Transcription regulation and membrane stress management in enterobacterial pathogens

Nan Zhang [1, *], Goran Jovanovic [1, *], Christopher McDonald [2], Oscar Ces [2], Xiaodong Zhang [3], Martin Buck [1,**]

[1] Division of Cell and Molecular Biology, Sir Alexander Fleming Building, Imperial College London, Exhibition Road, London, SW7 2AZ, UK

[2] Division of Chemistry, Imperial College London, Exhibition Road, London, SW7 2AZ, UK

[3] Department of Medicine, Sir Ernst Chain Building, Imperial College London, Exhibition Road, London, SW7 2AZ, UK

* These authors contributed equally to this work

** Author to whom correspondence should be addressed; E-Mail: m.buck@imperial.ac.uk; Tel.: +44 (0) 2075945443; Fax: +44 (0) 2075045419.

Abstract

Transcription regulation in a temporal and conditional manner underpins the lifecycle of enterobacterial pathogens. Upon exposure to a wide array of environmental cues, these pathogens modulate their gene expression via the RNA polymerase and associated sigma factors. Different sigma factors, either involved in general 'house-keeping' or specific responses, guide the RNA polymerase to their cognate promoter DNAs. The major alternative sigma54 factor when activated helps pathogens manage stresses and proliferate in their ecological niches. In this chapter, we review the function and regulation of the sigma54-dependent Phage shock protein (Psp) system – a major stress response when Gram-negative pathogens encounter damages to their inner membranes. We discuss the recent development on mechanisms of gene regulation, signal transduction and stress mitigation in light of different biophysical and biochemical approaches.

Introduction

Transcription of the Psp regulon in many important bacterial pathogens depends upon the major variant form of RNA polymerase (RNAP) containing the sigma54 factor as the dissociable promoter specificity factor. Working with Yersinia enterocolitica, Miller and colleagues identified psp genes as being virulence determinants in the mouse model, through using signature tag mutagenesis. In addition to directing RNAP to promoter sequences characterised by the consensus sequences TGCA around -12 and CTGGCAC around -24, the interactions between sigma54 and the core RNAP set the dependency of gene expression at the level of promoter DNA opening in the transition from a closed promoter complex (RP_C) to an open promoter complex (RP_O). The transition from RP_C to RP_O is catalysed by the ATPase function of transcription activators such as PspF acting directly upon the sigma54 Region I to relieve the inhibition imposed upon the -12 DNA fork junction {Bose, 2008 #10;Morris, 1994 #123}. The rearranged RNAP subsequently accepts the single-stranded template DNA at the active site for RNA synthesis. Thus the formation of RPo at for example the pspA and pspG promoters leads to expression of sets of psp genes used to manage membrane stress.

Implications of sigma54 in pathogenicity

The major alternative sigma54 factor is present in almost all diderm species with an outer membrane mainly consisted of lipopolysaccharides {Francke, 2011 #331}. It has been found to cross-regulate genes with other sigma factors in *Pseudomonas aeruginosa* {Schulz, 2015 #351}. Under stress conditions, sigma54 may repress sigma70-dependent transcription by blocking either a proximal sigma70 binding site or through interacting at the intragenic regions {Schafer, 2015 #348}.

Historically, sigma54 has been associated with nitrogen metabolism, alternative carbon source utility and motility {Buck, 2000 #16;Studholme, 2003 #157}. Recent studies have broadly expanded its functional repertoire in pathogenicity: biofilm formation and toxin production in *Bacillus cereus* ATCC 14579 {Hayrapetyan, 2015 #352}; internal colonization in *Vibrio parahaemolyticus* {Whitaker, 2014 #353}; antibiotics sensitivity in *Escherichia coli* {Liu, 2010 #354}; acid

