regulatory manner in E. coli.

Impaired phospholipid biosynthesis is a strong inducer of PspA in E. coli (Bergler et al., 1994). In turn, Kobayashi et al. (2007) provided evidence that the higher oligomeric form (36-mer) of purified PspA acts as an effector that binds phosphatidylglycerol (PG) and prevents proton leakage from membrane vesicles. In E. coli, the major phospholipid is phosphatidylethanolamine (~75 % of total IM lipids), which possesses a zwitterionic head group. The most abundant anionic IM phospholipids implicated in signalling and/or functioning of IM proteins are PG (~20 % of total IM lipids) and the diacid cardiolipin (CL) (~5 % of total IM lipids) (reviewed by Foss et al., 2011). The polar and lateral regions of the IM are rich in PG whilst the majority of CL resides in polar regions (Foss et al., 2011) (see Fig. S1a, available in the online Supplementary Material). Although PspA effector complexes do not directly bind CL in vitro (Kobayashi et al., 2007), the position of the PspBC-dependent PspA regulatory complexes close to the poles implies that CL and/or negative curvature might be involved in localization and function of the PspBC sensor and/or a PspA–PspBC complex. Indeed, the function of, for example, the Tat protein translocation system is CL-dependent (reviewed by Arias-Cartín et al., 2012; Berthelmann & Brüser, 2004), and PspB–PspA–Tat direct interactions have been reported (Mehner et al., 2012).

MreB is a bacterial actin and a key component of the bacterial cytoskeleton (reviewed by Typas et al., 2012). The helix-like circumferential dynamics and function of the lateral IM PspA effector complexes depend on MreB in E. coli (Engl et al., 2009). In E. coli, MreB assembles into short filaments that either bind directly to the cell membrane via its N-terminal amphipathic helix and move as independent units in directions that are perpendicular to the long axis of the cell (Salje et al., 2011) or co-localize and interact with RodZ (van den Ent et al., 2010) and organize the cell wall (PGL) synthesis machinery assembly, coupling their rotation in a helix-like circumferential fashion (van Teeffelen et al., 2011) (see Fig. S1b). The PG-dependent lipid helices were observed in Gram-negative and Gram-positive bacteria (Bará & others, 2008). Since PspA as an effector binds PG in vitro and PspA and PspB interact with MreB in vivo (Engl et al., 2009), the spatial organization and dynamics of PspA, MreB and anionic lipids might be interlinked. These relationships could be important in response to physico-chemical changes of the IM relevant to regulation and functioning of the Psp system.

