Managing membrane stress: the phage shock protein (Psp) response, from molecular mechanisms to physiology

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Abstract

The bacterial phage shock protein (Psp) response functions to help cells manage the impacts of agents impairing cell membrane function. The system has relevance to biotechnology and to medicine. Originally discovered in *Escherichia coli*, Psp proteins and homologues are found in Gram-positive and Gram-negative bacteria, in archaea and in plants. Study of the *E. coli* and *Yersinia enterocolitica* Psp systems provides insights into how membrane-associated sensory Psp proteins might perceive membrane stress, signal to the transcription apparatus and use an ATP-hydrolysing transcription activator to produce effector proteins to overcome the stress. Progress in understanding the mechanism of signal transduction by the membrane-bound Psp proteins, regulation of the *psp* gene-specific transcription activator and the cell biology of the system is presented and discussed. Many features of the action of the Psp system appear to be dominated by states of self-association of the master effector, PspA, and the transcription activator, PspF, alongside a signalling pathway that displays strong conditionality in its requirement.

Introduction

Much of our knowledge of the processes by which the information in DNA is accessed, replicated and expressed arises from founding basic studies of phage and bacterial biology. These studies gave rise to molecular biology as we now know it, and have had transformative and resonating impacts throughout all modern biology and biotechnology. Here, we present and discuss our knowledge of a bacterial stress response system in which the action of a filamentous phage secretin has given rise to a research area focused on establishing how extracytoplasmic stress is managed. What was once seen as an enigmatic relationship between *Escherichia coli* and the gene product IV (protein pIV) of filamentous phage f1 (Brissette *et al.*, 1990) can now be seen as providing both a paradigm for inner membrane (IM) stress and links to processes as far ranging as photosynthesis, salt tolerance in archaea and the pathogenesis of *Yersinia*, *Shigella* and *Salmonella* bacteria as well as making major contributions to establishing a second paradigm for gene activation in bacteria. Unless otherwise indicated, the review focuses on findings with *E. coli*.

Discovery of the Psp response

The Phage shock protein (Psp) response was originally discovered in Peter Model's laboratory at the Rockefeller University while studying filamentous phage f1 infection of *E. coli* (Brissette *et al.*, 1990). Upon phage f1 infection, the prolonged synthesis of the phage gene IV protein, pIV, induces continual and abundant production of an *E. coli*-encoded 25-kDa protein. pIV is the secretin required for phage extrusion from the cell and so the authors named the response the Psp response and the induced *E. coli* protein, accordingly, PspA. Subsequently, it was established that *pspA* is part of an operon, *pspABCDE* (Brissette *et al.*, 1991). The expression of the *psp* genes is driven by RNA polymerase (RNAP) containing the σ^{54} factor (Fig. 1) and is controlled at the transcriptional level by (1) positive and negative regulators (Weiner *et al.*, 1991) (see Psp regulon transcription
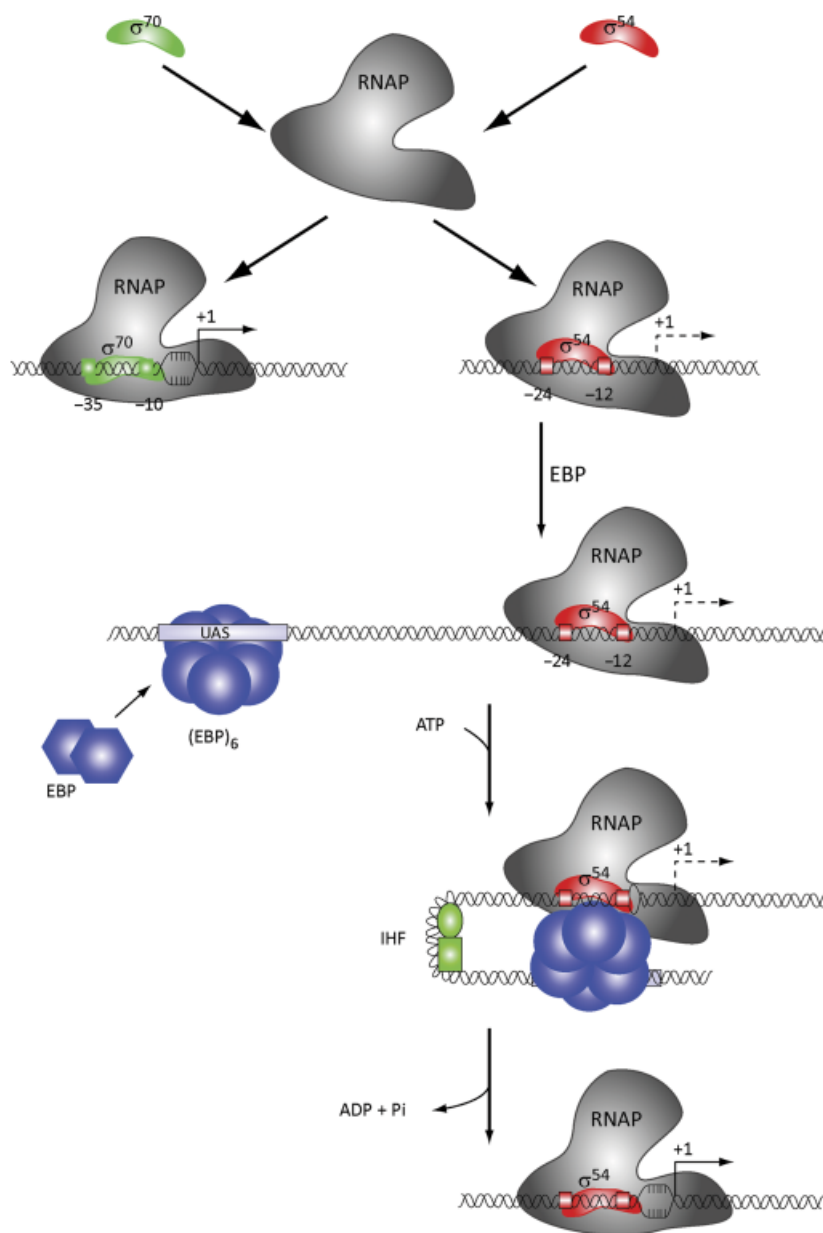
Fig. 1. Bacterial transcription activation. Two different routes to bacterial transcription initiation exist. The two types of σ factors (σ⁷⁰ or σ⁵⁴) interact with a common RNAP core enzyme, forming two distinct holoenzymes. In the presence of DNA, the σ⁷⁰-RNAP can spontaneously form the open promoter complex competent for transcription, whereas the σ⁵⁴-RNAP requires an additional factor, the bEBP, which hydrolyses ATP to catalyse the formation of the open promoter complex.

and its transcriptional activator PspF, and PspA, the negative regulator of PspF and PspBC-dependent signal transduction), (2) *cis*-acting elements (Weiner *et al.*, 1995) (see The specific domain organization of PspF) and (3) the transcriptional activator PspF (Jovanovic *et al.*, 1996) (see Psp promoters bind PspF and use σ⁵⁴-RNAP). Since then, considerable progress has been made in terms of *in vivo* and *in vitro* characterization of the regulation and function of the Psp response. Notably, the Psp response can be induced by numerous conditions that impair the membrane integrity (see Inducers of the Psp response) and is not restricted to *E. coli*. Most recently, the last member of the Psp response, PspG, physically separated, but coregulated

with the *psp* operon has been described (see Psp promoters bind PspF and use σ⁵⁴-RNAP) (Green & Darwin, 2004; Lloyd *et al.*, 2004).

Psp regulon transcription and its transcriptional activator PspF

Bacterial transcription

Transcription of DNA into RNA is a crucial and highly regulated cellular process requiring the multisubunit RNAP. Although the transcription machinery differs between eukaryotes and bacteria, the binding of RNAP to promoter

DNA and the transition from a closed-double stranded DNA promoter complex (the closed complex, RP_c) to an open melted-out complex (the open complex, RP_o) occurs universally. In bacteria, RNAP is composed of five conserved subunits: 2 α, β, β' and ω, which constitute the catalytic RNAP core enzyme (E). An additional dissociable subunit, sigma (σ), ensures promoter recognition specificity. There are two major classes of σ factors in bacteria, σ^{70} and σ^{54}, which confer different regulatory properties to the RNAP core enzyme (Fig. 1). The major σ^{70}-RNAP holoenzyme ($E\sigma^{70}$) recognizes -35 (TTGACA) and -10 (TATAAT) consensus DNA sequences and is constitutively active for transcription. $E\sigma^{54}$ recognizes -24 (GG) and -12 (GC) consensus sequences and requires specific activators, termed bacterial enhancer-binding proteins (bEBPs), to initiate transcription. These bEBPs (also called σ^{54} activators) are AAA+ proteins that use ATP hydrolysis (see AAA+ ATPase PspF is a bEBP) to isomerize the initial, transcriptionally inactive $E\sigma^{54}$-promoter DNA complex (RP_c) to a transcriptionally proficient open complex (RP_o) (Fig. 1) (Sasse-Dwight & Gralla, 1988, 1990; Morett & Buck, 1989; Popham et al., 1989; Merrick, 1993; Buck et al., 2000; Cannon et al., 2000; Iyer et al., 2004; Wigneshweraraj et al., 2005; Bose et al., 2008).

AAA+ ATPase PspF is a bEBP

AAA+ ATPases are P-loop ATPases that convert the chemical energy derived from NTP (e.g. ATP) into biological output, most often via mechanical force (and so are viewed as being mechanochemical enzymes) (Patel & Latterich, 1998; Ogura & Wilkinson, 2001). The P-loop (or the phosphate-binding loop) is an ATP- or a GTP-binding motif composed of a glycine-rich sequence that forms a loop inserted between a β-sheet and an α-helix. This P-loop interacts with the nucleotide and the Mg^{2+} to coordinate the β- and γ-phosphates of the NTP. The AAA+ ATPase are defined by a conserved region of about 200 amino acids (the AAA+ core), comprising an amino-terminal α/β subdomain and a carboxy-terminal α-helical subdomain. The

AAA+ core of bEBPs (Fig. 2) contains seven conserved motifs called C1–C7 (Morett & Segovia, 1993) including Walker A [or P-loop, consensus sequence GxxxxGK (T/S), where x represents any residue] and Walker B (consensus sequence hhhhDE, where h represents a hydrophobic amino acid) motifs, which are involved in ATP binding and hydrolysis, respectively, as well as a second region of homology (SRH) (Walker et al., 1982; Patel & Latterich, 1998; Vale, 2000; Ogura & Wilkinson, 2001; Lupas & Martin, 2002). AAA+ ATPases usually function as higher-order oligomers (commonly hexamers assembled from inactive dimers) that remodel their substrates in reactions consuming ATP (Dougan et al., 2002; Krzywda et al., 2002; Lupas & Martin, 2002; Zhang et al., 2002; Beuron et al., 2003; DeLaBarre & Brunger, 2003; Hanson & Whiteheart, 2005; Schumacher et al., 2006). The nucleotide-binding site is formed at the interface between adjacent subunits of the oligomer and is composed of the Walker A and B motifs of the same subunit. The trans-acting determinants from the adjacent subunit (e.g. the arginine finger residues) contribute to nucleotide hydrolysis (Patel & Latterich, 1998; Greenleaf et al., 2008). Substrate recognition, binding and remodelling are dependent on several critical functional motifs inserted into the AAA+ core. The nature and location of these insertions define the seven different subclades of the AAA+ protein family (Lupas & Martin, 2002; Erzberger & Berger, 2006), where bEBPs belong to subclade 6 of the AAA+ protein family (Erzberger & Berger, 2006). In the case of bEBPs, substrate recognition and remodelling require the action of two specific motifs inserted into the conserved AAA+ core called Loop 1 (inserted into α-helix 3) and Loop 2 (or Pre-Sensor I, inserted into α-helix 4). The substrate here is the RP_c and the specific target is the σ^{54} factor (see Contacting the σ^{54} factor).

The bEBPs are modular proteins, and in most cases, consist of three domains (Fig. 2): (1) an amino-terminal regulatory domain that controls the activity of (2) the central AAA+ ATPase and σ^{54}-interacting domain and (3) a carboxy-terminal DNA-binding domain [with a

Fig. 2. The domain organizations of the bEBPs. bEBPs are usually composed of three domains: (1) a regulatory domain 'R', which regulates the activity of, (2) the central catalytic AAA+ domain and (3) a DNA-binding domain 'D', which establishes promoter recognition specificity. Members of the family can lack the regulatory and/or the DNA-binding domain, but they all share a common AAA+ domain comprised of: a σ^{54}-interacting motif (GAFTGA) contained in Loop 1, Walker A and B motifs for ATP binding and hydrolysis, a Sensor I (SI) and Sensor II (SII) and a SRH containing the R-finger residue(s).
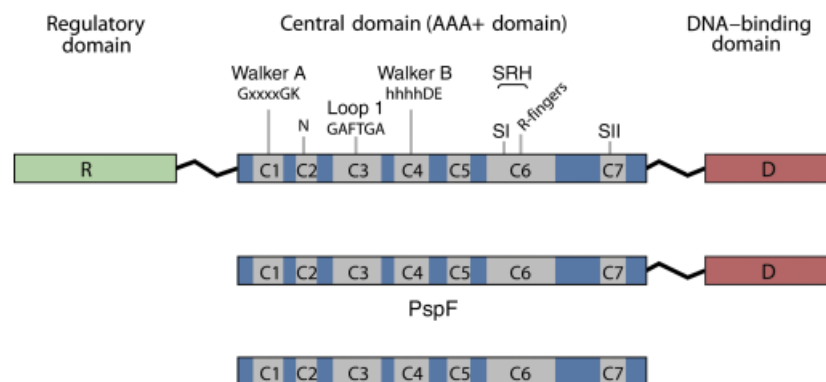
helix–turn–helix (HTH) feature] (Kustu *et al.*, 1989, 1991; Studholme & Dixon, 2003). The *cis*-acting regulatory domain is often part of a conventional two-component system (such as nitrogen regulatory protein C, NtrC; C4-dicarboxylic acid transport protein D, DctD) or belongs to another protein family (as xylene catabolism regulatory protein, XylR; nitrogen fixation protein A, NifA; zinc resistance-associated regulator, ZraR) and may be subject to modification [for instance, phosphorylation or small ligand binding (Dixon *et al.*, 1977; Shamanna & Sanderson, 1979; Wei & Kustu, 1981; Ronson *et al.*, 1987; Leonhartsberger *et al.*, 2001; Studholme & Dixon, 2003)]. The AAA+ ATPase domain is sometimes sufficient on its own (where dimerization determinants are operational in the AAA+ domain without assistance from the other domains) to catalyse ATP hydrolysis, contact and remodel σ^{54}. The activity of the AAA+ domain is often regulated by the amino-terminal regulatory domain (where present) either positively (NtrC) or negatively (DctD and NtrC1). The DNA-binding domain recognizes upstream activator sequences (UAS) within the promoter and therefore ensures the promoter recognition specificity of the activator (see Psp promoters bind PspF and use σ^{54}-RNAP) (Batchelor *et al.*, 2008, 2009; Hong *et al.*, 2009).