resistance in enterohemorrhagic *E. coli* {Mitra, 2014 #358}; dental pulp infection in *Lactobacillus rhamnosus* {Nadkarni, 2014 #359}; protein secretion and invasion in *Campylobacter jejuni* {Carrillo, 2004 #357;Hendrixson, 2004 #356}; intracellular trafficking within macrophages in *Burholderia cenocepacia* {Saldias, 2008 #360}; osmotolerance in *Listeria monocytogenes* {Okada, 2006 #361}; lipoprotein biosynthesis and virulence in *Borrelia burgdorferi* {Fisher, 2005 #362}; stress adaptation and virulence in *Edwardsiella tarda* {Liu, 2014 #363}; autolysis and biofilm formation in *Enterococcus faecalis* {Iyer, 2012 #364}; and type I and type IV pilli biogenesis in *Xylella fastidiosa* {da Silva Neto, 2008 #365}. Computational synteny analyses suggest that genes under regulation of sigma54 conform to a common theme, that is the transport and biosynthesis of molecules that constitute the bacterial exterior (extracellular polysaccharides, flagella, lipopolysaccharides, lipoprotein and the peptidoglycan cell wall, {Francke, 2011 #331}).

Domain reorganisation of sigma54 during transcription activation

A combination of single molecule biophysical methods has been used to probe the events taking the RP_C to RP_O at a few sigma54 test promoters. Kinetic studies on the *glnAP2* promoter using COSMO methodologies indicate that the transition from RP_C to transcript generation takes around ninety seconds, and activators interact with both RP_C and RP_O {Friedman, 2012 #59}. The Stockley and Tuma labs addressed the domain movement of sigma54 in relation to the promoter DNA and ATP hydrolysis using single-molecule FRET analysis {Sharma, 2014 #320}. At the point of ATP hydrolysis, sigma54 Region I moves nearly 30 Å towards the leading edge of the transcription bubble (approximately the same distance as from -12 to +1, Fig. 1). This downstream movement may have two functional consequences: Firstly, it may facilitate the removal of inhibitory interactions formed around the -12 fork junction DNA by the 'power stroke' action of multiple L1 loops on the PspF activator hexamer (Fig. 1). Secondly, the downstream movement brings sigma54 Region I in close proximity to the +1 site where it potentially constitutes an interaction network with the bridge helix and switch regions (Fig. 1B, {Zhang, 2015 #366}). Deletion of sigma54 Region I has been shown to rescue activation defects of certain bridge helix variants, reverse the regulatory effects of DksA bound to the secondary channel {Zhang, 2015

#366}, and bypass the activator requirement on several promoters *in vivo* {Schafer, 2015 #348}.

New structural insights of the RNAP-sigma54 transcription complex

The structure of the sigma54-containing RNAP and its co-complexes with promoter DNA and with the ATPase domain of PspF has been determined using combinations of single-particle cryo-electron microscopy and X-ray crystallography. It is clear that the closed or intermediate promoter complexes are maintained at the downstream promoter -12 by contacts made between several L1 loops and sigma54 Region I and at the upstream end by an L1 loop and the non-template -30 promoter DNA (Fig. 1A, {Bose, 2008 #10;Rappas, 2005 #132;Zhang, 2012 #231}). Recent structural elucidation of the RNAP-sigma54 holoenzyme by the Zhang lab shed new light on the sigma54 inhibitory mechanism. In order for the DNA template strand to be loaded into the active channel, the blocking sigma54 Regions I-III 'gate' must be shifted downstream (Fig. 2A). In contrast, the template strand can pass through the 'V'-shaped wedge formed between sigma70 regions 2 and 3 (Fig. 1C) or between the TFIIB core and linker regions. In line with the structural data, sigma54 variants that disrupt the Regions I-III 'gate' – such as deletion of Region I or mutations of Region III residue R336 – can spontaneously bypass the activation energy barrier {Chaney, 2001 #189;Schafer, 2015 #348}Also cite original Gralla Science paper and his PNAS paper too. The aromatic residues in sigma54 Regions I and III and the PspF L1 'GAFTGA' motif could potentially facilitate the DNA melting process.

As shown in Fig. 2, the downstream DNA channel is blocked by both sigma54 Region II and sigma70 region 1.1 (Fig. 2B and D). The core RNAP binding domain (CBD) on sigma54 contacts the β flap feature and blocks the RNA exit channel, and suggests that sigma54 must dissociate to allow the nascent RNA to extend beyond 8 nts (Fig. 2A). This obligatory dissociation of sigma54 in early transcription is notable, as it is not found with sigma70 where the CBD (σ2 region) binds to the surface of the core RNAP to allow its retention during elongation (Fig. 2C). Comparisons between sigma54 and sigma70 holoenzyme structures suggest that the two sigma factors employ different functional domains to contact similar regions on the core RNAP (Fig. 2).