In this study we applied genetic tools in combination with SMI to explore in vivo how PspA communicates with the IM stress signalling pathways and undergoes a switch from...
being a negative regulator to acting as an effector. We established a link between CL and induction of the Psp response on the one hand and the bacterial cytoskeleton and effector functions of PspA on the other. Our results provide evidence that membrane curvature, membrane lipid composition, bacterial actin MreB and cell wall biosynthesis machinery affect the dynamics of PspA upon IM stress.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** Bacterial strains and plasmids used in this work are listed in Table S1. New strains were constructed using Pl消失了 transduction (Miller, 1992) (e.g. MG1655 x MVA127 results in a strain MVA101; see Table S1). All strains were routinely grown under microaerobic conditions in Luria–Bertani (LB) broth or on LB agar plates at 37 °C (Miller, 1992). For microaerobic growth, overnight cultures of cells were diluted 100-fold (to OD₆₀₀ = 0.025) and shaken in universals at 100 r.p.m. For SM1 of eGFP–PspA and V–PspA, cells were grown in N₂–minimal medium supplemented with 0.4% (w/v) glucose as carbon source, 10 mM NH₄Cl as nitrogen source, and trace elements at 30 °C as described previously (Engl et al., 2009; Mehta et al., 2013). The expression of pIV from PMJR129 was induced by 1 mM IPTG for 10 or 20 min. The expression from pCA24N-based constructs (JW et al. described previously (Engl et al., 2009). For the experiment where the V–PspA expression was analysed after 10 or 20 min induction of pIV, IPTG (1 mM final) was added directly to the agarose pads simultaneously with the cells. V–PspA was visualized using wide field epifluorescence microscopy. The expression of pIV was tested independently in the same samples grown in medium (0, 10 or 20 min) using Western blot and pIV antibodies (see above). For the experiment with fosfomycin, the stressed ΔpspA cells expressing V–PspA were grown to OD₆₀₀ ~0.8, treated with 32 μg fosfomycin/ml for 10 min at 37 °C and imaged. The wide field or total internal reflection fluorescence (TIRF) SMF of live bacterial cells by means of a custom-built inverted epifluorescence/TIRF microscope based on a Nikon TE2000 optical system was as described (Mehta et al., 2013). TIRF microscopy was employed to limit the laser penetration to up to ~50–100 nm into the bacterial cell in order to differentiate between nucleoid- and membrane-associated V–PspA foci. The axial resolution for wide field imaging is ~1 μm, so that an entire E. coli cell with diameter ~500 nm was imaged; hence the foci within the same/similar Z (e.g. nucleoid-associated and polar membrane) can be in focus. The exposure time was 38 ms (for eGFP) or 15 ms (for Venus). A 100–1000 frame video sequence with 2 × 2 binning at a frame interval of 25 ms (for eGFP) or 30 ms (for Venus) was recorded. The DeltaVision OMX V3 system (Applied Precision) with 3 ms exposure time at a frame interval of 44 ms was used for photobleaching experiments of the rare V–PspA static foci observed in the nucleoid under non-stress conditions and of V–PspA foci at the poles under non-stress or stress conditions. Photobleaching traces of individual V–PspA static nucleoid foci under non-stress conditions and polar foci from non-stress and stressed cells were analysed and the oligomeric states were determined as described (Lenn et al., 2011; Mehta et al., 2013). The images were analysed using ImageJ (http://imagej.nih.gov/ij/) and Fili. The quantitative analysis of localization of V–PspA foci in the cell, the quantification of number of foci per cell and the total raw intensity profiles were performed using ImageJ software. Cells expressing eGFP–PspA or V–PspA were analysed to determine the intensity of foci at specific cellular locations. For the spatial analysis of a signal, a line approximately 16 pixels wide was drawn along the longitudinal axis of cells of similar lengths to cover the entire cell, and the intensity values were obtained, yielding pixel-by-pixel intensity values across the cell length from pole to pole. When the differences in intensity values were high, for comparison we normalized the data points representing the mean intensity. The apparent 2D diffusion analysis (diffusion coefficient D) of V–PspA foci determined using Matlab (Mathwork) scripts was as described (Mehta et al., 2013).

**RESULTS**

**IM stress causes formation of V–PspA high-order oligomers in polar membrane regions and an increase of V–PspA complexes in lateral membrane regions**

To assess and quantify the spatial and temporal dynamics of V–PspA under increasing IM stress conditions, we used analyses were performed on a Bench Pro 4100 Card processing station (Invitrogen). Proteins were detected using the ECL plus Western Blotting Detection kit (GE Healthcare) and images were visualized using the Bio-Rad GelDoc and ChemiDoc Imaging system with Image Lab software. The quantitative analyses were performed using ImageJ software.
a strain lacking the native *pspA* gene and expressing chromosomal single-copy P*pspA*–V–PspA (Mehta *et al.*, 2013; see also Fig. 1a) in the absence or presence of increasing amounts of pIV. pIV is an outer membrane secretin and its mislocalization into the IM induces the PspBC-dependent Psp response (Joly *et al.*, 2010). The V–PspA (or eGFP–PspA) fusion exhibits reduced (50%) negative control activity compared with WT PspA (Engl *et al.*, 2009; Mehta *et al.*, 2013) and this accounts for the moderately elevated expression of both V–PspA and PspBC under non-stress conditions in a Δ*pspA* mutant (Jovanovic *et al.*, 2014).

Consequently, in wide field SMI we observed V–PspA complexes (2±0.3 foci per cell, *n*=50) in the polar region of the cell and some lateral membrane foci (Fig. 1b, Video S1). The pIV-induced IM stress and its time-dependent increase (Fig. 1f) led to a gradual elevation in total fluorescence intensity of V–PspA (Fig. 1g) and increased numbers of dynamic and static lateral V–PspA complexes (Fig. 1c, d, g, Videos S2 and S3). Notably, V–PspA expression, localization and dynamics in stressed cells closely resembled those of plasmid-borne eGFP–PspA as expressed in non-stressed Δ*pspA* cells (Fig. 1e, g, Video S4).