Commonly, bEBPs have three domains (Fig. 2); however, some can lack the regulatory domain (PspF, *Pseudomonas syringae*: HrpR and HrpS) or the HTH DNA-binding domain (*Helicobacter pylori*: FlgR; *Chlamydia trachomatis*: CtcC). In the absence of the regulatory domain, controlling the catalytic activity of the AAA+ domain usually depends on *trans*-acting factors [PspA in the case of PspF; see PspA, the negative regulator of PspF (Brissette *et al.*, 1991; Elderkin *et al.*, 2002, 2005; Joly *et al.*, 2009) and HrpV in the case of

HrpRS (Preston *et al.*, 1998)]. The bEBPs lacking an HTH domain activate the transcription of a limited number of genes that need to be expressed simultaneously (Fig. 2).

Psp promoters bind PspF and use σ^{54}-RNAP

The *psp* regulon in *E. coli* comprises seven genes: the *pspABCDE* operon at 29.44 min (expressed from the *pspA* promoter) and *pspF* (*pspF* promoter) and *pspG* at 91.84 min (*pspG* promoter, Fig. 3). The transcription from the *pspF* promoter is dependent on σ^{70}-RNAP (Jovanovic *et al.*, 1996), whereas transcription from the *pspA* and *pspG* promoters is dependent on σ^{54}-RNAP (Weiner *et al.*, 1991; Lloyd *et al.*, 2004). In contrast to the *pspA* promoter, which contains two UAS (UAS I and II, between position -80 and -126 relative to the transcription start site) (Dworkin *et al.*, 1997; Jovanovic *et al.*, 1999), the *pspG* promoter contains only one UAS (UAS II, at position -78 to -92) to which PspF specifically binds (Fig. 3) (Weiner *et al.*, 1995; Jovanovic *et al.*, 1996; Lloyd *et al.*, 2004). In addition, integration host factor (IHF)-binding sites are present between the UAS and the σ^{54}-RNAP-binding site in *pspA* (position -60 to -25) and *pspG* (position -44 to -31) promoters (Weiner *et al.*, 1995; Lloyd *et al.*, 2004). IHF binding enables (1) DNA looping, (2) the efficient binding of PspF to the UAS and (3) prevents cross-activation with heterologous σ^{54}-RNAP activator, thereby facilitating specific interactions between PspF and the RP_c to contribute to the induction of *psp* genes transcription (Fig. 3) (Weiner *et al.*, 1995; Dworkin *et al.*, 1997, 1998; Jovanovic & Model, 1997a).

Notably, UAS I and II of the *pspA* promoter partially overlap with the σ^{70}-dependent *pspF* promoter, allowing an autogenous negative regulation of *pspF* expression through
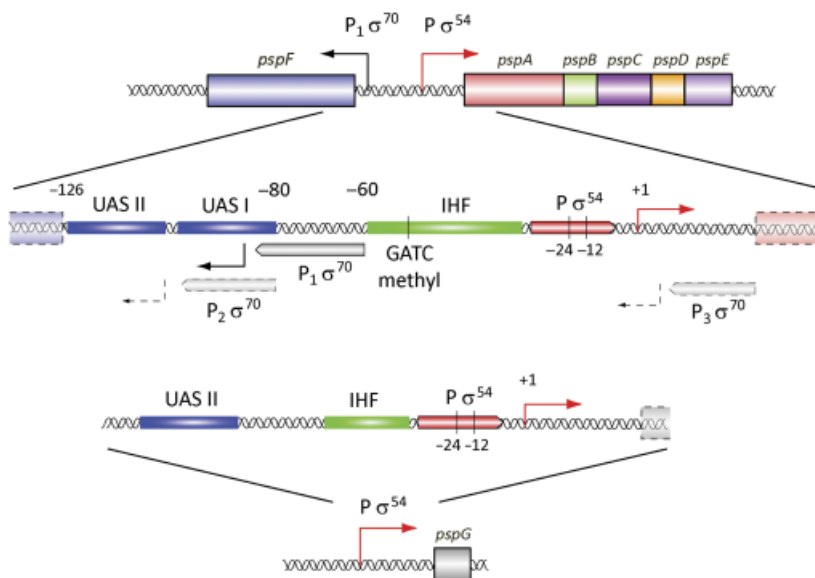

Fig. 3. The Psp regulon in *Escherichia coli*. The Psp regulon comprises seven genes. The *pspA* gene encodes a peripheral IM protein of 222 amino acids, *pspB* and *pspC* encode integral IM proteins (PspB: 74 amino acids and PspC: 119 amino acids), *pspD* encodes IM-associated PspD (73 amino acids), *pspE* encodes a periplasmic rhodanese PspE (104 amino acids), *pspF* gene encodes for bEBP PspF (325 amino acids) and *pspG* encodes an integral IM protein PspG (80 amino acids).

the binding of PspF to UAS I and II (Fig. 3) (Jovanovic *et al.*, 1997). Because of its autoregulation and its weak promoter, PspF is produced at a constantly low intracellular level. Its mRNA, however, is stabilized by repetitive reiterative IHF bacterial interspread mosaic elements positioned downstream of the *pspF* gene (Jovanovic & Model, 1997b). Strikingly, *pspF* transcription is inhibited by 6S RNA, suggesting a role of 6S RNA in limiting the extent of the Psp response under conditions of nutrient deprivation at an elevated pH (Trotochaud & Wassarman, 2006).

Maxson & Darwin (2006a) reported the existence of σ^{70}-dependent promoters in front of the *psp* operon, suggesting an additional level of transcriptional regulation complexity for the *Yersinia enterocolitica psp* operon that may function to allow constitutive basal-level expression of *psp* genes, and potentially, additional regulation. In contrast, *psp* operon transcription in *E. coli* appears to be uniquely controlled by σ^{54}-dependent promoters, further demonstrated by experiments showing that a *gIII* gene (encoding phage protein PIII) placed under the control of the *pspA* regulatory region is not transcribed when PspF activity is negatively controlled by PspA [see PspA, the negative regulator of PspF (Rakonjac *et al.*, 1997)]. Psp proteins in some bacteria and archaea are expressed independent of the σ^{54} and PspF transcription system, and many aspects of their expression and control remain to be elucidated (e.g. in *Bacillus subtilis*, the Archaea, and see Psp beyond the enterobacteria and conservation of Psp proteins).

The specific domain organization of PspF

PspF is a 325-amino-acid cytoplasmic protein composed of two functional domains: (1) an amino-terminal AAA+ domain with ATPase activity and (2) a carboxy-terminal HTH domain responsible for DNA binding. Unlike most bEBPs (Fig. 2), PspF lacks a regulatory domain (Jovanovic *et al.*, 1996), and the activity of its AAA+ ATPase domain is negatively regulated *in trans* by an additional factor PspA (see PspA–PspF and a regulated inhibitory complex) (Dworkin *et al.*, 2000; Elderkin *et al.*, 2002, 2005; Joly *et al.*, 2009).

The AAA+ domain of PspF (residues 1–275) comprises the seven bEBPs' conserved motifs C1–C7 (Morett & Segovia, 1993) (Fig. 2 and see AAA+ ATPase PspF is a bEBP) including: Walker A (residues 37–45) and Walker B (residues 103–108) motifs, and a SRH (residues 142–169; Fig. 4b and c) (Zhang *et al.*, 2002; Schumacher *et al.*, 2004, 2006; Joly *et al.*, 2008a). PspF also contains (Fig. 4b and c) a conserved aspartate (N64, acting as part of the 'glutamate switch'), Loop 1 (residues 79–94, containing the σ^{54}-interacting 'GAFTGA' motif) and Loop 2 (or Pre-Sensor I insertion, residues 131–139, proposed to facilitate Loop 1 positioning) (Bordes *et al.*, 2003; Joly *et al.*, 2007, 2008a; Zhang & Wigley, 2008; Burrows *et al.*, 2009c).

The HTH domain of PspF (residues 292–325) recognizes and binds to the UAS sites present in the *pspA* and *pspG* promoters, thereby specifically activating σ^{54}-dependent transcription from only these two promoters (Fig. 3 and see
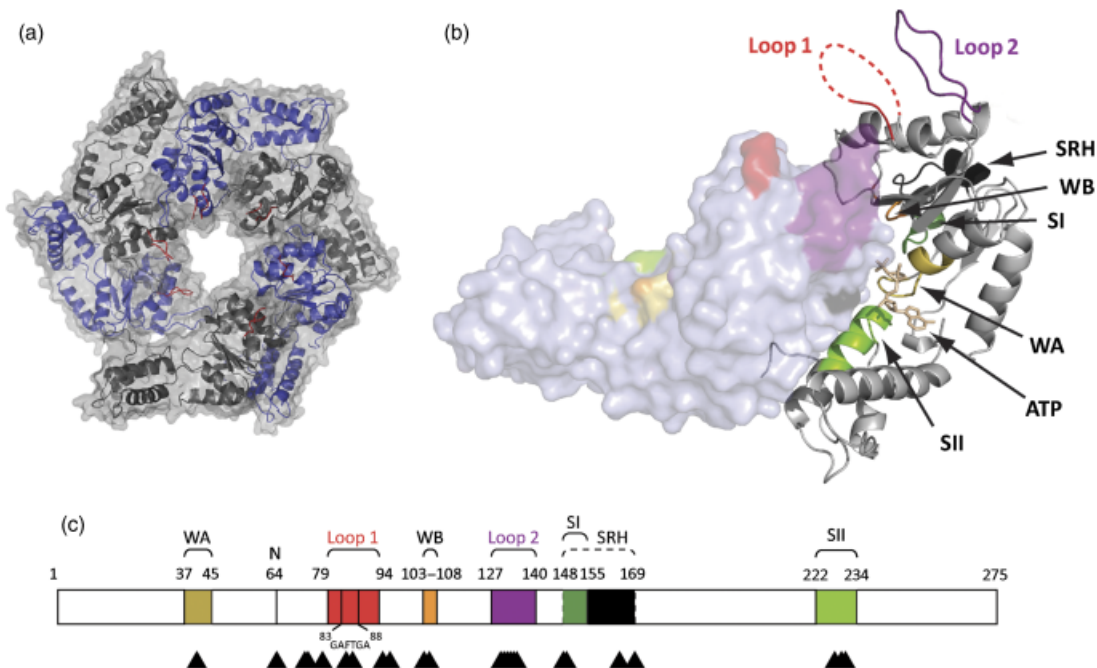

Fig. 4. PspF$_{1-275}$ structure and motif organization. (a) Model of the PspF$_{1-275}$ hexamer, (b) model of a PspF$_{1-275}$ dimer structure and (c) schematic organization of the PspF$_{1-275}$ functional motifs. WA represents Walker A, WB represents Walker B, SI represents Sensor I, SII represents Sensor II and SRH represents the Second Region of Homology. Triangles represent substitutions made in conserved motifs (see Table 1 for details).

Psp promoters bind PspF and use σ^{54}-RNAP) (Jovanovic et al., 1996, 1999; Dworkin et al., 1997; Lloyd et al., 2004).

PspF self-association

PspF oligomerization (to form a hexamer, Fig. 4a) is required for the formation of its functional ATPase catalytic sites located at the interface between adjacent subunits (Schumacher et al., 2004; Joly et al., 2006). The ATPase activity of the AAA+ domain of PspF is not linearly, but sigmoidally dependent on the protein concentration, strongly suggesting cooperation between subunits for catalytic activity. Hexamer formation by the PspF AAA+ domain is also favoured by ATP and ADP (Schumacher et al., 2004; Joly et al., 2006). The PspF self-association and assembly pathway (in terms of the number and nature of intermediates) is unknown as is the influence of the HTH domain on the self-assembly process. Substitutions in many of the conserved motifs in PspF affect the oligomerization properties of its AAA+ domain. Indeed, substitution of the Walker A residue (K42A) abolishes hexamer formation (resulting in only an apparent monomer–dimer form) while substitutions of the Walker B residues (D107A or E108A) or of the putative arginine finger residues (R162A or R168A) favour (apparently constitutive) hexamer formation, establishing an important relationship between the oligomeric state of PspF and features of its ATP-binding pocket (Table 1 and see Role of 'cis' and 'trans' residues in ATPase activity) (Schumacher et al., 2004; Joly et al., 2007).