A transcription inhibitor – Gp2 – encoded by the bacteriophage T7 has been shown to significantly affect RP_O formation in the sigma70-containing but not the sigma54-containing RNAP complexes {Wigneshweraraj, 2004 #382}. Recent structural elucidation of the Gp2-sigma70 holoenzyme suggests that Gp2 bridges between the β' jaw domain and sigma70 region 1.1 through electrostatic interactions, thereby preventing the egress of region 1.1 from the active channel and blocking the promoter DNA from entering {Bae, 2013 #303}. In comparison, the β' jaw domain in the sigma54-containing RNAP changes its conformation upon activator binding and ATP hydrolysis ({Wigneshweraraj, 2004 #382}), which could attribute to its insensitivity towards any Gp2 inhibition.

Describing the conformational change pathway to RP_O will require further structural determinations, and in particular complexes with fully and partially opened promoter DNA templates, with and without the bound activators and at high resolution. Recent advances in detector technology and image processing algorithms promise a major continuing role for cryo-electron microscopy in such structural studies.

Introduction of the Psp regulon and its signals

The sigma54-dependent Psp regulon in enterobacterial pathogens is triggered by extracytoplasmic stresses that damage the inner membrane (IM) integrity, and is recognised as of major importance in biofilm formation, virulence, macrophage infection, antibiotics resistance and persistence {Darwin, 2013 #372;Joly, 2010 #208;Rowley, 2006 #371}. A large variety of stimuli can cause inner membrane stresses and lead to dissipation of the proton motive force (pmf) and changes in the redox states {Darwin, 2005 #45;Joly, 2010 #208;Model, 1997 #118}. These include mislocalisation of outer membrane secretins (filamentous phage protein pIV and its bacterial homologues such as YscC, PulD, OutD), malfunction of the protein translocation systems, blockage of phospholipids and peptidoglycan biogenesis, antimicrobials that target membrane biosynthesis, and exposure to bile salts or the intracellcular environment of phagocytic cells. The Psp response is also induced upon exposure to organic solvents (e.g., ethanol and methanol), hyperosmotic shock, extreme temperatures as well as incorporations of large protein assemblies,

protonophores and fatty acids into the IM. These stimuli may perturb the IM something missing in this sentence? potential (depolarisation), the net charge on anionic lipids (redox states), membrane fluidity/rigidity, phospholipids packing and the stored curvature elastic (SCE) stress.

In *E. coli*, there are seven genes present in the Psp regulon (Fig. 3A): the *pspABCDE* operon at 29.44 min (under the *pspA* promoter), divergently oriented *pspF* (*pspF* promoter), and *pspG* at 91.84 min (*pspG* promoter). Transcription from the *pspA* and *pspG* promoters is dependent on sigma54 and the PspF transcription activator {Lloyd, 2004 #308;Weiner, 1991 #368}. Notably, the two promoters possess an altered -12 recognition element (GT or GA) in place of the usual consensus GC element. The PspF activators hexamerise at their cognate upstream activating sequence (UAS) and are brought to close proximity to the sigma54-containing RNAP by DNA looping as facilitated by the integration host factor (IHF) {Lloyd, 2004 #308}. In contrast to the single UAS site present on the *pspG* promoter (between -78 and -92), the *pspA* promoter contains two UAS sites (between -80 and -126, Fig. 3A). The greater transcription strength of the *pspA* promoter may be particularly suited for controlling PspA levels to achieve its negative repressor function (acting on PspF) as well as its effector function (acting on the IM) under stress conditions {Lloyd, 2004 #308;Seo, 2007 #373}. Binding of the PspF hexamers to the UAS sites is a prerequisite for activation of the *pspA* operon. However, it collides with the *pspF* gene transcription in the opposite orientation (Fig. 3A). So the PspF expression is under control of an autonomous negative feedback loop. Congruent with this observation, microarray analysis indicates that under IM stresses (pIV secretin production) the *pspA* and *pspG* promoters are strongly induced while the *pspF* promoter remains largely unaffected (Ref).