![](image)

**Fig. 1.** The IM stress-dependent subcellular distribution of V–PspA. (a) Construct expressing chromosomal V–PspA under control of the *pspA* promoter (P*pspA*) in a Δ*pspA* strain (MVA127). (b–d) Wide field SMI of MVA127 cells expressing V–PspA (white foci) in the absence of stress (+vector, pGZ119EH, after 20 min) (b) and upon stress (+pIV-cam, pMJR129) with pIV expression induced for 10 min (c) or for 20 min (d) before imaging. (e) Wide field SMI of eGFP–PspA (pEC1) in a non-stressed Δ*pspA* strain (MG1655 Δ*pspA*). In (b–e) representative images are shown. Bars, 1 μm. Schematics represent non-quantitative interpretations of the images, depicting localizations and dynamics of V–PspA or eGFP–PspA: black dots, membrane foci; grey dot, less frequently observed membrane focus; arrows, dynamic membrane foci. (f) Western blot to show the level of pIV expression (band ~46 kDa) in cells from (c, d) and control pIV level in cells at 0 time point without IPTG (“). LC, loading control is the protein band from crude cell extract, which shows non-specific cross-reaction with the pIV antibody (α-pIV). M, molecular mass marker. Below: the quantification of pIV protein levels presented in arbitrary units. (g) Left: graph of total fluorescence intensity of V–PspA and eGFP–PspA foci (*n*=50) from (b–e). Error bars, ±SE. Right: x-axis, lateral membrane (M-lateral) localization of the V–PspA foci (*n*=50) from (b–d) and eGFP–PspA foci (*n*=50) from (e); y-axis, percentage of all foci analysed.
The total fluorescence intensity represents the number of V–PspA molecules. The very significant increase in total fluorescence intensity between 10 and 20 min induction of pIV (Fig. 1g, left) reflected a moderate increase in pIV level (Fig. 1f) and a small increase in the number of lateral V–PspA foci (10 min, 7 ± 0.6; 20 min, 8 ± 0.7; n=50) (see also Fig. 1g, right). However, the formation of foci containing high-order oligomeric V–PspA effectors may have been the main factor contributing to total fluorescence intensity.

Using wide field SMI and photobleaching we determined the oligomeric states of V–PspA under non-stress or stress growth conditions. In the non-stressed state V–PspA imaged as low-order (3–10-mer) assemblies in rare central static nucleoid-associated foci (Fig. 2a), presumably interacting with PspF and forming a PspA-PspF inhibitory complex at psp promoter(s) as shown by Mehta et al. (2013). This is in good agreement with in vitro data establishing that at least three PspA proteins are necessary to bind PspF for negative control (Zhang et al., 2013). We also determined that in non-stressed cells V–PspA is a low-order assembly at polar foci, likely representing the PspA-PspBC membrane regulatory complex (Fig. 2b). During stress, the V–PspA in polar complexes was found as both low- (5- or 6-, up to 12-mer) and high-order assemblies (up to 25-mer; photobleaching and maturation of Venus fluorescence protein may limit the observed number of V–PspA subunits, especially in foci representing high-order oligomers) (Fig. 2b). It seems that the IM stress signals lead to a distinct dynamic response following an increase in V–PspA expression and its high-order oligomer assembly in polar membrane regions.

**Efficient PspA-negative control is required for native basal level expression and spatial distribution of V–PspA**

To determine the behaviour of V–PspA when expression of PspBC is natively controlled, the P_PspA–V–PspA was integrated as a single chromosomal copy into WT cells expressing native PspA (Fig. 3a). Normal regulation of psp expression, characterized by a low level of stable V–PspA, was observed under non-stress conditions whereas elevated levels were seen in pIV-stressed cells (Fig. 3b). When V–PspA and native PspBC were expressed at a basal level in non-stressed WT cells, V–PspA localized either as a single nearly static central nucleoid [by means of interacting with PspF (Mehta et al., 2013; Jovanovic et al., 2014); visible in wide field but not visible in TIRF] or as a static polar membrane complex (visible in both wide field and TIRF) (Fig. 3c). This is in agreement with V–PspF imaging in live cells revealing a single central nucleoid V–PspF-PspA inhibitory complex that communicates in a PspBC-dependent fashion with the polar membrane region by fast relatively free diffusion (Mehta et al., 2013). Under stress, when expression of V–PspA is induced, we observed polar regulatory complexes and the appearance of static and dynamic lateral effector V–PspA complexes (Fig. 3d, Video S5). Therefore, with the negative control imposed by native PspA we could clearly visualize V–PspA exhibiting the ‘off’ and ‘on’ states of the Psp response.