PspF ATPase activity

Role of '*cis*' and '*trans*' residues in ATPase activity

In PspF, coordinated movements and interactions between cis- and trans-acting residues are expected to occur for and during ATPase activity. The conserved residues constituting the nucleotide-binding pocket are thought to be required for sensing the presence of ATP as well as in ATP hydrolysis (Zhang et al., 2002; Rappas et al., 2005, 2006, 2007; Schumacher et al., 2006; Joly et al., 2007, 2008a).

The conserved lysine (K42 in PspF) in the Walker A motif (Fig. 4b and c and see AAA+ ATPase PspF is a bEBP) forms ionic interactions with the oxygen of the β- and γ-phosphate. Typically, the conserved threonine (T) or serine (S) residue coordinates the metal Mg^{2+} ion; however, in PspF, this function is attributed to the glutamate E43 (Walker et al., 1982; Patel & Latterich, 1998; Ogura & Wilkinson, 2001; Zhang & Wigley, 2008). The substitution of K42 by alanine completely abolishes PspF's ATPase activity by drastically reducing ATP binding (Table 1) (Schumacher et al., 2004). Strikingly, this variant is unable to oligomerize and remains as a monomer–dimer (Schumacher et al., 2004; Joly et al., 2009).

The Walker B motif (Fig. 4b and c and see AAA+ ATPase PspF is a bEBP) provides a more direct contribution to hydrolysis. The conserved aspartate (D107 in PspF) is involved in Mg^{2+} coordination and the glutamate (E108 in PspF) behaves as a catalytic base in the activation of a water molecule, which subsequently attacks the γ-phosphate of ATP (Walker et al., 1982; Patel & Latterich, 1998; Vale, 2000; Ogura & Wilkinson, 2001; Buck et al., 2006; Erzberger & Berger, 2006; Rappas et al., 2006; Ogura et al., 2008; Zhang & Wigley, 2008). In PspF, the substitution of D107 by alanine or glutamate (D107A or D107E) favours the formation of a constitutive hexamer with drastically reduced ATPase activity, but largely unchanged ATP binding (Table 1) (Schumacher et al., 2004; Joly et al., 2007). Substitution of E108 by alanine or glutamine (E108A and E108Q) also favours the formation of a constitutive hexamer with reduced ATPase activity, but with increased ATP binding (Table 1). The E108D substitution, in contrast, has almost no effect on the oligomer formation or nucleotide binding and maintains a significantly higher ATPase activity compared with A or Q substitutions, suggesting that the charge of the 108 residue is important for activity (Joly et al., 2007). Similar properties were observed for Walker B substitution in NtrC (Rombel et al., 1999).

The asparagine residue (N64), first reported in PspF, contributes to the ATPase activity and appears to sense, and relay to the helix 3 containing the σ^{54}-interacting Loop 1, the presence of ATP in the nucleotide-binding pocket (Figs 4c and 5b) (Joly et al., 2008a). This key residue is also proposed to be the (final) allosteric target of the negative regulator (of PspF activity – PspA) (Fig. 5b and see PspA, the negative regulator of PspF) (Joly et al., 2008a).

The 'arginine finger' motif (or R-finger, Fig. 4c) has been reported in several AAA+ ATPases to have a direct involvement in ATPase activity and to act from the adjacent subunit to that which binds ATP (Greenleaf et al., 2008; Ogura et al., 2008; Augustin et al., 2009). This R-finger has been proposed to participate in the catalysis of ATP hydrolysis by stabilizing the accumulating negative charge of the ATP transition state. In PspF, the putative R-finger motif is thought to be composed of two arginine residues R162 and R168, but their precise roles in ATP hydrolysis remain unclear (Table 1) (Schumacher et al., 2006).

The 'Sensor I' motif in AAA+ proteins (Fig. 4b and c) is commonly a conserved polar residue (T148 and N149 in PspF). Its side chain is thought to contact the γ-phosphate of ATP during binding and hydrolysis, allowing the distinction between bound ATP and ADP (Table 1) (Lupas & Martin, 2002).

The 'Sensor II' motif (Fig. 4b and c) that is present in many AAA+ proteins (and is one of the factors differentiating the AAA and from the AAA+ families) comprises a conserved arginine (R227), which is thought to contact the γ-phosphate

Table 1. PspF substitutions and associated activities

| Residues	Substitutions	Major phenotypes	References
Walker A			
Lys[42]	K42A	Reduced nucleotide binding – no hexamer (dimer) – no activities	Schumacher *et al.* (2004)
PspA-negative regulation motif			
Trp[56]	W56A	Activities as WT but no binding of PspA	Elderkin *et al.* (2005)
Glutamate 'switch'			
Asn[64]	N64A	Increased nucleotide binding – reduced ATP hydrolysis – slightly reduced OC formation – binding of PspA, but no negative regulation of ATPase	Joly *et al.* (2008a)
	N64D	No hexamer – no activities	Joly *et al.* (2008a)
	N64Q	Increased nucleotide binding – reduced ATP hydrolysis – reduced OC formation – binding of PspA, but no negative regulation of ATPase	Joly *et al.* (2008a)
	N64S	WT like ATP hydrolysis – constitutive hexamer – slightly reduced OC formation – binding of PspA, but no negative regulation of ATPase	Joly *et al.* (2008a)
GAFTGA			
Phe[85]	F85A	Reduced ATP hydrolysis – no σ^{54} interaction – no OC formation	Zhang *et al.* (2009)
	F85Y	Reduced ATP hydrolysis – σ^{54} interaction – reduced OC formation	Zhang *et al.* (2009)
	F85W,H,I	WT like ATP hydrolysis – no σ^{54} interaction – no OC formation	Zhang *et al.* (2009)
	F85C,E,L,Q,R	Reduced ATP hydrolysis – no σ^{54} interaction – no OC formation	Zhang *et al.* (2009)
Thr[86]	T86A	WT like ATP hydrolysis – no σ^{54} interaction – no OC formation	Bordes *et al.* (2003)
	T86S	WT like ATP hydrolysis – σ^{54} interaction – no OC formation, but rescued by mutants of σ^{54} region I (G4L)	Dago *et al.* (2007)
Walker B			
Asp[107]	D107A	Decreased ATP hydrolysis – constitutive hexamer – σ^{54} interaction – reduced OC formation	Schumacher *et al.* (2004), Joly *et al.* (2007)
	D107E	Decreased ATP hydrolysis – increased ATP binding – σ^{54} interaction – constitutive hexamer – reduced OC formation	Joly *et al.* (2007)
Glu[108]	E108A	Decreased ATP hydrolysis – increased ATP binding – constitutive hexamer – stable σ^{54} interaction in the presence of ATP – reduced OC formation	Joly *et al.* (2007)
	E108D	Decreased ATP hydrolysis – increased ATP binding – stable σ^{54} interaction in the presence of ATP – reduced OC formation	Joly *et al.* (2007)
	E108Q	Decreased ATP hydrolysis – increased ATP binding – constitutive hexamer – stable σ^{54} interaction in the presence of ATP – reduced OC formation	Joly *et al.* (2007)
R-finger			
Arg[162]	R162A	Decreased hydrolysis – decreased ATP binding – no OC formation	Schumacher *et al.* (2004)
Arg[168]	R168A	Decreased hydrolysis – decreased ATP binding – constitutive hexamer – no OC formation	Schumacher *et al.* (2004)
Sensor I			
Thr[148]	T148A	Slightly reduced ATP hydrolysis – σ^{54} interaction – no OC formation	Schumacher *et al.* (2007)
Asn[149]	N149A, S	Slightly reduced hydrolysis – σ^{54} interaction – reduced OC formation	Schumacher *et al.* (2007)
Loop 2			
Arg[131]	R131A	Reduced ATP hydrolysis – σ^{54} interaction – no OC formation	Burrows *et al.* (2009c), Rappas *et al.* (2006)
Val[132]	V132A	Reduced ATP hydrolysis – σ^{54} interaction – no OC formation	Burrows *et al.* (2009c)
Gly[133]	G133A	Reduced ATP hydrolysis – no OC formation	Burrows *et al.* (2009c)
Ser[135]	S135A	Reduced ATP hydrolysis – no OC formation	Burrows *et al.* (2009c)
Gln[136]	Q136A	Reduced ATP hydrolysis – σ^{54} interaction – reduced OC formation	Burrows *et al.* (2009c)
Pro[137]	P137A	Reduced ATP hydrolysis – σ^{54} interaction – reduced OC formation	Burrows *et al.* (2009c)
Pro[137]	P137T	Increased ATP hydrolysis – σ^{54} interaction – reduced OC formation	Burrows *et al.* (2009c)
Leu[138]	L138A	Reduced ATP hydrolysis – no OC formation	Burrows *et al.* (2009c)
Gln[139]	Q139A	Reduced ATP hydrolysis – no OC formation	Burrows *et al.* (2009c)
Sensor II			
Arg[227]	R227A	Reduced ATP hydrolysis – no OC formation	Rappas *et al.* (2006)
Lys[230]	K230A	Reduced ATP hydrolysis – no OC formation	Rappas *et al.* (2006)
Asn[231]	N231A	Reduced ATP hydrolysis – no OC formation	Rappas *et al.* (2006)
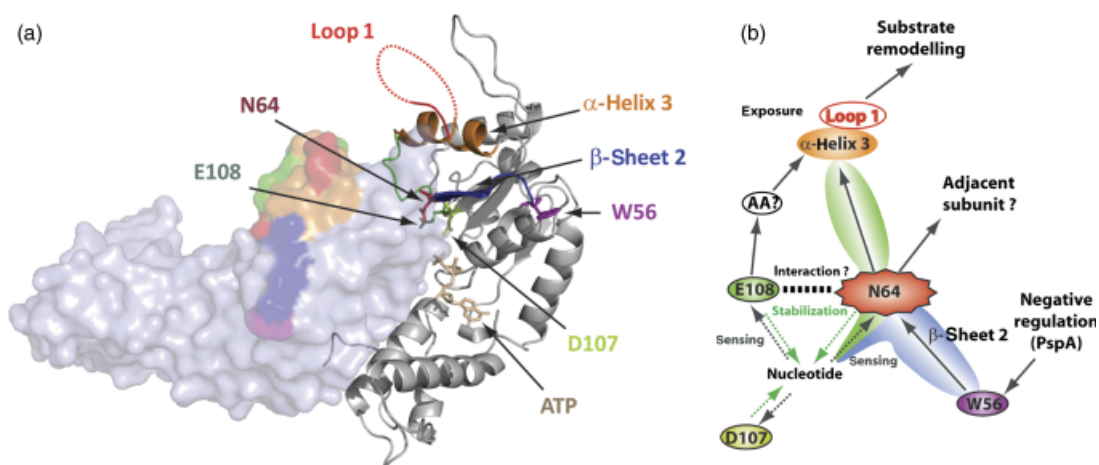
Fig. 5. Intramolecular pathway for PspF-positive and -negative control. (a) Structural model and (b) schematic representation of two pathways regulating PspF activity. PspA-negative regulation is proposed to occur by the sensing of PspA binding to PspF residue W56, which will relay the presence of bound PspA via the β-sheet 2 (in blue) to the N64 residue (in red). The side chain orientation of N64 is proposed to be changed, resulting in the decrease of ATPase activity. In contrast, the N64 residue is proposed to be involved in the signal coupling between ATP and exposure of the Loop 1 by sensing directly the presence of bound ATP and relay the signal via the linker 1 (in green) to the α-helix 3 (in orange), resulting in the exposure of Loop 1 (in red). For both pathways, the key residue is N64.

of ATP and to translate changes occurring in the ATP-binding pocket into conformational 'movement' between the α/β and the α subdomains further amplified at the level of the hexamer (Table 1) (Ogura & Wilkinson, 2001).

Different types of ATPase cycles

The activity of AAA+ proteins is directly linked to their ability to hydrolyse ATP once oligomerized. For PspF, its ATPase activity is inhibited by its cognate negative regulator PspA. The nature of the ATP hydrolysis cycle used by particular AAA+ ATPases is of prime importance in understanding the regulation of their activity and how the mechanochemical energy is used to remodel their various substrates. Certain remodelling substrates may benefit from a particular type of ATPase cycle and so it is crucial to understand which ATP hydrolysis cycle is occurring in a particular AAA+ ATPase. One of the more general unresolved questions is whether and how nucleotide binding and hydrolysis is coordinated within hexameric AAA+ ring structures for their biological output. The precise contribution of each step of the ATP hydrolysis cycle and the number of ATP molecules needed for the formation of RP_o by σ^{54}-RNAP is unclear, although as indicated below, some insights into the type of ATPase cycle PspF uses are emerging.

Evidence for four different types of ATPase cycles (Fig. 6) has been reported for AAA+ ATPases: stochastic [e.g. for ClpX (Martin et al., 2005)], synchronized or concerted [e.g. for SV40-LTag (Gai et al., 2004)], rotational [e.g. for F1-ATPase (Boyer, 1997)] and sequential [e.g. for P4 packaging ATPase (Mancini et al., 2004)]. Founded on studies of the

nucleotide occupancy, two basic models may serve to distinguish how AAA+ molecular machines function (Ades, 2006). The homogeneous nucleotide occupancy observed in a large number of AAA+ crystal structures (Lenzen et al., 1998; Zhang et al., 2000; Gai et al., 2004) suggests a concerted ATP hydrolysis cycle, where all subunits hydrolyse ATP simultaneously. Other structures showed substoichiometric and mixed nucleotide occupancy within the hexameric ring (Bochtler et al., 2000; Wang et al., 2001), supporting either a sequential or a rotational hydrolysis mechanism where heterogeneous nucleotide occupancy is coordinated between subunits.