Activation of the Psp regulon by PspF

The PspF activator unlike many other activators of the sigma54 RNApolymerase lacks an N-terminal regulatory domain and so it is active *per* default (Jovanovic et al, 1996, JBac). Rather, the catalytic AAA^+ ATPase domain of PspF is negatively regulated *in trans* by PspA to achieve control of transcription outputs {Joly, 2009 #207}. The concentration-dependent oligomerisation of PspF plays a key role in both ATP

hydrolysis and energy coupling to the RP_C remodeling event. The hydrolytic state of ATP bound at the hexameric interface is sensed by the Walker motifs and arginine hand and relayed to L1 loop movement by the 'Glutamate Switch' pair (E108-N64 in PspF) via the W56 loop and Linker 1 (Fig. 3B, {Joly, 2012 #85;Zhang, 2008 #233}). Disruption of the 'Glutamate Switch' leads to phenotypes that decouple the inhibitory interactions from DNA melting {Darbari, 2014 #369}. After hydrolysis, the 'ADP-switch' pair (E43-Y126 in PspF) is thought to facilitate the ADP release {Joly, 2012 #85}.

In the absence of ATP, the AAA+ domain of PspF self-associates into heptamers with low nucleotide-binding affinities (Fig. 1A, {Zhang, 2014 #345}). The cooperative binding of nucleotides in at least two adjacent subunits causes the L1 loops from these subunits to engage sigma54 Region I (Fig. 1A). This functional asymmetry in PspF subunits may well correlate with a heterogeneity in nucleotide occupancy and be key to achieving an ATPase driven remodeling of RPc {Joly, 2006 #210}. The 'GAFTGA' motif in PspF L1 loop, along with aromatic residues in sigma54 Regions I and III, might be presented in a way to facilitate promoter DNA melting. The shedding of the seventh PspF subunit and the opening of the PspF hexameric ring might occur in the transition state (Fig. 1A, {Zhang, 2014 #345}). The observed nucleotide-driven functional asymmetry and partial sequential hydrolysis model may conform to a ubiquitous mechanism as employed by the sigma54 activator family, or at least by PspF and NtrC1 {Sysoeva, 2013 #370;Zhang, 2014 #345}.

Negative regulator function of PspA

PspA is a bifunctional inner membrane binding protein: it negatively regulates the PspF activator under non-stress conditions and switches to an effector function when the IM is stressed. Like its homologue Vipp1 that is essential in thylakoid biogenesis and photosynthesis in cyanobacteria, green algae and plants {Westphal, 2001 #374}, PspA is composed of helical domains (HD 1-4, Fig. 3C). The last helical domain (HD4, residues 187-222) has been implicated in higher-order oligomerisation associated with PspA's effector function. Deletion of this domain does not seem to affect PspA's ability to bind and inhibit PspF *in vitro* {Elderkin, 2005 #194}. Recent structural elucidation of a PspA fragment (residues 1-144, Fig. 3C) revealed a coiled-

coil topology similar to the M-domain of the unfoldase ClpB {Osadnik, 2015 #375}. The HD1 (residues 1-64) of PspA contains two N-terminal amphipathic helices (AHa, residues 2-19; AHb, residues 25-42, Fig. 3C) (ref). It has been proposed that the AHa domain can sense anionic lipids and membrane curvature caused by lipid-packing defects. AHa may then wedge its hydrophobic residues (Fig. 3D) into the lipid bilayer to alleviate the membrane stress {McDonald, 2015 #376}. The AHb domain of PspA has been implicated in binding to the 'YLW56' hydrophobic patch on the surface of PspF, so shutting down the ATPase activity and uncoupling across the hexameric AAA+ ring the hydrolysis-driven L1 loop movement (ref+{Zhang, 2013 #377}).