**Fig. 2.** The stoichiometry of V–PspA before and after IM stress. The stoichiometry was determined by photobleaching the V–PspA in MVA127 cells. The distribution of stoichiometries was calculated from data obtained for V–PspA. (a) Nucleoid-associated foci (non-stress, n=14) and (b) polar IM-associated foci under non-stress (n=66) or stress (+pIV, pGJ4; n=67) conditions. Schematics (as in Fig. 1); circles, nucleoid-associated foci; black dots, membrane foci; arrow, dynamic membrane focus.
**CL is implicated in a PspBC-dependent induction of the Psp response**

The anionic phospholipid CL has mainly a membrane curvature-associated polar localization (Renner & Weibel, 2011). PspA does not appear to bind membrane vesicles containing CL (Kobayashi et al., 2007), but PspA is tethered by and co-localizes with static PspBC foci in curved polar membrane regions (Yamaguchi et al., 2013; Mehta et al., 2013). This suggests that localization of the inhibitory PspA-PspF complex or PspB within the PspA-PspBC regulatory complex at the poles of cells may depend on PspA interacting with the PspBC bound to CL-rich membrane domains. We examined the contribution of CL to PspA activities and to cell growth under IM stress in a Δcls mutant (non-polar mutation) containing a significantly reduced amount of CL (Tan et al., 2012).

We determined that the Δcls mutation does not induce the Psp response per se and decreases basal level expression or induction of pspA under stress (Fig. 4a). In addition, the induction of pspA under stress is to some extent PspBC-independent in a Δcls mutant (Fig. 4a). In the absence of negative control, in ΔpspA cells the Δcls mutation did not impact on σ54-dependent transcription of the pspA gene (Fig. S2a), suggesting that observed differences in pspA expression in Δcls mutants reflect a change in the PspA negative control function. Additionally, the overexpression of either PgsA, implicated in biosynthesis of PG and CL or Cls, required for CL biosynthesis, did not induce the Psp response (Fig. S2b). We note that overexpression of phospholipid synthases had little impact on the overall pool of individual phospholipids within *E. coli* (reviewed by Raetz, 1986).

Growth in a stressed Δcls mutant was delayed but the final growth yield was not significantly reduced compared with WT cells (Fig. 4b). As a control, a ΔpspF mutant lacking any induction of the Psp response exhibited growth that was severely impaired (Fig. 4c). Notably, the increased amount of PG seen in a Δcls mutant (Tan et al., 2012) and the fact that PspA effectors bind PG may account for cell adaptation and normal growth upon IM stress, even though the induction of the Psp response is decreased in Δcls mutants.

To determine whether CL affects the subcellular localization of V-PspA, we imaged V-PspA in an *E. coli* Δcls mutant background under non-stress or stress conditions using the wide field and TIRF modes of SMI. The majority of non-stressed Δcls mutants showed only nucleoid-associated foci (Fig. S3a, b, Video S6). The total intensity of V–PspA foci in stressed Δcls cells (Fig. S3c) correlated well with the level of pspA induction upon stress (Fig. 4a) and the amount of effector complexes was in accordance with their growth being similar to WT (Fig. 4b). As seen for the PspBC- and CL-independent induction of pspA (Fig. 4a), in stressed ΔpspBC Δcls mutants the expression of V-PspA is induced and the formation of the lateral effector but not polar complexes can be readily observed (Fig. 4f).

Although we failed to construct a stable and functional PspB– or PspC–eGFP/Venus fusion protein to directly

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**Fig. 3.** The negative control of *psp* and spatial distribution of V–PspA. (a) Construct expressing chromosomal V–PspA under control of the *pspA* promoter *(P_

pspA)* in *pspA*+ cells (MVA101). (b) Left: Western blot to show the V–PspA fusion (~53 kDa) stability and expression in MVA101 before and after stress (+pIV, pGJ4). α-GFP JL8, Venus antibody; M, molecular mass marker. Middle: LC, loading control, Coomassie-stained MVA101–/+pIV samples. Right: quantification of V–PspA protein expression presented in arbitrary units (a.u.). (c, d) Wide field SMI of non-stressed MVA101 cells expressing V–PspA (c) and with induced expression of V–PspA upon stress (+pIV, pGJ4) (d). Representative images are shown. Bars, 1 μm. Schematics (as in Fig. 1): circle, nucleoid-associated focus; black dots, membrane foci; arrow, dynamic membrane focus.
localize the Psp sensor(s) in a WT or cls mutant, it has been established that PspBC recruits the PspA-V-PspF complex to the IM in E. coli and co-localizes with PspA in the polar membrane regions of Y. enterocolitica cells (Yamaguchi et al., 2013). Therefore, our results strongly suggest that the PspBC-dependent induction of the Psp response under stress and the localization of V–PspA within polar IM regulatory complexes is influenced by the presence of CL.