In the case of PspF, it has been shown that the hexamer is simultaneously occupied by ATP and ADP, a finding inconsistent with the synchronized model in which all subunits of the hexamer are simultaneously occupied by the same form of nucleotide (Joly et al., 2006). In addition, the stochastic model is not supported because it appears that some of the nucleotide-binding sites are not independent, given the observation that ADP or an excess of ATP, either stimulated or inhibited, respectively, the ATPase activity of the hexamer. Subsequently, it would seem that the PspF ATPase cycle is either rotational or sequential (Fig. 6c and d) (Joly et al., 2006).

Contacting the σ^{54} factor

'GAFTGA' motif of PspF

The interaction between the RP_c (see Bacterial transcription) and its cognate bEBP (see AAA+ ATPase PspF is a bEBP) occurs via the direct contact between the consensus GAFTGA sequence and region I of σ^{54}. The 'GAFTGA
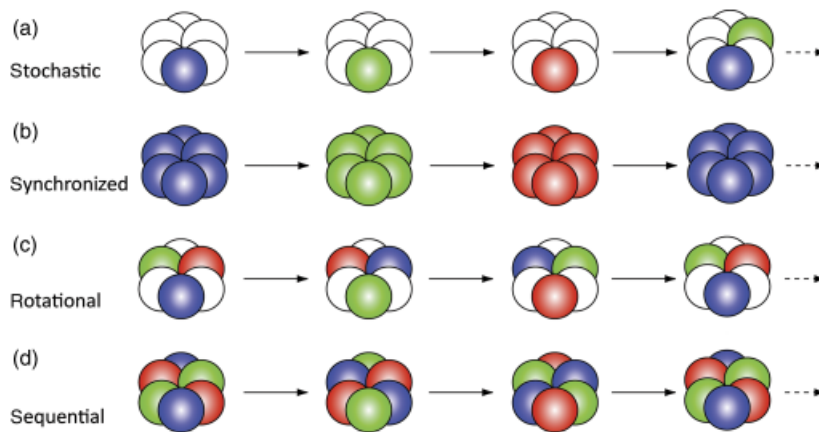
Fig. 6. ATP hydrolysis cycles. Four different models are presented for the cycle of ATP hydrolysis in AAA+ proteins. (a) Stochastic model, where all the subunits are independent for ATP hydrolysis, (b) synchronized, all the subunits are active and occupied by the same nucleotide form at the same time, (c) rotational, only three subunits are active and always occupied by a different nucleotide form at the same time and (d) sequential, all the subunits are active and opposite subunits are occupied by the same nucleotide form at the same time. No colour represents an inactive subunit, blue represents a nucleotide-free subunit form, green represents an ATP-bound subunit form and red represents an ADP-bound form.

motif' is located within Loop 1 and presented to the surface of bEBP hexamers (Figs 2, 4 and 5) (Rappas *et al.*, 2005; De Carlo *et al.*, 2006). The threonine in this GAFTGA motif is crucial for maintaining a productive interaction with σ⁵⁴ as its substitution by alanine (T86A in the case of PspF) results in the loss of σ⁵⁴ interaction (Bordes *et al.*, 2003; Lee *et al.*, 2003; Sallai & Tucker, 2005; De Carlo *et al.*, 2006). Interestingly, the T86S variant promotes tighter σ⁵⁴-binding interactions, but reduces the transcription activation activity (Bordes *et al.*, 2003; Dago *et al.*, 2007). It seems that the threonine of GAFTGA is involved in a direct interaction with σ⁵⁴ as well as in RP$_c$ activation. The highly conserved phenylalanine (of the GAFTGA motif) is also important. Substitution of F85 (in PspF) with tyrosine (the most frequent naturally occurring substitution, e.g. in XylR) still allows PspF to interact with σ⁵⁴, but largely, in a DNA conformation-dependent manner, fails to activate transcription efficiently (Zhang *et al.*, 2009). These examples highlight the importance of the GAFTGA motif. It appears that more than a simple binding interaction between PspF and σ⁵⁴ is required for transcription activation. A second step (after the initial interaction) involving the GAFTGA motif and potentially interactions with promoter DNA is possible, especially given the results of PspF to DNA proximity studies (Bose *et al.*, 2008).

Regulation of σ⁵⁴ contact

Nucleotide-associated activities

Specific nucleotide-bound states of PspF are expected to be directly associated with different stages of its engagement with the RP$_c$ as well as the subsequent transcription inter-

mediates formed along the pathway to the RP$_o$ (Fig. 1 and see Bacterial transcription). The current model describing the nucleotide-dependent regulation of PspF activity is as follows: in the ATP-bound state, the GAFTGA σ⁵⁴-interacting motif contained in Loop 1 is exposed (Fig. 4b). This loop 1 exposure is mediated by sensing of the nucleotide-bound state via residue E108, which will position the γ-phosphate of the ATP in close proximity to asparagine 64 (N64) (Joly *et al.*, 2007, 2008a). N64 will 'sense' the presence of ATP and relay this information to Loop 1 located within helix 3 via the linker 1 (Rappas *et al.*, 2006; Joly *et al.*, 2008a). The relocation event is proposed to be aided by Loop 2, but precise Loop 2 contributions remain unclear (Fig. 4b) (Burrows *et al.*, 2009c). In the ADP-bound state, the side chain of the residue E108 rotates about 90°, the γ-phosphate position of the ATP is not occupied anymore, the N64 side chain is relaxed and the Loop 1 reverts to a buried conformation, releasing the interaction with σ⁵⁴ (Rappas *et al.*, 2006). To date, the precise mechanism by which these two states (ATP- vs. ADP-bound) interconvert is unknown. The buried conformation associated with the ADP-bound state has been observed for NtrC1 (Lee *et al.*, 2003) and a general glutamate switch mechanism has been proposed for AAA+ proteins (Zhang & Wigley, 2008).

Use of nucleotide analogues

To identify, monitor and study nucleotide-bound states of PspF, several nucleotides and their analogues are currently being used.

ATP – (see Different types of ATPase cycles and Regulation of σ⁵⁴ contact) The PspF-ATP-bound state is proposed to be responsible for contacting the RP$_c$. This is supported

by structural data obtained on NtrC1-ATP (Chen *et al.*, 2007) and on PspF-ATP obtained by soaking the crystal with ATP (Rappas *et al.*, 2006), where the conformation of Loop 1 leads to its more exposed state (than is observed in the ADP-bound state). Functional data support this mode of engagement with RP_c, in particular, the observation of a stable complex between PspF E108 variants (each with very low ATPase activity) and σ^{54} in the presence of ATP.

AMPPNP – A ground-state nonhydrolysable ATP analogue. With NtrC1, it supports the formation of a stable complex with σ^{54} (Chen *et al.*, 2007). This stable complex is not observed with PspF, probably because the affinity of PspF for AMPPNP is too low (Joly *et al.*, 2006). Soaking apo-PspF$_{1-275}$ crystals with AMPPNP-induced side chain orientations similar to those observed in the ATP-bound formed (Rappas *et al.*, 2006).

ADP-BeF$_x$ – A ground-state analogue formed *in situ* in the nucleotide-binding pocket from ADP, NaF and BeCl$_2$. This nonhydrolysable nucleotide analogue supports the formation of a stable complex between NtrC1 or PspF and σ^{54} (Chen *et al.*, 2007; Burrows *et al.*, 2009b).

AMP-AlF$_x$ – A ground-state analogue formed *in situ* in the nucleotide-binding pocket from AMP, NaF and AlCl$_3$. This nonhydrolysable nucleotide analogue allows the formation of a stable complex between PspF and σ^{54} (Joly *et al.*, 2008b; Burrows *et al.*, 2009b). Interestingly, structural data suggest that this predicted ADP analogue is a true ATP analogue with the γ-phosphate position in the nucleotide-binding pocket occupied by the AlF moiety. Repositioning of the phosphate group of the AMP from the α to the β position (corresponding to the position occupied by the phosphate in the ATP-bound state) is observed (Joly *et al.*, 2008b).

ATP[γS] – A slowly hydrolysed ATP analogue. Recent studies with PspF showed that slowing down the rate of ATP hydrolysis using ATP[γS] allows the identification of transcriptional intermediate complexes (Burrows *et al.*, 2009a).

ADP-AlF$_x$ – An ATP hydrolysis transition state formed *in situ* in the nucleotide-binding pocket from ADP, NaF and AlCl$_3$. This nonhydrolysable nucleotide analogue allows the formation of a stable complex between NtrC1 or PspF and σ^{54} (Chaney *et al.*, 2001; Chen *et al.*, 2007; Burrows *et al.*, 2009b). Using this nucleotide analogue, Rappas *et al.* (2005) reported the first structural information regarding the complex formed between a bEBP and σ^{54} using cryo-electron microscopy (cryoEM). Recently, new structural information between PspF and Eσ^{54} has been obtained (Bose *et al.*, 2008).

ADP – +Pi, a product of ATP hydrolysis. A low ADP concentration stimulates ATPase activity (see Different types of ATPase cycles) (Joly *et al.*, 2006). When PspF is bound to ADP, the GAFTGA motif is proposed to be in a buried conformation [as observed in NtrC1-ADP (Chen *et al.*,

2007)], which does not support σ^{54} interaction (Rappas *et al.*, 2006).

From structural studies of metal fluoride analogues with ADP, the BeF moieties are usually in a tetrahedral conformation (and hence are ATP ground-state analogues), while the AlF moieties can adopt a planar conformation (transition-state analogues). Nevertheless, the geometry adopted by the fluorides also determines the nucleotide-bound state mimicked: trigonal ($x = 3$, ATP transition state) or quadrangular ($x = 4$, ATP ground state). Both have been reported, but in the case of PspF, the structural information available concerning these moieties does not allow a strict assignment ($x = 3$ or 4) (Chen *et al.*, 2007; Joly *et al.*, 2008b).

PspA, the negative regulator of PspF

For many bEBPs, the activity of the catalytic AAA+ ATPase domain is regulated by an *in cis* regulatory domain. PspF lacks such a regulatory domain and is regulated *in trans* by PspA (note that *E. coli* strains lacking *pspA* have a constitutively active PspF), which binds to and controls the activity of the PspF AAA+ ATPase domain, providing the first studied example of an *in trans* regulated bEBP (Figs 2, 7 and 5). PspA strongly inhibits PspF ATPase activity, but whether this is sufficient to fully account for the loss of RP_o formation is not yet established (Fig. 5).

PspA predicted structure and domain organization

PspA is a peripheral IM-associated protein comprised of 222 amino acids (in *E. coli*). PspA is involved in two different aspects of the Psp response: negative regulation of PspF and an effector function of the Psp response (see Generation of the Psp system-inducing signal). *In silico* analysis predicts that PspA forms a helical coiled-coil structure comprising four helical domains (HD1–HD4) (Dworkin *et al.*, 2000; Elderkin *et al.*, 2005; Joly *et al.*, 2009). Prokaryotic coiled-coil protein clusters typically comprise membrane-bound proteins and signal transducers, as well as membrane-spanning transporters and secretion proteins (Liu & Rost, 2001). Clusters specific to prokaryotes include for example the methyl-accepting chemotaxis proteins. Low-resolution structural studies suggest that *in vitro*, PspA can form a high-order oligomer described as a 36-mer ring or a clathrin-like structure (Hankamer *et al.*, 2004; Standar *et al.*, 2008; Joly *et al.*, 2009). In both cases, PspA forms a complex with an apparent molecular weight of about 2 MDa (Hankamer *et al.*, 2004; Kobayashi *et al.*, 2007; Standar *et al.*, 2008). A protein fragmentation approach, combined with *in vitro* studies, demonstrated that each of the putative helical domains of PspA plays a role in the formation of the 36-mer. Where HD2–HD3 supports the formation of an apparent hexamer, the HD1–HD2–HD3 fragment, in

contrast, forms a dimer, implying that HD1 negatively affects HD2–HD3 oligomerization. The presence of HD4 (HD1–HD2–HD3–HD4; full-length PspA) results in the formation of the 36-mer, suggesting that HD4 counteracts the effect of HD1 (Elderkin *et al.*, 2005; Joly *et al.*, 2009).

PspA–PspF and a regulated inhibitory complex

Complex composition

The mechanism by which PspA negatively regulates the PspF ATPase activity by directly binding to it is rather complex. Using PspA fragments, Joly *et al.* (2009) have shown that binding of various HD1 combinations (e.g. full-length PspA, HD1–HD2–HD3, HD1–HD2) to PspF results in different degrees of ATPase inhibition. In addition, HD4 appears to be dispensable for PspF negative regulation, but is required for a cooperative inhibition of PspF ATPase activity in the context of full-length PspA. Taken together, these results demonstrate that a functional interaction between PspF and PspA involves several different interacting PspA HDs (see PspA predicted structure and domain organization). Alanine substitution of the surface-exposed trypto-phan residue at position 56 of PspF abolishes the interaction between PspA and PspF, resulting in unregulated PspF activity with little (if any) other consequences on PspF activity (*in vivo* and *in vitro*) (Elderkin *et al.*, 2005). W56 is clearly a specific binding determinant for PspA and may be a direct contact site (Fig. 5).