The dynamics of PspA and PspF was studied in live cells using single molecule tracking and photobleaching approaches. Under non-stress conditions, up to six PspA monomers are recruited to inhibit one PspF hexamer at the nucleoid {Joly, 2009 #207;Osadnik, 2015 #375}Mehtra Nat coms ref OK here too. Occasionally, the PspA-F inhibitory complexes are transiently recruited to the inner membrane cardiolipin-rich polar regions by PspB and PspC for stress sensing – this leads to a partial release of PspF and thus a basal level of *psp* gene expression. Indeed, over-production of PspB and PspC induces the Psp regulon in the absence of stress signals (ref). The PspA-F inhibitory complexes or PspF hexamers rarely occupy both *pspA* and *pspG* promoters simultaneously (Mehta et al). The heterogeneous promoter occupancy is likely due to the limited amount of intracellular PspF activators. Such limitation could potentially lead to variations in *psp* gene expression – hence differential stress responses – within a population of cells. Joly *et al* showed that the PspA-F inhibitory complex can still engage sigma54 {Joly, 2009 #207}. It is possible that the elongated PspA folds back on the outer rim of the PspF hexamer so as to vacate for sigma54 interactions. Similar domain folding events have been observed in the N-terminal regulatory domains of other sigma54 activators such as NtrC and NorR {Bush, 2012 #242;De Carlo, 2006 #47}.

Effector function of PspA

Under membrane stress conditions, PspA dissociates from the inhibitory complexes made with PspF to form higher-order oligomers (≥ 36mers) with a calculated mass of 1034 kDa, thus releasing PspF for transcription activation (Fig. 4, {Hankamer, 2004

#199}). Such large PspA assemblies have been studied using cryo-electron microscopy and by single molecule photobleaching (refs) . They are proposed to bind to liposomes, thereby directly suppressing a proton leakage {Kobayashi, 2007 #214}. Binding of the PspA higher-order oligomers to the IM is facilitated by its AHa domain that upon membrane association folds from a random coil into an α-helix (Fig. 3CD and Fig. 4). The PspA-IM interaction via AHa and the PspA-F interaction via AHb (i.e., effector function vs. negative regulator function) are mutually exclusive. This functional exclusivity is thought to be maintained by a highly conserved residue P25 that connects the two amphipathic helices in PspA (Fig. 3C, ref). It seems that the higher-order oligomeric state is an intrinsic property of PspA when PspF is not present. PspA has been shown to form active oligomeric effectors in heterologous systems (such as bacteria, archaea and plants) that carry only PspA homologues (ref).

Work with purified components has established that the stored curvature elastic stress (SCE) and the presence of anionic phospholipids are important for membrane binding and the effector function of PspA. An increased SCE stress introduced into vesicles by lipid packing defects causes a release of PspA-F inhibitory complexes and elevates PspA-lipids interactions via AHa *in vitro* (McDonald et al., 2015). An addition of conically shaped lipid II to the anionic lipids-rich microdomains during peptidoglycan biosynthesis has the same effect as membrane curvature on the lipid bilayer (Vanni et al., 2013, Antonny et al., 1997) and so may increase the SCE stress.

Depolarization of the plasma membrane may induce a nano-scale reorganisation of the negatively charged anionic lipids microdomains containing phosphatidyl-glycerol (PG) and phosphatidyl-serine (PS), and trigger a nano-clustering of membrane-targeting PspA proteins via electrostatic interactions. An SCE stress and a negatively charged lipid bilayer drive the association of PspA to the IM independently, and they do not seem to reinforce one another. For instance, the negative charge on anionic lipids dampens the SCE stress sensing by PspA (ref).

The PspA higher-order oligomers also play a key role in the maintenance of torque tension stored in the IM within a critical range. The presence of ordered anionic lipids microdomains introduces curvature, restricted lateral diffusion and

high stresses to the IM. It has been shown that insertions of randomly aggregated synthetic PspA AHa peptides could further elevate stress and eventually lead to a membrane phase transition to a porous state (ref). However, the full-length PspA protein or the high-order PspA scaffold may provide a regular dispersion of the AHa domains and limit IM contacts.