Apparently, CL depletion affects the membrane in numerous ways (reviewed by Arias-Cartín et al., 2012) and could therefore indirectly influence PspB and PspC activity. Relevant to the Psp response, CL-associated flotillin YuaG (FloT) from Bacillus subtilis functionally organizes the bacterial membrane and interacts with proteins involved in membrane-related signalling and protein secretion (Donovan & Bramkamp 2009; Bach & Bramkamp, 2013). However, the PspA homologue LiaH or its regulator(s) has not been found to interact with YuaG.

Intriguingly, there is a potential flotillin 1 homologue in E. coli, an IM protein YqiK, which might act in scaffolding of detergent-resistant microdomains under specific stress conditions (Hinderhofer et al., 2009; López & Kolter, 2010). Therefore, to expand upon our results with CL, we assessed the contribution of YqiK to the induction, function and spatial organization of the Psp proteins. Lack of YqiK (ΔyqiK; non-polar mutation) did not induce pspA per se (Fig. S4a) but, although with less pronounced effect, reduced the level of induction of pspA, as also seen in a Δcls mutant (Fig. S4a). As a control, we showed that the yqiK mutation did not impact on deregulated PspF-dependent transcription of pspA (Fig. S2a) and the overexpression of YqiK did not induce pspA (Fig. S2b).

In line with the morphology of cells expressing YqiK and localization of YqiK-YFP in E. coli (López & Kolter, 2010),
our imaging results showed that overexpression of YqiK-GFP yielded enlarged cells and displayed IM localizations in distinct, relatively static (only local movement was seen) complexes in polar and lateral regions of the cell (Fig. S4c, d). The growth of ΔyqiK under stress was not substantially different from WT (Fig. S4b). The same was true for subcellular localization of V-PspA (Fig. S4e, Video S7). Hence, there are similarities between cls and yqiK mutants and it seems that YqiK supports signalling for the induction of the Psp response.

**RodZ affects the Psp response and membrane localization of V-PspA**

The effector function, lateral membrane localization and dynamics of PspA depend on MreB, while the induction of the Psp response and localization of PspA regulatory complexes are MreB independent (Engl et al., 2009). MreB’s circumferential movement along the long axis of the cell is driven by the process of PGL biosynthesis itself (White et al., 2010; van Teeffelen et al., 2011; Kawai et al., 2011; Garner et al., 2011; Domínguez-Escobar et al., 2011; Olshausen et al., 2013), and the association of MreB with the cell wall biosynthesis apparatus is via RodZ protein (Bendezú et al., 2009; van den Ent et al., 2010).

To address the potential link between PspA and RodZ (via MreB), we assayed activities and spatial distribution of PspA in rodZ mutants (ΔrodZ, non-polar mutation). We established that the absence of RodZ does not in itself induce pspA expression but allows a strong induction of pspA upon IM stress, which notably is PsbC-independent (Fig. 5a). As controls, lack of RodZ did not influence the PspF-dependent transcription of pspA (Fig. S2a) and overexpression of RodZ did not induce pspA (Fig. S2b). However, despite the induction of pspA under IM stress being elevated in a ΔrodZ mutant, the growth was impaired to an extent seen for the cells lacking a Psp response (Fig. 5b; and see Fig. 4c).

In ΔrodZ mutants with no rod-shaped morphology, it was hard to observe distinct V–PspA foci in the absence of IM stress (Fig. 5c). Under stress, the V–PspA expression was induced in ΔrodZ cells and we observed distinct dynamic effector complexes (Fig. 5d, Video S8). The same was true for plasmid-borne eGFP–PspA (Fig. 5e). The movement of eGFP–PspA in ΔrodZ mutants remained MreB-dependent since in the double ΔmreB ΔrodZ mutant the eGFP–PspA foci were static (Fig. 5f). However, RodZ did contribute to MreB-dependent spatial distribution of eGFP–PspA, which may be of importance for the function of the PspA effectors in WT cells. In the ΔmreB mutant, eGFP–PspA formed distinct static membrane foci (Engl et al., 2009; see Fig. S5a) while in the double ΔmreB ΔrodZ mutant the eGFP–PspA foci localized in one membrane macro-domain (Figs 5f and S5b). Similarly, the eGFP–PspA foci in a ΔmreB ΔyqiK double mutant, lacking MreB and potential flotillin, localized in one static horseshoe-shaped macro-feature (Fig. S5c, d), suggesting MreB, RodZ and YqiK may be involved in organizing membrane regions for the intrinsic interactions of PspA complexes with the IM. Interestingly, in plant cells the Vipp1 homologue of PspA was very dynamic under osmotic stress and formed lateral membrane filament-like structures (Zhang et al., 2012) resembling those of eGFP–PspA in ΔmreB ΔrodZ and ΔmreB ΔyqiK mutants.