Nucleotides (ATP, ADP) do not antagonize or reinforce PspA's action on PspF, suggesting that a signal other than the ATP/ADP ratio (as a measure of energy charge) exists to allow the regulated release of the negative regulation of PspF (Joly *et al.*, 2009). Interestingly, the complex formed between PspF and PspA can also accommodate the nucleotide-dependent binding of σ^{54}. Hence, a ternary PspA–PspF–RP$_c$ complex might exist, in which PspF is preloaded with ATP (set for ATP hydrolysis). This 'loaded complex' might facilitate transcription activation and thus a fast response upon the release of PspA-negative regulation triggered by extracytoplasmic cues associated with impairment of the integrity of the IM (see Signalling pathways for stress perception).

Release of the PspA–PspF inhibitory complex

The release of the PspA–PspF inhibitory complex is a key regulatory point for induction of the Psp response under stress conditions. Recently, significant progress has been made in understanding how PspF-negative regulation imposed by PspA is established and then might be released. Direct biochemical experiments indicate that the inhibited PspA–PspF complex likely contains six PspF and six PspA subunits (Joly *et al.*, 2009). Thus, potentially an oligomeric

form of PspA directly associated with and responsible for PspF regulation is not a 36-mer, but a 6-mer (Fig. 7). Whether PspA exists as a hexamer when bound to PspF remains to be shown (potentially through electron micro-scopy studies). Kobayashi *et al.* (2007) provided evidence that only the higher-oligomeric form of PspA can block proton leakage from liposomes and vesicles. This suggests that a switching mechanism exists between the PspA-nega-tive regulator (6-mer) and effector (36-mer) functions based on changes in the oligomerization state. Further evidence for such a mechanism arises from *in vivo* data on the number of PspA molecules in complexes. Subcellular locali-zation studies identified two classes of PspA complexes: lower- and higher-order oligomers (Engl *et al.*, 2009). Notably, under pIV-dependent induction of *psp* expression, significant negative regulation of the Psp system is still present (Engl *et al.*, 2009; Jovanovic *et al.*, 2009), suggesting that inhibitory PspA–PspF regulatory complexes and PspA effector complexes can coexist.

The pivotal event in releasing negative regulation that PspA imposes on PspF is believed to be the signalling of stress to PspA (and see Signalling pathways for stress perception). Although in principle PspF could itself be a direct target for the relief of negative control, no evidence exists to support such a mechanism, and the absence of a defined regulatory domain in PspF is taken as evidence against such a mechanism. The complexity of signalling to PspA upon stress depends on the particular stimulus and can be conditionally dependent on the growth conditions (see also Signalling pathways for stress perception). Induc-tion of *psp* by ethanol or osmotic shock partially requires the PspB and PspC proteins (Weiner *et al.*, 1991), positive regulators generally accepted as having a sensory function (reviewed in Model *et al.*, 1997; Darwin, 2005b). The most extensively used and the best-characterized *psp*-inducing stimulus in *E. coli* is the prolonged production of a secretin (e.g. pIV, PulD, YscC). Psp induction by pIV (and other secretins) strictly requires the action of both PspB and PspC sensors under aerobic and microaerobic growth (Weiner *et al.*, 1991; Jovanovic *et al.*, 2009). While extreme heat, ethanol and osmotic shocks cause transient *psp* induction, production of the pIV secretin leads to continual induction (Brissette *et al.*, 1990). PspA synthesis is detected 4 min after the production of pIV and the maximal rate reached by 10 min, well ahead of the steady-state level of pIV produc-tion (30 min) (Brissette *et al.*, 1990). Loss of pIV production and decay of the inducing signal is reflected by a decline in PspA synthesis within a few minutes and is more rapid than could be accounted for by dilution (Brissette *et al.*, 1990). The signal originating either due to extreme heat shock (48–50 °C) in aerobiosis (Weiner *et al.*, 1991) or due to pIV secretin stress in anaerobiosis (Jovanovic *et al.*, 2009) may well be recognized directly by PspA (see also Signalling
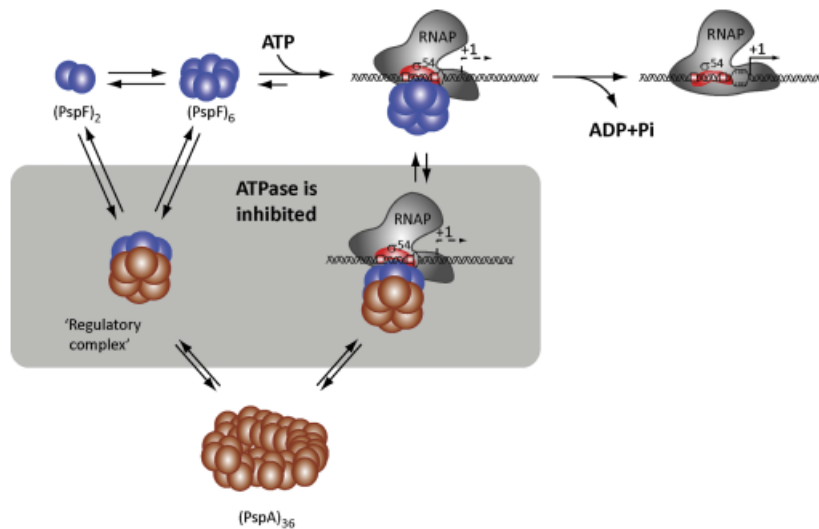
Fig. 7. PspF-negative regulation by PspA. Under nonstressful growth conditions, PspF ATPase activity is negatively regulated via the direct interaction between PspF and PspA. Biochemical experiments have shown that the PspA–PspF regulatory complex is composed of about six PspA and six PspF subunits, whereas the purified PspA forms a 36-mer complex. The interaction between PspF and PspA does not abolish the interaction between PspF and the RP$_c$.

pathways for stress perception) since PspBC are not required for Psp response induction under such conditions.

PspBC–A interaction

PspB and PspC are membrane-bound proteins that contain putative leucine zipper motifs (Fig. 8a). To date, no evidence of post-translational modifications of these proteins before or upon stress has been reported. Therefore, it has been proposed that PspB, PspC and PspA protein–protein inter- actions underpin the mode of signal transduction from PspBC sensors to PspA (Adams *et al.*, 2003). Indeed, PspA–PspC, PspB–PspC and PspB–PspA (in the presence of PspC) pairwise interactions have been demonstrated (Fig. 8c) (Adams *et al.*, 2003; Maxson & Darwin, 2006b). Overproduction of PspC induces *psp* expression and co- overproduction of PspB and PspC facilitates this, effectively as a nonstress growth condition (see Signalling pathways for stress perception and Table 2) (Weiner *et al.*, 1991; Maxson & Darwin, 2006b). The action of PspC as a PspA-binding protein suggests a direct effect of the PspBC sensors on PspA, leading to the release of negative regulation. For negative regulation, PspA may remain bound at the IM or may be released into the cytoplasm to interact with PspF. Determining where the inhibited state PspF–PspA complex forms and persists is a challenge. Control of the PspF–PspA negative regulatory interaction may be achieved through the formation of a large inclusive complex containing PspF, PspA, PspB and PspC or a series of subcomplexes (including e.g. PspBC and PspF–PspA) to achieve signal transduction to PspA and the release of negative regulation. The cellular localization where such signal transduction complexes are formed and their precise compositions are for the most part unknown. Whether or not multivalent interactions are possible between PspF, PspA, PspB and PspC is not yet

known. Clearly, under conditions where Psp response in- duction requires PspBC (e.g. with pIV, aerobically and microaerobically; see Signalling pathways for stress percep- tion), communicating the Psp inducing signal to PspF–PspA is likely to require a PspA–PspBC interaction at some stage at the IM, but only if there is no cytoplasmic factor signalling between PspBC and PspA.

Signalling pathways for stress perception

PspBC-dependent signal transduction

Gueguen *et al.* (2009) showed that for *Y. enterocolitica*, the presence of PspB increases the level of PspC. Some variant forms of PspB and PspC constitutively activate *psp* expres- sion in the absence of stress, suggesting that the predicted transmembrane and cytoplasmic domains are important for sustaining the negative regulation of PspF. In addition, they showed that the PspC periplasmic domain, specifically the leucine zipper motif, is implicated in the activation of *psp* expression. They proposed that this part of PspC is involved in signal detection, supporting the model of Adams *et al.* (2003). However, PspC variants that constitutively activate *psp* expression still required the positive function of the periplasmic domain. An alternative model for signal detec- tion and activation of *psp* expression directly involving the periplasmic domain of PspC in signal transduction to PspA overcomes the issue of a necessary positive role for the periplasmic domain.

Jovanovic *et al.* (2006) have proposed that ArcB, the sensor kinase of the two-component ArcAB system [a master regulator controlling the switch from aerobic to anaerobic respiration and fermentation (Malpica *et al.*, 2006)], is required for full pIV-dependent *psp* expression in *E. coli* and that *psp* expression-inducing stresses could
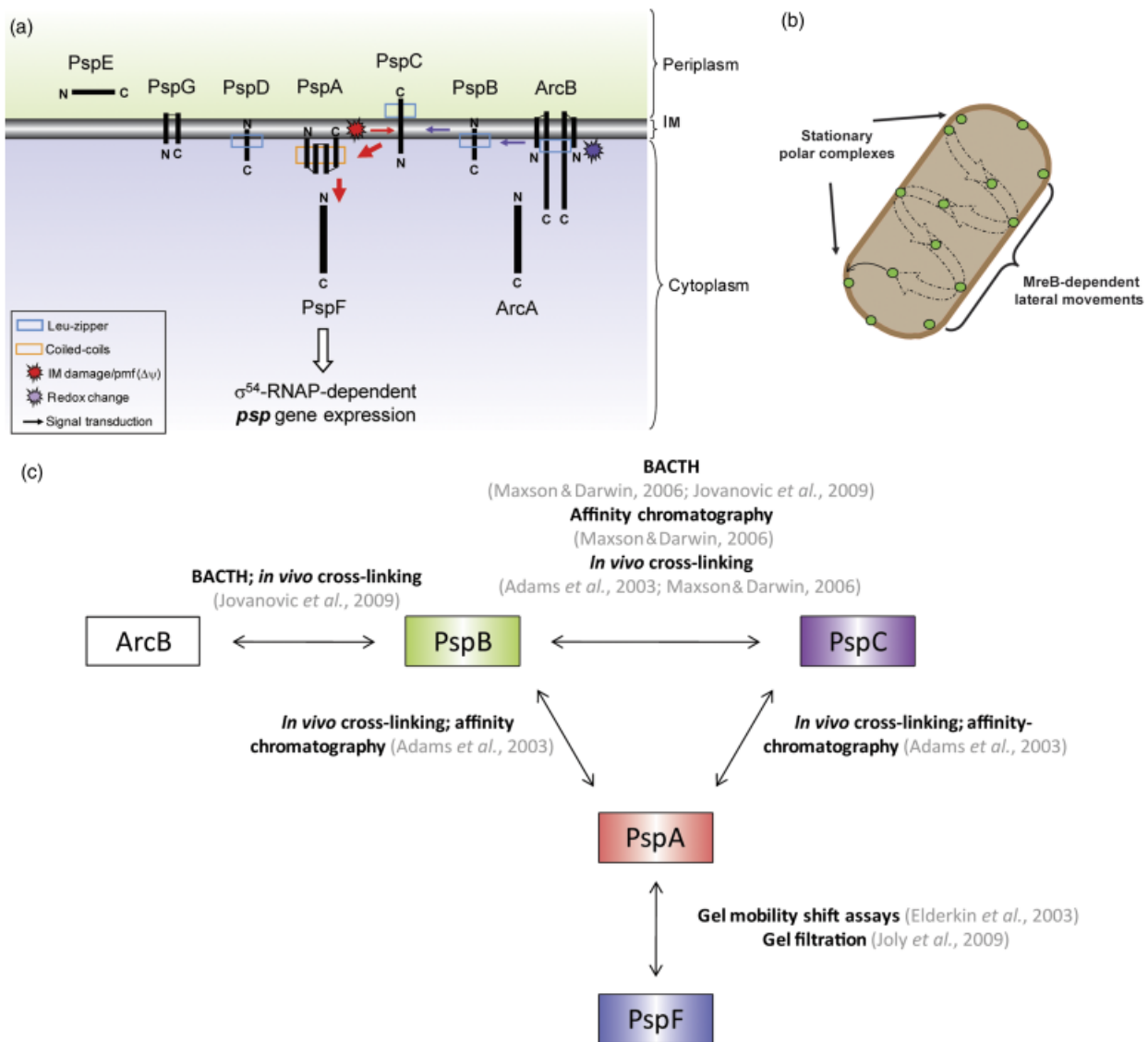
Fig. 8. Localization, topologies and interplay of Psp proteins. (a) Schematic representation of Psp (reviewed in Model *et al.*, 1997; Darwin, 2005b) and Arc (reviewed in Malpica *et al.*, 2006) protein topology and interplay in signal transduction. N, N-terminus; C, C-terminus; purple arrows, redox-dependent signal transduction; red arrows, IM damage- and/or pmf (Δψ)-dependent signal transduction; thick red arrows, release of the PspA–PspF inhibitory complex. (b) Subcellular localizations of PspA and PspG. PspA and PspG localize into distinct classes, stationary polar clusters (potentially involved in the regulation of *psp* expression) and mobile complexes displaying MreB-dependent movements along the length of the cell required for PMF maintenance (Engl *et al.*, 2009). (c) To date, pairwise binary interactions between PspA–PspF, PspA–PspB, PspA–PspC, PspB–PspC as well as PspB–ArcB have been observed using bacterial two-hybrid (BACTH), *in vivo* cross-linking, affinity chromatography and gel filtration analysis. Whether these interactions occur within a Psp multiprotein complex remains to be demonstrated.