The effects of anionic lipids interactions and SCE stress observed with PspA also apply to Vipp1 (a PspA homologue in cyanobacteria) and its AHa domain. However, differences in effector functions between PspA and Vipp1 have been observed. For instance, Vipp1 can substitute for PspA in *E. coli*, but PspA can only partially replace Vipp1's function (ref). Vipp1 is primarily involved in anionic lipids interactions, which is consistent with its proposed function of thylakoid membrane fusion. This preference to anionic lipids recognition is likely to attribute to a higher percentage of cationic residues in Vipp1 (residues 20-24, ref). PspA primarily senses the SCE stress with less an involvement in anionic lipids interactions (ref). Despite these differences, it is clear that a ubiquitous membrane stress response amongst functional homologues is employed across multiple domains of life, from enterobacteria to plants.

In *E. coli*, the PspA higher-order oligomers move along a helical path, and evidence from interaction studies suggests this may well occur by forming direct complexes with MreB. The actin-like MreB is essential in maintaining peptidoglycan biosynthesis and cell morphology and is implicated in diffusional restriction along the plasma membrane (ref). The MreB protein may well carry PspA along the IM to deliver it to distinct membrane regions. Many of these regions are marked by flotillin YqiK and RodZ and contain the peptidoglycan biosynthesis machineries (ref). The restricted diffusion, along with the transient protein-protein and protein-lipid interactions, within these lipid microdomains is important for IM engagement by PspA effectors and the SCE stress mitigation process. In *Bacillus subtilis*, the PspA homologue LiaH displays MreB-independent dynamics in the cytoplasm prior to stress and assembles with LiaI to form static complexes along the membrane upon membrane stresses (ref).

Signal transduction via PspB and PspC

PspB and PspC are inner membrane proteins that positively regulate the Psp regulon and under microaerobic growth link the control of the Psp regulon to respiration and the ArcAB two component system. They sense and transduce the IM stress signals to PspA following a direct and stable engagement with the PspA-F inhibitory complex (Fig. 4). The co-localisation and function of PspB and PspC in the IM polar regions depend on flotillin YqiK and anionic phospholipid cardiolipin (which is preferentially found in the curved polar IM regions). PspC is a polytopic protein and its C-terminus (Ct) appears to be able to undergo topological changes upon membrane stresses to facilitate its role in stress signaling (ref). Under non-stress conditions, the PspC Ct stays in the periplasm. A change in the head-group charges of the anionic lipids located in the IM inner leaflet triggers membrane depolarisation or a drop in pmf and PspC is thought to sense this signal and flips its Ct domain from the periplasm to the cytoplasm for PspB interaction. This inactive complex of PspB-C only allows transient interactions with the PspA-F inhibitory complex and permits a basal level of *psp* expression. Upon experiencing a second stress signal, such as an SCE stress, the PspC Ct domain switches to stably interact with PspA, possibly via the AHb domain. The stable binding of PspA to PspC has two functional consequences: (1) The PspA-F inhibitory complex is either dismantled or cannot reform to allow a high level of *psp* gene expression, and (2) PspA is encouraged to bind to the curved polar inner membrane regions and form higher order oligomers (36mers) as effectors.

The signal transduction pathway leading to increased psp promoter activity may occur in a PspB/C-dependent or independent manner that varies with the severity of IM stresses. For instance, if the stress signal is weak to moderate, such as the IM mislocalisation of pIV secretins, the PspB and PspC sensors are targeted for *psp* gene activation. In contrast, severe signals that significantly depolarise the IM or greatly increase the membrane SCE stress (e.g., extreme temperature, hyperosmotic or ethanol shock) are directly recognised by the PspA-F inhibitory complex without the intermediate step of PspB/C consultation. This PspB/C-bypass response has also occurs when the secretin IM mislocalisation is combined with a blockage in peptidoglycan biosynthesis or when the quantity of secretin pIV and fatty acids are elevated above a threshold in anaerobiosis (ref). Overall, the PspA-F inhibitory

complex may serve as a minimal regulatory unit and an emergency shortcut in dealing with severe IM stresses in enterobacteria.