Notably, even when the cell shape was perturbed, gross regulation of pspA transcription was retained. It appears that, so long as PspA can localize at the IM, then negative control can be relieved. V–PspA expression was induced by IM stress in ΔpspBC ΔrodZ cells (Fig. 5g; see also Fig. 5a). Even though the rodZ mutants do not have conventional polar membrane regions, the action of PsbC seems to be critical for V–PspA IM localization since the absence of PsbC in ΔrodZ mutants caused complete alteration of IM localization of V–PspA effector complexes. V–PspA then mainly decorated the entire IM (Fig. 5g), suggesting an additional role of PsbC in organizing some PspA effector complexes at the IM. Something similar has been observed in cells expressing the eGFP–PspAΔA25–40 variant, with diminished interaction with PsbC (Jovanovic et al., 2014). The resulting disorganization of the PspA effector that we saw here in the complete absence of PsbC and when the cell shape was perturbed was even more pronounced.

**Block in cell wall synthesis elevates the Psp response to the IM stress and changes dynamics of the lateral membrane PspA effectors**

To address whether a block in PGL synthesis directly affects the Psp response, we treated unstressed or stressed WT cells with different sublethal doses of fosfomycin to inhibit bacterial cell wall biosynthesis by inactivating the cytoplasmic enzyme MurA that catalyses the first essential step in PGL biosynthesis (Brown et al., 1995). We showed that a short (10 min) incubation with fosfomycin at 0.25 or 0.5 MIC increased the basal level transcription and pV-dependent stress induction of pspA (Fig. 6a). Notably, the corresponding growth of non-stressed cells was better than that of the stressed cells (Fig. 6a). It appears that defective PGL biosynthesis causes an additional IM stress and this may correspondingly account for a ΔrodZ mutant elevating the Psp response to pIV (see Figs 5a and 6a).

To assess the localization and dynamics of the V–PspA effectors in stressed WT cells in the presence of fosfomycin, we used wide field SMI. Potentially, a block in PGL biosynthesis could lead to stalling of the membrane V–PspA effectors if their movement is tightly and solely coupled to the cell wall synthesis dynamics via RodZ and MreB. The observed RodZ-independent dynamics of V–PspA and eGFP–PspA (Fig. 5d, e) and MreB-dependent movement of eGFP–PspA (Fig. 5a) suggest that the subpopulation of V–PspA should not be affected. Counterintuitively, fosfomycin treatment (0.5 MIC, see above) sped up the diffusion of V–PspA in ΔpspA mutants producing pIV (mean D=0.02 µm² s⁻¹) compared with untreated stressed cells (mean D=0.01 µm² s⁻¹) (Fig. 6b).
images show a mixture of cells with normal and more rounded shape (Fig. 6c) found to exhibit $9 \pm 0.3$ (n=50) V–PspA foci per cell. It is possible PspA became less constrained in its lateral membrane movements upon blocking of PGL synthesis.

**DISCUSSION**

Peripheral IM-binding proteins are emerging as providing important examples of where their localization and hence functionalities are directly sensitive to membrane phospholipid composition, membrane curvature and the bacterial cytoskeleton (Foss et al., 2011). Here, we establish the functional relationships and interdependences between Psp response, bacterial membrane and cytoskeletal elements to provide insights into how cell structure relates to the perception and management of IM stress by PspA.

The IM stress induces strong $psp$ expression, and consequently we see the formation of high-order oligomeric PspA effectors in polar regions together with an increase of PspA lateral effector complexes (Fig. S6). Potentially, the level of the signal and corresponding induction of PspA expression correlate with the strength of stimulus and thus also membrane damage. There is growing evidence that the integration of several signals induces the Psp response. Changes in membrane potential leading to a drop in pmf, as well as changes in redox state or mode of respiration were found to be either conditional or insufficient to signal the IM damage (Jovanovic et al., 2009; Wang et al., 2010; Engl et al., 2011).