initially activate ArcB. However recently, Seo *et al.* (2007) have reported ArcB-independent induction of *psp* expression in both *E. coli* and *Y. enterocolitica*. Such a discrepancy might be explained by the differences in the growth conditions used in the two experimental settings. Recent data now indicate that Arc involvement is indeed conditional, thereby reconciling both observations (Jovanovic *et al.*, 2006, 2009; Seo *et al.*, 2007). ArcAB appears to have a major involvement in signal propagation for induction of the Psp

response under microaerobic growth, but not when cells are grown aerobically or anaerobically (Fig. 9). The results suggest two ways to transduce the Psp response signal via the Arc system under microaerobic growth: (1) activated ArcB itself increases *psp* expression and (2) the phosphorelay from ArcB to ArcA seems to be important for signal amplification. Bacterial two-hybrid protein interaction results indicate that the cross-talk between the ArcAB and the Psp system may be mediated via a direct protein–protein

Table 2. *psp*-inducing conditions

Conditions	Organism	Target/effect	References
Filamentous phage infection (f1, Ike, Pf3)	*E. coli*	Energy generation	Brissette *et al.* (1990), Russel & Kazmierczak (1993)
Lytic phage infection (PRD1)	*E. coli*	Energy generation	Poranen *et al.* (2006)
Secretin production (pIV, PulD, YscC, OutD)	*E. coli* S. typhimurium K. oxytoca Y. enterocolitica	Energy generation	Darwin & Miller (2001), Gao & Xu (2009), Hardie *et al.* (1996b), Lloyd *et al.* (2004), Possot *et al.* (1992), Russel & Kazmierczak (1993)
Late stationary growth phase at pH9, ageing cells (*rpoS* mutant facilitates induction)	*E. coli*	Cell envelope; energy generation	Weiner & Model (1994), Saint-Ruf *et al.* (2004)
Stationary growth phase (*rpoE* mutant facilitates induction)	*S. typhimurium*	Cell envelope; energy generation	Becker *et al.* (2005)
Alkaline shock (pH 9)	*B. subtilis* S. lividans	Energy generation	Vrancken *et al.* (2008), Wiegert *et al.* (2001)
Media downshifts	*E. coli*	Energy generation	Model *et al.* (1997), Weiner & Model (1994)
Ionophores – protonophores [carbonylcyanide *m*-chlorohydrazone (CCCP), 2,4-dinitrophenol (DNP), carbonyl cyanide *p*-trifluoro-methoxyphenylhydrazone (FCCP)].	*E. coli* S. typhimurium S. lividans	Energy generation; uncoupling agents that discharge a transmembrane electrochemical gradient of protons	Becker *et al.* (2005), Kobayashi *et al.* (2007), Vrancken *et al.* (2008), Weiner & Model (1994)
F_1F_0 ATPase mutants	*Y. enterocolitica* S. typhimurium	Energy generation	Becker *et al.* (2005), Maxson & Darwin (2004)
Overexpression of ATP synthase from thermophilic *Bacillus* PS3 in *E. coli*	*E. coli*	Energy generation	Kobayashi *et al.* (2007)
Contact-dependent growth inhibition	*E. coli*	Cell envelope; energy generation	Aoki *et al.* (2009)
Verapamil, dibucaine	*E. coli*	Abolish PMF and decrease intracellular ATP concentration	Andersen *et al.* (2006)
Addition of CORM-3 [Ru(CO)$_3$Cl(glycinate)]	*E. coli*	Inhibits bacterial Aerobic respiration	Davidge *et al.* (2009)
biofilm formation	*E. coli* P. aeruginosa	Cell envelope; energy generation	Beloin *et al.* (2004), Mace *et al.* (2008)
Persister *E. coli* cells	*E. coli*	Multidrug tolerance, biofilm formation	Keren *et al.* (2004)
Macrophage infection and swarming	*S. enterica* S. flexneri	Energy generation	Eriksson *et al.* (2003), Lucchini *et al.* (2005)
Ethanol (5%, 10%); methanol (5%, 10%)*	*E. coli* E. coli O157:H7 S. typhimurium S. lividans	Cell envelope; fatty acid composition; Cell envelope proteins	Brissette *et al.* (1990), Chiou *et al.* (2004), Hassani *et al.* (2008), Vrancken *et al.* (2008)
Hydrophobic organic solvents (*n*-hexane, cyclooctane)	*E. coli* B. subtilis	Cell envelope	Kobayashi *et al.* (1998), Mascher *et al.* (2004)
Hyperosmotic shock (sucrose, NaCl)	*E. coli* S. lividans H. volcanii	Cell envelope	Bidle *et al.* (2008), Brissette *et al.* (1990), Vrancken *et al.* (2008)
Extreme heat shock (48–50 °C)	*E. coli* S. typhimurium	Cell envelope	Brissette *et al.* (1990), Hassani *et al.* (2009)
Sodium azide, CCCP, Sec mutants, Tat mutants, YidC mutants	*E. coli*	Block of protein secretion	DeLisa *et al.* (2004), Jones *et al.* (2003), van der Laan *et al.* (2003)
Free fatty acids (e.g. octanoic acid); fatty acid elongation mutant (*fabI*)	*E. coli*	Cell envelope; disorder phospholipids and act as an uncoupler	Kleerebezem *et al.* (1996), Weiner & Model (1994)

Table 2. Continued.

Conditions	Organism	Target/effect	References
Diazaborine, cerulenin	E. coli	Cell envelope; inhibition of fatty acids and phospholipid biosynthesis	Bergler et al. (1994)
Globomycin	E. coli	Cell envelope; inhibition of the processing of lipoproteins	Bergler et al. (1994)
Lipoprotein Llp mislocalization, HslJ mislocalization*	E. coli	Cell envelope; energy generation	Robichon et al. (2003)
Overexpression of mislocalized OM porins mutant (LamB, PhoE)	E. coli	Cell envelope; energy generation	Carlson & Silhavy (1993), Kleerebezem et al. (1996), Kleerebezem & Tommassen (1993)
glmS mutant	Y. enterocolitica	Cell envelope biosynthesis	Maxson & Darwin (2004)
Overexpression of YedR	E. coli	Envelope stress, integral IM protein, induction of capsule production dependent on Rcs proteins	Bury-Mone et al. (2009)
SDS	S. lividans	Cell envelope	Vrancken et al. (2008)
Antibiotics bacitracin, vancomycin, nisin, ramoplanin	S. lividans, B. subtilis	Inhibitors of cell wall biosynthesis, interfere with the lipid II cycle in the cytoplasmic membrane	Mascher et al. (2004), Vrancken et al. (2008), Wiegert et al. (2001)
rpoE mutants	S. typhimurium	Cell envelope, energy generation	Becker et al. (2005)
rpoE mutants+divalent Cu, Cd, Zn	E. coli	Cell envelope, energy generation	Egler et al. (2005)
Divalent metal chelator TPEN	E. coli	Energy generation	Sigdel et al. (2006)
Lack of HSP synthesis (σ^{32} mutants)	E. coli	Prolonged Psp response under stress	Brissette et al. (1991), Weiner et al. (1991)
Low level expression of CobS from Salmonella and archaea in E. coli	E. coli	Metabolism, coenzyme B_{12} synthesis	Maggio-Hall et al. (2004)
Overexpression of PspC (PspBC)	E. coli, Y. enterocolitica	Loss of psp-negative control	Maxson & Darwin (2006a), Weiner et al. (1991)
Overproduction of PspF	E. coli	Activation of psp promoters	Jovanovic et al. (1996)
Overproduction of either ArcB* or ArcA	E. coli	Activation of PspBC sensors	Jovanovic et al. (2009)
Respiratory chain redox system mutants*	E. coli	Change in intracellular redox potential	(C. Engl, G. Jovanovic, M. Buck, unpublished data)

*Our unpublished data.
Gram-negative bacteria: *Escherichia coli*, *Escherichia coli* O157:H7, *Salmonella typhimurium*, *Salmonella enterica*, *Shigella flexneri*, *Yersinia enterocolitica*, *Klebsiella oxytoca*, *Pseudomonas aeruginosa*. Gram-positive bacteria: *Bacillus subtilis*, *Streptomyces lividans*. Archaea: *Haloferax volcanii*. Searching this database (http://genexpdb.ou.edu/index.php) yielded 20 examples of changes in *pspA* expression. Many can be rationalized as reflecting biofilm formation, high temperature, exposure to ethanol, changes in respiration activity, carbon source dependences and growth into the stationary phase.

interaction between ArcB and PspB (via PspB's leucine zipper motif) (Fig. 8c) (Jovanovic *et al.*, 2009). ArcAB-dependent signalling via PspB requires the presence of PspC in order to transduce the signal to PspA and hence release the negative regulation of PspF (see Complex composition and Release of the PspA–PspF inhibitory complex) (Jovanovic *et al.*, 2009).

Lack of the Arc system reduces, but does not abolish pIV-dependent induction of *psp* expression in microaerobiosis (Jovanovic *et al.*, 2009). Clearly, the ArcAB-dependent signal transduction pathway may provide only one part of the signal required for elevating the level of Psp response sufficiently to maintain and conserve the proton-motive force (PMF) under stress conditions (see Generation of the Psp system-inducing signal). Under pIV-induced stress, more than one signal could be generated and recognized by PspBC sensors (Jovanovic *et al.*, 2006, 2009). Therefore, a 'double check-point' might exist to regulate pIV-dependent induction of *psp* expression (Fig. 8a). Such a configuration might improve the specificity of Psp response to extracytoplasmic stress signals. Also, dual sensing by PspBC would help the Psp system to establish specificity to stimuli, as described for unorthodox two-component systems that maintain their robustness to noise (Kim & Cho, 2006).

In anaerobiosis, pIV-dependent *psp* expression is largely independent of PspBC and ArcAB proteins, indicating that
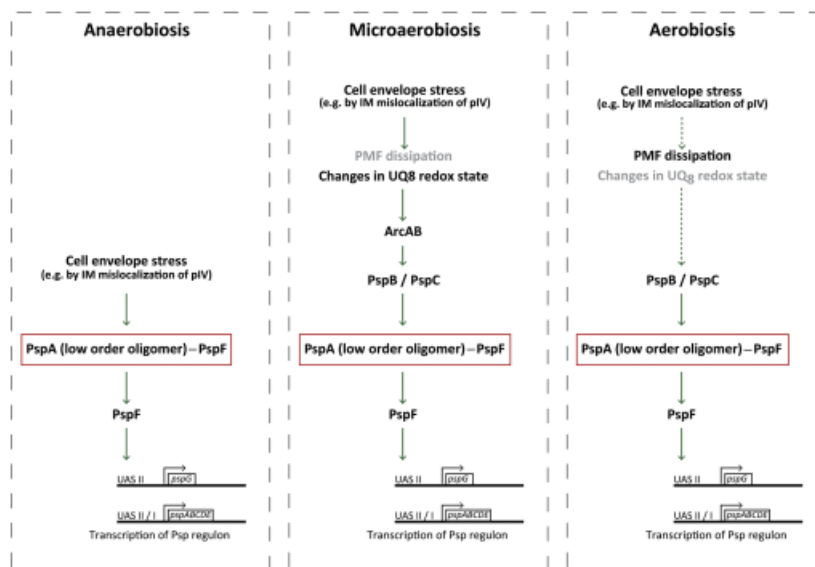
Fig. 9. The signalling pathway of the Psp response. Current findings lend support to a complex aeration-dependent regulation of the Psp response in *Escherichia coli*. Under anaerobic growth, the PspA–PspF inhibitory complex (red box) appears to be able to sense the cell envelope stress independent of the positive regulators PspBC and ArcAB. Under microaerobic growth, pIV production causes a continuous change in the redox state of the UQ_8 pool. This change may be sensed by ArcAB or other factors, required for Psp induction under microaerobic growth. The cross-talk between ArcAB and Psp may be mediated via protein–protein interaction of ArcB and PspB. PspBC then transduces the signal to the PspA–PspF complex and causes the release of PspF for the activation of Psp transcription. Induction of the Psp response under aerobic growth requires PspBC, but is independent of the ArcAB system. Additionally, the change in the redox state of the UQ_8 observed under microaerobic growth is less pronounced under aerobic growth and hence it is unclear whether this change is involved in Psp signal transduction under these conditions.

the repressive PspF–PspA complex might itself be able to recognize inducing signals, or that PspBC and ArcAB are substituted by other proteins (Fig. 9). PspBC are also only partially required for the induction of *psp* expression when induced by ethanol treatment or hyperosmotic shock, and redundant upon induction by extreme heat shock (Weiner *et al.*, 1991). The functioning of a minimal sensory complex involving PspA is consistent with the observation that so far homologues of PspA without PspBC have been found in many other organisms (see Psp beyond the enterobacteria and conservation of Psp proteins) (Westphal *et al.*, 2001; Bidle *et al.*, 2008; Vrancken *et al.*, 2008).