Additional Psp effectors to mitigate membrane stresses

In *Yersinia enterocolitia*, the PspB and PspC sensors function as effectors in dealing with secretin-induced membrane stresses (Maxson and Darwin 2006; Horstman and Darwin 2012). In *E. coli*, members of the Psp regulon also participate in the conservation of energy and pmf. PspD is a peripheral IM protein that can support and to some extents substitute PspA for the effector function {Jovanovic, 2006 #379}. PspE is a *bona fide* periplasmic rhodanase (Adams *et al*., 2002; Cheng *et al*., 2008; Chng *et al*., 2012). Under IM stress conditions, it may function to repair the damaged Fe-S clusters present in the IM respiratory enzymes and/or support the disulfide bond formation in the increasingly oxidative environment in the periplasm. PspG interacts with PspC in the static polar complexes and migrates to specific membrane regions in an MreB-dependent fashion as seen for PspA {Engl, 2009 #380;Jovanovic, 2010 #381}. The effector function of PspG is needed to fully ameliorate the IM stress in *E. coli* {Engl, 2009 #380;Jovanovic, 2010 #381;Jovanovic, 2006 #379;Lloyd, 2004 #308}. When over-produced, PspG and PspA jointly down-regulate cell motility that consumes the pmf. They also bring down the glycerol shift in order to promote the synthesis of glycerol-3-phosphate which can be used in phospholipids biogenesis and replacement of damaged anionic lipids during membrane stresses (Jovanovic *et al*., 2006; Bury-Mone *et al*., 2009). In addition, they up-regulate the polyamine spermidine production by nearly 70-fold in order to slow down protein production {Jovanovic, 2006 #379}.

Psp proteins in pathogenicity and antimicrobial responses

The Psp response was initially discovered in enterobacteria following the production of filamenous phage secretin pIV. Since then, it has been implicated in support of cellular growth, adaptation, and delivery of toxins and virulence factors in many pathogens. Many of the outer membrane associated secretion systems, such as T2/3/4 SS, are affected by the Psp response genes (ref). For enterobacterial pathogens, surviving the antibacterial action of bile salts is of the uppermost

importance. *E. coli* mutants that lack the *psp* genes are not resistant to bile salts and show impaired growth in the stationary phase (ref). When *Salmonella* consumes phospholipids in bile salts, production of PspA protects its inner membrane and potentially elevates the synthesis of phospholipids (Caetano *et al*., 2011). The Psp response, including PspA and its homologues, is also required for intracellular adaptation, virulence and survival of proteobacteria, particularly important in mycobacteria infection and survival inside the macrophages (Darwin PloS Path; new papers Burkhod + Mycobacterium 2x).

The Psp response genes are involved in biofilm formation and chronic infections of multi-drug resistant enterobacterial persister cells (Keren *et al*., 2004; Shah *et al*., 2006; Ma *et al*., 2009; Dhamdhere and Zgurskaya, 2010). Persistence can be achieved by abolishing the glycerol shift (aerobic respiration) and increasing phospholipids synthesis in glycerol-3-phosphate dehydrogenase (*glpD*) mutants. This type of persistence is congruent with the metabolic fine-tuning of Psp effectors (Girgis *et al*., 2012). The indol-induced formation of persister cells depends on the induction of *psp* and *oxyR* genes, when the target protein Flu – a membrane antigen responsible for biofilm formation – is absent (Vega *et al*., 2012 Vega; Kint *et al*., 2012). Notably, an increased production of PspA and PspG leads to a 5-fold down-regulation of Flu and a 13-fold up-regulation of YodA (another OxyR target, Jovanovic *et al*., 2006). YodA and Psp are important in cadmium-resistance, divalent cation transport and virulence in the absence of sigmaE in *S. typhimurium* (Wang and Crowley, 2005; Becker et al. 2005). In addition, synthesis of PspA and PspG up-regulates the transport and biosynthesis of polyamine spermidine in order to arrest cells in a metabolically stagnant state and polyamines are required for virulence of e.g., *Salmonella enterica* (Jelsbak *et al*., 2012).