Particular changes in properties of the membrane are likely to contribute to the origin of the IM stress signal(s) and the sites of PspA effector action. It is clear that there are at least two different pathways involved in IM stress signalling to PspA. One mode employs signal transduction to the PspA-PspF inhibitory complexes via PspBC sensors and the other acts through direct binding of PspA-PspF to a stress-related IM determinant (Fig. S6). Dual control of the $\sigma^B$ regulon, which responds to outer-membrane stress (Lima et al., 2013), has some similarity with the dual modes (PspBC-dependent and PspBC-independent) of IM binding of PspA upon stress.

Our results connect the CL-containing polar regions of the cell with the respective localizations of PspA and PspBC,
PspBC signalling, IM binding of PspA and stress-dependent formation of the high-order oligomeric PspA effectors (Fig. S6). The PspBC sensors localized in polar regions transduce the IM stress signal(s) to the PspA-PspF inhibitory complex, releasing negative control of \( psp \). PspC may sense a change in IM charge, membrane potential or curvature via CL, leading to a switch in PspC topology needed for recruiting PspA to the PspBC complex (Jovanovic et al., 2010; Flores-Kim & Darwin, 2012) (see also Fig. S6). The protein Opi1 has been shown to exhibit similar behaviour, with its signalling dependent on intracellular pH and the protonation state of phosphatidic acid phosphate head groups (Young et al., 2010), and Fis1 binding to lipid vesicles is increased upon protonation and concentration of anionic lipids (Wells & Hill, 2011). The PspBC complex may integrate the threshold level signals to modulate the Psp response to a range of stimuli. This can lead to reduction of noise and less pronounced oscillations of \( psp \) induction, in agreement with the mechanistic model of the Psp response (Toni et al., 2011).

Our results also strongly suggest that upon stress in WT cells PspBC are also involved in connecting the PspA effector complex via MreB to particular cell features defined by RodZ, YqiK activities and anionic-lipid-rich membrane domains.

We showed here that the \( psp \) inducing plIV-originated signal(s), besides using the PspBC signalling pathway, can also act via a PspBC-independent mode. In addition, plIV production in a \( \Delta \)pspA background (or when PGL biogenesis is blocked) facilitates the IM stress and elevates the importance of the PspBC-independent response. Severe stresses, such as extreme osmotic shock, 50 °C heat shock, 10% ethanol, have been shown to transiently induce \( psp \) in a PspBC-partial or -independent manner (reviewed by Model et al., 1997). Therefore, extreme stimuli may cause PspA to bypass the PspBC signalling and directly respond to changes in the IM (see Fig. S6).

The several lines of evidence suggest that CL-associated protein translocation systems and adjacent PG-rich

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**Fig. 6.** Fosfomycin treatment increases IM stress and dynamics of V–PspA. (a) The expression of \( pspA \) was determined in three independent experiments (i–iii) in WT + vector (MVA44 + pBR325D, pale grey bars) and stressed WT + plIV (MVA44 + pGJ4, dark grey bars) cells in the absence (0) or presence of fosfomycin at different concentrations (1 MIC = 64 μg ml\(^{-1}\)) (see Methods for details). The expression of \( pspA \) in non-stressed or stressed cells treated with fosfomycin is significantly different from untreated cells (non-stressed, \( P<0.01 \); stressed, \( P<0.005 \)) as determined by one sample t-test analysis of \( \beta \)-galactosidase activity. The line graph below shows the corresponding OD values of non-stressed (i–iii, thin lines) and stressed (i–iii, bold lines) cells in the absence or presence of fosfomycin. (b) The distribution of diffusion coefficients for V–PspA in \( \Delta pspA \) cells (MVA127) under stress (+plIV, pGJ4) and in the absence (white bars, \( n=709 \)) or presence (grey bars, \( n=1106 \)) of 32 μg fosfomycin ml\(^{-1}\) (0.5 MIC) after 10 min of growth. The data are presented as normalized distributions of the diffusion coefficients (μm\(^2\) s\(^{-1}\)) obtained as described by Mehta et al. (2013). (c) Example of wide field SMI of V–PspA in \( \Delta pspA \) cells under stress and in the presence of fosfomycin as in (b). Bar, 1 μm.
domains might be targets for the PspA effector complexes positioned in polar IM regions of the cell (Fig. S6). Depletion or defects in all protein translocation systems (Sec, Tat, YidC, SRP) induce PspA (reviewed by Joly et al., 2010). Importantly, a PspBC-dependent PspA–Tat interaction (Mehner et al., 2012) suggests that the PspA effector function could involve direct repair of the CL-associated translocon system(s). The functions of PspA in repairing Tat defects can be substituted by Vipp1 in E. coli, while PspA partially substitutes for the same defect in the absence of Vipp1 (DeLisa et al., 2004). Moreover, PspA stimulates protein export in E. coli (Kleerebezem & Tommassen, 1993) and a PspA homologue improves the pmf-dependent Tat- and Sec-supported heterologous protein secretion in Streptomyces (Vrancken et al., 2007). As shown for PspA, the Tat and YidC homologues have been found in bacteria, archaea and chloroplasts. Clade PspA (CL0235) has two members, PspA/IM30 and Snf7. Snf7 is a family of proteins involved in protein sorting and transport from the endosome to the vacuole/lysosome in eukaryotic cells that play an important role in the degradation of both lipids and cellular proteins (Peck et al., 2004). Therefore, one major and conserved function of PspA and its homologues may be to maintain the activities of protein translocation systems.