Inducers of the Psp response

Secretins are an important family of outer membrane (OM) proteins involved in filamentous phage extrusion, type II and type III secretion systems and type IV pili biogenesis (Genin & Boucher, 1994). The secretin pIV encoded by the filamentous phage f1 was the first identified inducer of the Psp response (Brissette *et al.*, 1990; Russel & Kazmierczak, 1993). Its characterization provides insights into the nature of the pIV-dependent stress. pIV has been well characterized due to its role in phage f1 infection and as a model OM-localized secretin. Following phage assembly, the mature phage particles are exported from the cell via an exit

channel, which facilitates the crossing of the IM and OM barrier without killing the bacterium (Russel, 1994b; Feng *et al.*, 1999). The pIV protein forms the OM part of the exit channel (Russel, 1994b). The multimeric structure of pIV has been revealed using a combination of scanning transmission electron microscopy (Linderoth *et al.*, 1997) and cryoEM (Opalka *et al.*, 2003). The pIV secretin is a gated (Russel *et al.*, 1997) homomultimeric channel consisting of 14 subunits. The channel is 24 nm long, with a pore size of 6.0–8.8 nm. The sequence and structure of pIV are homologous to bacterial OM secretins of the type II and type III secretion systems (Russel, 1994b). pIV homologues include PulD, an OM secretin from *Klebsiella oxytoca*, OutD from *Erwinia chrysanthemi* or HrpH from *P. syringae* (Russel, 1994b), XpsD from *Xanthomonas campestris*, ExeD from *Aeromonas hydrophila* and MxiD from *Shigella flexneri*. Notably, PulD and OutD also induced the Psp response (Possot *et al.*, 1992; Russel & Kazmierczak, 1993), and in *Y. enterocolitica psp*, expression is induced by synthesis of the Ysc type III secretion system (Darwin & Miller, 2001). Moreover, overproduction of YscC, the OM secretin of the Ysc type III secretion system, alone resulted in increased *psp* expression (Darwin & Miller, 2001).

Indications on how pIV (and other OM secretins) might induce the Psp response were drawn from studies where pIV was found to be evenly distributed between the IM and the

OM [25% of pIV in each fraction and the remainder in the soluble (8%) and aggregated (42%) fraction] when using a Triton X-100-based cell fractionation method (Russel & Kazmierczak, 1993). The variants of pIV, which either remained within the cytoplasm, or were unable to multimerize, or produce continually open or closed pIV channels did not induce *psp* expression (Russel & Kazmierczak, 1993; Russel, 1994a). Furthermore, phage f1 encode a pIII protein that makes the bacterial OM permeable, but does not in itself induce *psp* expression (Brissette *et al.*, 1990; Russel, 1994b; Ogura & Wilkinson, 2001). Additionally, multimeric (but not monomeric) PulD activated *psp* expression when mislocalized to the IM in the absence of its cognate chaperone-pilotin PulS (Hardie *et al.*, 1996a, b; Guilvout *et al.*, 2006). PulS chaperone overexpression abolishes the induction of *psp* expression by PspD. pIV does not have a known cognate chaperone, but PulS overproduction was able to abolish the induction of *psp* expression by a PulD-pIV chimeric protein (Daefler *et al.*, 1997). Finally, the unprocessed form of OM LamB situated as a multimer in the IM (Carlson & Silhavy, 1993) or a sorting signal-deficient form of either the OM lipoprotein LLp retained in the IM (Robichon *et al.*, 2003) or the OM lipoprotein HslJ (G. Jovanovic & M. Buck, unpublished data) induces *psp* expression. Hence, it seems likely that it is the location of some OM proteins (such as pIV) as multimers within the IM that stimulates *psp* expression. In addition to the mislocalization of OM secretins into the IM, the Psp response is also induced by a variety of conditions expected to impact upon the IM (see Table 2).

Generation of the Psp system-inducing signal

To date, the mechanism of signal perception by the Psp system as well as the nature of the signal is not well understood (Darwin, 2005b, 2007). Taken together, the agents that induce the Psp response can be assumed to have the potential to affect the integrity of the IM. Therefore, the signal that induces Psp response could be directly related to the physical/chemical properties of the membrane, determined by its phospholipids. A number of observations implicate phospholipids in the induction of *psp* expression. Blocking the synthesis of phospholipids or the exogenous application of free fatty acids strongly induces *psp* expression (see Table 2). Induction of PspA and modifications of the membrane fatty acid composition were observed following treatment with *psp*-inducing agent ethanol (Chiou *et al.*, 2004). In addition, tolerance to ethanol can be achieved in cells enriched with phosphatidyl-serine (PS) (Mishra & Prasad, 1988). Interestingly, a block in protein translocation known to induce *psp* expression can be overcome in IM vesicles by the introduction of phosphatidyl-glycerol (PG) and PS or *in vivo* by increasing the production of the

phosphatidyl-glycerol-phosphate synthase (Kusters *et al.*, 1991). Potentially, the composition of fatty acids and the consequent anion : zwitterion ratio of phospholipids in the membrane may be important determinants for generating the signal that induces *psp* expression. Kobayashi *et al.* (2007) showed that PspA binds PG and PS, indicating the importance of lipid binding in repairing membrane damage and so in directly counteracting the consequences of *psp*-inducing stresses.

In a number of cases, it has been shown that agents that impair the integrity of the IM also decrease the level of PMF, for example pIV production in cells lacking the Psp response, as well as a treatment with the proton ionophore carbonylcyanide *m*-chlorohydrazone (CCCP) (Weiner & Model, 1994; Jovanovic *et al.*, 2006), knocking out σ^E in *Salmonella enterica* (Becker *et al.*, 2005) or the expression of a mislocalized form of the OM porin PhoE (Kleerebezem & Tommassen, 1993; Kleerebezem *et al.*, 1996). Depletion of YidC also dissipates the PMF because YidC is required for proper IM assembly of the cytochrome *o* oxidase and the F_1F_0-ATPase (van der Laan *et al.*, 2003; Celebi *et al.*, 2006; Kol *et al.*, 2008). It is therefore proposed that dissipation of PMF may serve as the inducing signal to increase the Psp response (Darwin, 2005b, 2007), or that a decline in PMF is closely related to the signal or the activities of systems sensed by the PspBC proteins (e.g. performance of the respiratory chain, Fig. 10). The requirement of ArcB activation for Psp signal transduction under microaerobic growth, however, implies that at least under such conditions, the primary signal for Psp induction might act upstream of ArcB. Several studies point to the quinone pool as a candidate for such a signal, because ArcB can be activated by changes in the redox state of both ubi- and menaquinones (Georgellis *et al.*, 2001; Malpica *et al.*, 2004; Bekker *et al.*, 2009), and because cells deleted for *ubiG*, part of the ubiquinone-biosynthesis pathway, showed reduced *psp* expression (Jovanovic *et al.*, 2006). A recent study (C. Engl, G. Jovanovic & M. Buck, unpublished data) using direct quinone measurements in: (1) electron transport mutants (each with different ubiquinone contents) and (2) in cells with an intact electron transport chain, but under pIV stress, demonstrated a correlation between the onset of the Psp response caused by pIV and changes in the redox state (ubiquinone : ubiquinol ratio; Figs 8a and 9). Strikingly, pIV production appears to directly impact on the cells' redox state. The study further indicates that PMF dissipation alone is not sufficient to induce the Psp response (C. Engl, G. Jovanovic & M. Buck, unpublished data). The electron transport mutants showed different levels of *psp* expression; however, their PMF levels remained similar to those of the wild-type strain, where the Psp response is not induced. Individual dissipation of the PMF components $\Delta\psi$ or ΔH^+ either by the K^+-ionophore valinomycin or by acetate did not increase *psp* expression in
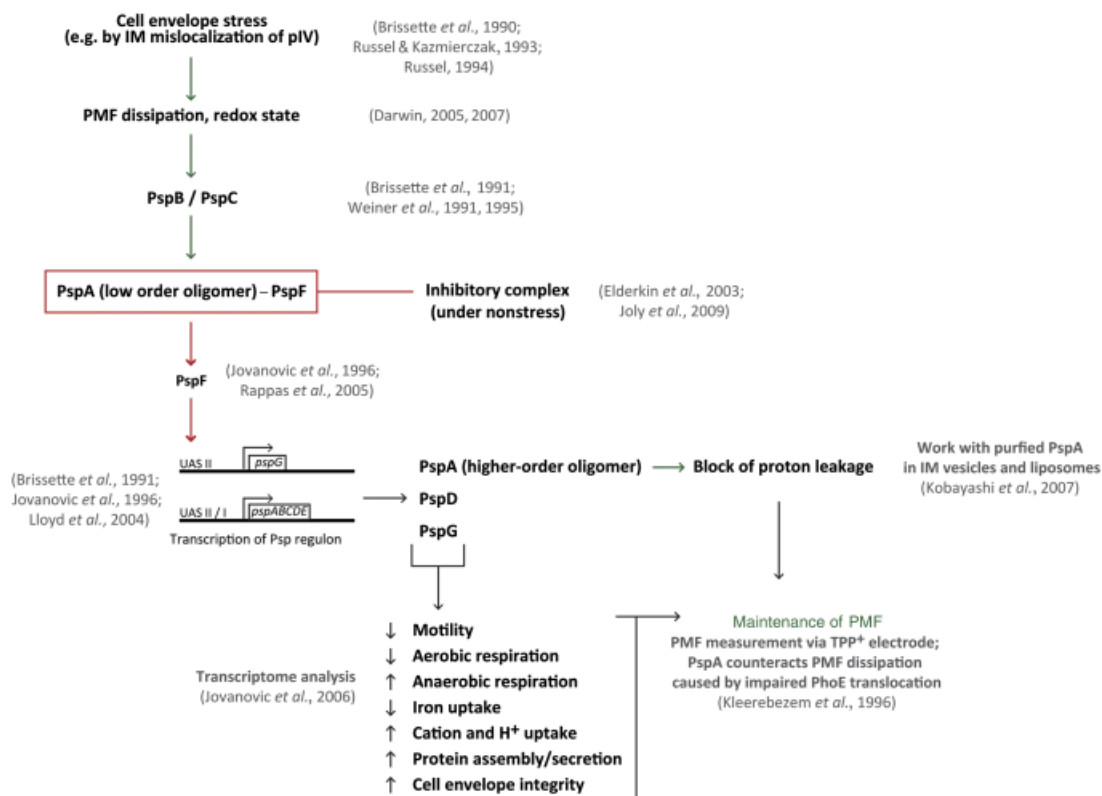
Fig. 10. Regulation and function of the Psp response. The Psp response is induced upon cell envelope stress in aerobiosis, which may cause dissipation of PMF and potentially a change in the cell's redox state. The inducing signal (green arrows) is sensed by PspB/PspC and relayed to the PspAF inhibitory complex. Subsequently, PspF is released and activates the transcription of the *psp* genes (red arrows). Psp effectors (PspA, PspD, PspG) help to maintain PMF under stress by adjusting the transcription profile of genes involved in the indicated cellular processes (only major changes are given). Additionally, PspA (as a high-order oligomer) is proposed to directly block proton leakage. References to the publications supporting the proposed signal transduction pathway are shown in grey.

wild-type *E. coli* strains. Such outcomes, indicating that a decline in PMF is not sufficient to induce the Psp response, might explain the following paradox:

The Psp response is continually induced in pIV-producing cells even though the Psp response functions to maintain PMF. If the PMF dissipation was the only inducing signal, the observed PMF maintenance by the Psp response would be expected to cause a loss of the inducing signal and hence decrease *psp* expression [inconsistent with experimental observations; recall that a decline in PMF is not seen in cells expressing pIV unless PspF is deleted (Jovanovic *et al.*, 2006)]. It is more likely that the Psp response cannot directly or fully confront (and so diminish) the inducing signal; otherwise, the Psp response would not be continually induced in pIV-producing cells. In contrast to the decline in PMF, the changes in the redox state caused by pIV prevail even when the Psp response is 'on', supporting the idea that these changes may contribute to the signalling activity and are needed for continued *psp* expression under pIV stress.

The activities of the electron transport chain, the state of ubiquinones and respiration activities may impact on the induction of Psp response via the actions of and dependencies on ArcAB. Notably, induction of *psp* expression and a significant downregulation of aerobic respiration and PMF occur simultaneously in cells undergoing contact-dependent growth inhibition (Aoki *et al.*, 2009). Furthermore, the specific inhibition of *E. coli* aerobic respiration upon delivery of CO to the terminal oxidases induces *psp* expression (Davidge *et al.*, 2009).

Biological function of the Psp response

PMF maintenance under envelope stress

Although bacteria lacking the Psp response are still viable, their tolerance to envelope stresses that decrease the energy state of the cell is markedly reduced (Model *et al.*, 1997; Darwin, 2005b, 2007). For example, cells deleted of *psp* genes exhibit impaired growth in the stationary phase at

alkaline pH (Weiner & Model, 1994) or on bile salts (Adams *et al.*, 2003). Additionally, in the absence of a functional Psp response, the efficiency of PMF- and ATP-dependent protein secretion is decreased (Kleerebezem & Tommassen, 1993; Kleerebezem *et al.*, 1996). These observations imply that the Psp response functions to help maintain the energy state of the cell under stress (Kleerebezem *et al.*, 1996; Model *et al.*, 1997; Darwin, 2005b, 2007; Jovanovic *et al.*, 2006).