The PspA homologue LiaH is present in Gram-positive pathogens and directly protects cells from antibiotics that work on cell walls or peptidoglycan (e.g., bacitracin, nisin, ramoplanin, vancomycin and cationic peptides, Jordan *et al*., 2008; Joly et al., 2010; Wolf *et al*., 2010). LiaH confers resistance to daptomycin – a class of antibiotics that binds to phosphatidyl-glycerol and reorganises the membrane architecture (Pogliano *et al*., 2012). Given the structural similarity between the AHas of LiaH and PspA, it is possible that LiaH remodels the membrane using this domain

to counter the effects of daptomycin. Interestingly, LiaH is strongly induced by biosurfactants (rhamnolipids, ref). Biosurfactants are produced by Gram-negative bacteria to assist growth competition, and by inference, the Psp response might be involved in intercellular competition and contact-dependent growth inhibition.

The highly hydrophobic IbsC peptides that are toxic for cells can induce the Psp response in *E. coli* (ref). After penetrating the membrane, these cationic antimicrobial peptides sequester anionic phospholipids and cause displacement of membrane proteins required for biogenesis and respiration (Epand *et al*., 2011). The specialised microdomains harbouring PG, PS and CL and Psp proteins might serve as potential targets for treating chronic persistent infections (Allison *et al*., 2011; Hurdle *et al*., 2011). Finally, the vesicle destabilising properties of PspA and Vipp1 AHa-derived peptides, in particular the Vipp1 AHa cationic peptide, could be used specifically for antimicrobial therapies, as the eukaryotic membranes carry a much lower net negative charge (C.M., G.J., O.C., M.B., unpublished data).

Potential cross-regulations with other cell envelope networks

The integrity of the cell envelope is constantly monitored by at least five multi-component signal transduction systems; alongside the Psp system, there are Cpx, sigmaE, Rcs and Bae systems. Although these systems respond to stresses acquired in different envelope compartments, it is reasonable to speculate a level of their interplay might well occur when common cellular processes – such as motility, biofilm formation, virulence and antimicrobial resistance – are targeted. Although the Cpx system responds to accumulation of misfolded cell envelope proteins in the periplasm, recently it has been associated with sensing the IM surface charge as influenced by altered lipid composition and perturbations in the IM. In addition, the Cpx response leads to changes in the peptidoglycan structure. Similar to the Psp system, it regulates chemotaxis, motility, adhesion, biofilm formation, and T3/4 SS (ref). The sigmaE system mainly controls the outer membrane (OM) homeostasis. The extracellular sigmaE senses the accumulation of unassembled OM proteins (e.g., porins) and LPS in the periplasm and regulates cellular responses that ameliorate envelope perturbations. It has been established in *S. typhimurium* that the sigmaE system employs the Psp response to facilitate resistance against heavy metals (ref).

Moreover, under severe cell envelop damages, all three Psp, Cpx and sigmaE systems are induced and their responses are likely to be interlinked in order to minimize the envelope damage (Darwin). The Rcs system responds to damages in the OM and peptidoglycan (ref). Activation of the Rcs response down-regulates gene expression in motility and virulence but is required for normal biofilm formation and periplasmic space content quality control. Under osmotic stress conditions, the Rcs system enhances capsule production to protect the OM while the Psp system protects the IM. The Bae system specifically responds to alterations and damages of the envelope caused by toxic agents and so as to up-regulate efflux pumps to remove the toxins (ref). It also target drug efflux systems and so is implicated in multi-drug transport and resistance (ref).

Conclusions

The major alternative sigma54 factor is a global regulator of genes involved in metabolism and virulence in pathogens. Sigma54-dependent transcription requires activation by ATPase activators that form homo- or hetero-hexamers in respond to their cognate regulators. These regulators are often part of a two-component system that helps cells sense environmental cues. The Psp response we described in this chapter is an example of how pathogens employ the sigma54-dependent transcription system to perceive and ameliorate various membrane stresses. The success of this system lies in a coordinated cascade of protein expressions and a delicate balance between regulator and effector functions. Depending on the severity of membrane stresses, the Psp system can decide whether or not to take an emergency shortcut by bypassing several sensors and effectors. This autonomous pathway selection represents a level of cellular intelligence and is vital for the survival of pathogens in their niches. Understanding the function of the Psp response, and by extension the sigma54-dependent transcription system, is of broad interest not only for detecting the complex cellular regulatory circuitries but also for developing novel antimicrobial targets and therapeutic cationic peptides with bactericidal properties and has been greatly advanced through findings arising from biophysical and structural biology.