MreB defines the spatial distribution of lateral membrane PspA effectors and, together with its IM-binding partner RodZ, may be implicated in targeting cell wall synthesis machinery, conferring an adaptation of cells to IM stress (Fig. S6). Potentially, sites in lateral membrane regions where the cell wall synthesis machinery is assembled can be targeted by MreB-guided PspA effectors in order to support lipid II-dependent PGL biosynthesis and elongation of the lateral cell wall under IM stress. On that note, the expression of IM protein MurG, found to interact with MreB and to be essential for the lipid II-dependent cycle of PGL synthesis (see Fig. S1b), is upregulated in E. coli cells overexpressing PspA (Jovanovic et al., 2006). In Gram-positive bacteria, lantibiotics and bacitracin interfere with cell wall synthesis by binding lipid II and strongly induce expression of the PspA homologue LiaH and its recruitment to membrane (Typas et al., 2012; Domínguez-Escobar et al., 2014). However, LiaH dynamics in B. subtilis were found to be independent of MreB and cell wall synthesis (Domínguez-Escobar et al., 2014).

The cell wall synthesis machinery in E. coli may also serve to attract and functionalize the lateral membrane PspA effectors, leading to IM repair upon stress. Genomic analyses showed that RodZ function is conserved and unique to bacteria and that rodZ and pgsA (required for PG and CL biosynthesis) genes are often adjacent, suggesting they are functionally linked (Alyahya et al., 2009). Note that PG has been found to directly bind PspA high-order oligomers which repair membrane damage in vitro (Kobayashi et al., 2007). Also, gene-to-metabolite correlations suggest that in E. coli PG plays a critical role for membrane balance (Takahashi et al., 2011). Notably, other envelope-associated complexes may well function through MreB and with changes in lipid organization impact upon the localization and dynamics of PspA.

Blocks in lipid biosynthesis induce the expression of PspA (Bergler et al., 1994), suggesting that Psp through its effector function(s) may act to modulate lipid metabolism. As observed by using psaA or pspG mutants or overexpressing PspA or PspG (additional IM effector; Lloyd et al., 2004; Jovanovic et al., 2006; Engl et al., 2009), these Psp effectors have the potential to diminish the expression of genes implicated in the glycerol shift and aerobic respiration and upregulate expression of genes that favour glycerol 3-phosphate conversion into phospholipids (Jovanovic et al., 2006; Bury-Moné et al., 2009). As we noted above, the increased amount of PG in the Δcls mutant we used here may contribute to the IM stress adaptation when induction of Psp is reduced. Intriguingly, in E. coli, overexpression of the foreign protein MGS, which binds anionic lipids, greatly elevates PG production (Ariöz et al., 2013), raising the possibility that the IM binding of highly expressed native PspA effectors may trigger a cellular signal for the stimulation of anionic lipid synthesis to repair and/or exchange PG (and CL).

In summary, the studies presented here show a functional link between CL, PspBC-dependent signalling and polar IM localization of PspA. Upon IM stress the PspA regulator switches to high-order oligomeric effector complexes in polar regions of the cell whilst employing bacterial actin MreB to target lateral membrane regions, some of which are marked by cell wall biosynthesis machinery protein RodZ. Further experiments are needed to reveal which properties of the IM change in a stress-specific manner to directly signal the membrane stress to PspA and to unravel the molecular mechanism of IM repair.

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