Role of PspA in PMF maintenance

When measuring the membrane potential in living cells using a tetraphenylphosphonium (TPP$^+$)-sensitive electrode, Kleerebezem *et al.* (1996) showed that PspA counteracts proton loss caused by translocation of a mutant form of the OM porin PhoE. These results were supported by the observation that purified PspA blocks proton leakage of membrane vesicles reconstituted from total lipids from *E. coli* (Fig. 10) (Kobayashi *et al.*, 2007). Furthermore, PspA can directly and specifically bind to the IM phospholipids PG and PS (Kobayashi *et al.*, 2007). How PspA repairs proton leakage is still unknown. The authors (Kobayashi *et al.*, 2007), however, proposed that under membrane stress, PspA forms a scaffold covering the cytoplasmic face of the IM by binding to PG and PS in the lipid layer. This may for example change the rigidity of the IM and as such facilitate PMF maintenance (Kobayashi *et al.*, 2007). Recently, scaffold-like networks composed of homomultimeric PspA have been reported from structural studies (Standar *et al.*, 2008). In contrast, a subcellular localization study on PspA does not readily support these findings (Engl *et al.*, 2009). PspA (and PspG) is spatially organized into distinct classes (Fig. 8b). Some complexes, localized in the polar region of the cell, were rather immobile while other complexes displayed a range of curved and linear motions along the length of the cell. It was hypothesized that the polar localized complexes may directly communicate with other systems in this area of the cell to modulate PMF-consuming processes such as chemotaxis or to promote pathogenicity via for example type III secretion (Engl *et al.*, 2009). Lateral trafficking of PspA appeared to be linked to the cytoskeleton as such trafficking was absent in cells treated with the compound A22 (which inhibits the MreB protein), indicating that the movement of PspA is facilitated by the MreB scaffold (Engl *et al.*, 2009). This localization study lends support to a role for the Psp response in cell wall biogenesis in conjunction with other MreB-associated proteins, such as penicillin-binding proteins, in order to repair stress-induced membrane damage and maintain PMF. Indeed, PMF was not maintained when PspA and PspG were unable to move along the length of the cell (Engl *et al.*, 2009).

Transcriptome studies

To unravel the genetic program that might be involved in the Psp response, microarray analyses were performed under different growth conditions (Lloyd *et al.*, 2004). Transcriptional profiling revealed that the synthesis of pIV secretin in *E. coli* and *S. enterica* serovar Typhimurium leads to a highly restricted response limited to the upregulation of the *pspABCDE* operon genes and *pspG* (Lloyd *et al.*, 2004). This result was confirmed in *Y. enterocolitica* cells expressing the secretin YscC (Seo *et al.*, 2007). Elevated expression of *psp* genes, or indeed any other genes, when pIV was produced was not observed in Δ*pspF* or Δ*pspBC* mutants (Lloyd *et al.*, 2004), consistent with (1) the requirement for the PspF- and PspBC-positive regulators in pIV-dependent microaerobic induction of Psp response (see Psp promoters bind PspF and use σ^{54}-RNAP and PspBC-dependent signal transduction) and (2) a very restricted transcriptomic response to secretin-induced stress in *E. coli*.

Subsequent differential transcription assay experiments (Fig. 10) of *psp* mutants in bacteria cultivated under normal growth conditions in minimal media (in the absence of an applied stress) revealed that PspA and PspD caused the downregulation of genes implicated in chemotaxis and the PMF-consuming process (e.g. motility) and upregulation of genes (e.g. *gad*) involved in a low pH response (Jovanovic *et al.*, 2006). A cell motility effector function for PspA and PspD as well as for PspG has been shown (Model *et al.*, 1997; Lloyd *et al.*, 2004; Engl *et al.*, 2009; Jovanovic *et al.*, 2009). Interestingly, overproduced PspA and to some extent PspG strongly affected the expression of genes that would facilitate the uptake and biosynthesis of polyamine spermidine (Jovanovic *et al.*, 2006). Polyamines are reported to play roles in bacterial pathogens and link to biofilm formation, escape from phagolysosomes, toxin activity and protection from oxidative and acid stress (reviewed in Shah & Swiatlo, 2008). Spermidine transport may also be important for type III secretion system expression, including one bona fide *psp* expression inducing secretin, in some Gram-negative pathogen bacteria (Zhou *et al.*, 2007). Survival in an acidic environment under nutrient limitation (a condition that induces *psp* expression) is a key property of intestinal pathogens and commensals. Polyamines seem to be major mediators in the response to acid stress in bacteria. In *E. coli*, the *gadA* and *gadB* genes products (upregulated by PspA) contribute to acid resistance and their expression is regulated by polyamines (Jung & Kim, 2003). The *gad* genes and GABA (involved in spermidine biosynthesis) are also implicated in a response to high pH during anaerobiosis (Yohannes *et al.*, 2004), which might explain the strong upregulation of genes (*yodA*, *yfiD*) involved in a high pH response when PspG is overproduced (Jovanovic *et al.*, 2006). Notably, genes of the Puu system involved in

spermidine synthesis and pH resistance are adjacent to the *psp* operon in *E. coli*. It seems that under certain growth conditions, the expression of *psp* genes might be part of the activities of larger adaptive networks.

Jovanovic *et al.* (2006) have established the potential role of the PspA, PspD and PspG proteins in modulating the metabolism of glycerol-3-phosphate (G-3-P) and consequently respiration and phospholipids biosynthesis. The absence of PspA and PspD (results obtained with knockout mutants in the absence of pIV-induced stress) caused the downregulation of genes involved in glycerol utilization and usage of G-3-P in the glycerol shift (as occurs in aerobic respiration). PspG, when considerably overproduced, upregulates genes that facilitate the conversion of dihydroxyacetone-phosphate to G-3-P (decreasing the glycerol shift) (Jovanovic *et al.*, 2006). Consistent with this impact on G-3-P-associated genes were the results obtained in transcriptional profiling of cells overproducing PspF (Bury-Mone *et al.*, 2009). These results suggest that Psp effectors cause a reduction in G-3-P use as an electron donor for the aerobic respiratory chain in order to increase the pool of G-3-P for phospholipids biosynthesis catalysed by PlsA, whose expression is under the positive control of σ^E (Bury-Mone *et al.*, 2009). Such gene expression changes could help manage the severe envelope stress (caused by a *glmS* mutation) or energy stress (caused by verapamil or dibucaine treatment) when Psp and σ^E systems are each induced (Maxson & Darwin, 2004; Andersen *et al.*, 2006).

Changes in PspG levels were found to influence iron usage and fine-tune the expression of the genes involved in the switch from aerobic to anaerobic (mostly formate–nitrate) respiration (Jovanovic *et al.*, 2006). Consistently, formate neutralization genes were downregulated upon overproduction of PspG (Jovanovic *et al.*, 2006) and genes controlling the formate hydrogenase system and formate transport were derepressed upon induction of Psp response by PspF overproduction (Bury-Mone *et al.*, 2009). Consequently, electron transport from formate to cytochrome *b* and quinones in the formate–nitrate respiratory chain could help maintain the PMF.

PspE as a rhodanese

The biological target of the periplasmic protein PspE, under *psp* expression-inducing conditions, is unknown. PspE has a rhodanese activity (Adams *et al.*, 2002; Cheng *et al.*, 2008) and may serve as a detoxification enzyme for cyanide or heavy metal cations such as cadmium, both of which induce the *psp* expression (see Table 2). Alternatively, it may directly repair the Fe–S clusters active in the electron transport chain. In *E. coli*, PspE can contribute 85% of the total rhodanese activity, with the GlpE protein contributing the remaining (Cheng *et al.*, 2008). Upregulation of the *glpE*

gene upon PspG overproduction (Jovanovic *et al.*, 2006) suggests that increased rhodanese activity may be linked to *psp* expression-inducing conditions.

PspBC as effectors

PspB and PspC were proposed to be dual function components of the Psp system in *Yersinia*, acting both as positive regulatory sensors of *psp* expression and effectors during secretin-induced stress (Maxson & Darwin, 2006b). The authors have shown that PspB and PspC play an effector role in supporting the growth of *Y. enterocolitica* when the YsaC secretin is overexpressed (Maxson & Darwin, 2006b). While PspB and PspC work together in the Psp signal transduction pathway, they can also act independently to support growth under stress conditions in at least one organism (Maxson & Darwin, 2006b). In a subsequent study, the regulatory and effector functions of PspC were uncoupled (Gueguen *et al.*, 2009). In *E. coli*, in the absence of PspC, growth is decreased to some extent under normal growth conditions (G. Jovanovic, C. Engl & M. Buck, unpublished data). Interestingly, overproduction of PspC, but not PspBC significantly impairs the growth of *E. coli* in the absence of stress (G. Jovanovic, C. Engl & M. Buck, unpublished data). This is consistent with the observation that in *E. coli*, PspBC constitutes a toxin–antitoxin pair (Brown & Shaw, 2003).

Psp in the virulence of pathogenic Gram-negative bacteria

A Psp system is found in many Gram-negative pathogens (Darwin, 2005b, 2007) and is induced by OM secretins that are components of the type II and type III secretion systems. These systems are used during the infection process for cell adherence and for the export of virulence factors into the extracellular milieu or into target cells. The Psp response may therefore protect the cell envelope of bacterial pathogens during infection. Indeed, mutation of *pspC* in *Y. enterocolitica* resulted in attenuated virulence and impaired growth (Darwin & Miller, 2001). Furthermore, *psp* expression is elevated during swarming of and macrophage infection by *S. typhimurium* and *S. flexneri* (Eriksson *et al.*, 2003; Wang *et al.*, 2004; Lucchini *et al.*, 2005) and during biofilm formation in *E. coli* (Beloin *et al.*, 2004). Also, induction of PspA production by heat shock increased the virulence of *S. typhimurium* in mice (Hassani *et al.*, 2009).

The Psp response may also be of importance for the medical treatment of Gram-positive and Gram-negative bacteria. Double deletion of *rpoE* and *pspA* in *Salmonella* makes the bacteria more sensitive to the protonophore CCCP or BPI (bactericidal permeability increasing protein), leading to the death of bacteria (Becker *et al.*, 2005). At antimicrobial levels of CORM-3 [Ru(CO)$_3$Cl(glycinate)], a compound that targets CO to the terminal oxidases, thereby

inhibiting the bacterial aerobic respiration, the production of PspA and PspD is induced (Davidge *et al.*, 2009). Importantly, the *E. coli* persister cells that form biofilm and develop multidrug tolerance have induced *psp* expression (Keren *et al.*, 2004). The resistance to antibiotics and stresses associated with these nongrowing bacteria is facilitated by energy production-dependent mechanisms (Ma *et al.*, 2009). Notably, some multidrug resistance pumps use PMF to drive drug efflux, suggesting a potential role of Psp response in microbial drug response. Taken together, these observations suggest that mutagenesis of *psp* and associated genes could give rise to recombinant pathogenic enterobacteria with diminished virulence, which might be used for the production of live-attenuated vaccines.

Psp in biotechnology

Overproduction of PspA has been shown to stimulate efficient Sec protein export in Gram-negative bacteria (Kleerebezem & Tommassen, 1993) and both Sec- and Tat-dependent protein secretion in Gram-positive bacteria (Vrancken *et al.*, 2007). PspA overproduction considerably improves the secretion yield of recombinant proteins (Aldor *et al.*, 2005; Vrancken *et al.*, 2007). In addition, in Gram-positive bacteria *Streptomyces lividans*, used for the production of small bioactive molecules (e.g. antibiotics) and enzymes, and *B. subtilis* used in food production (e.g. for an immune-stimulatory effect), when treated with antibiotics (e.g. nisin, used in dairy products industry), both organisms strongly induce the production of PspA homologues (Wiegert *et al.*, 2001; Mascher *et al.*, 2004; Vrancken *et al.*, 2008). This indicates the possible benefits of using PspA overexpression for improved production of proteins and molecules of interest in biotechnology and medicine.

Ethanol can be found in foods and in food-processing environments and can be used as a preservative in the food-processing industry, in addition to being an important biofuel. Food-borne pathogens, particularly Gram-negative pathogenic bacteria, can adapt to survive exposure to organic solvents and sublethal concentrations of ethanol by introducing phenotypic modifications in the cellular fatty acid composition. This phenotype and tolerance to ethanol is closely associated with the production of PspA (Chiou *et al.*, 2004), and so PspA can be exploited with respect to food protection as a target against pathogenic bacteria. Additionally, the Psp system-dependent response (and tolerance) to ethanol and methanol (see Inducers of the Psp response and Table 2) is of relevance for the production of biofuels.

The positive regulation of the *pspA* promoter has been exploited. The advantage of the highly regulated *pspA* σ^{54} promoter has been used successfully for the construction of phagemid vector (Rakonjac *et al.*, 1997) for phage-display technology and the selection of transiently interacting or toxic proteins (Beekwilder *et al.*, 1999).

Psp beyond the enterobacteria and conservation of Psp proteins

In the Psp system, the proteins work together in order to fulfil their physiological roles. Despite, or rather because of the diversity of interactions outlined above, the system is only functional if the necessary components are all present. Hence, an evolutionary or a comparative perspective can provide additional insights into the system's workings.

Phylogenetic spread of the Psp system

An *in silico* search within available genomic sequences (Darwin, 2005b; Huvet *et al.*, 2009) as well as experimental data (Westphal *et al.*, 2001; Vrancken *et al.*, 2007, 2008; Bidle *et al.*, 2008) show that the *psp* regulon is not just highly conserved among Gram-negative bacteria, but many of its parts are present in a number of organisms throughout all three domains of life. Darwin (Darwin, 2005b) reported Psp systems in *Gamma-*, *Delta-* and *Alphaproteobacteria*. However, while PspFABC were always conserved, other Psp proteins appeared to be dispensable. It was therefore concluded that the minimal Psp system comprises PspFABC (Darwin, 2005b). Recent *in silico* analysis of the evolutionary divergence of members of the Psp response across 129 bacterial species also found a high degree of coconservation of PspA and PspF (Fig. 11); other Psp proteins, in contrast, appear to be far less conserved (Huvet *et al.*, 2009). Other organisms show an even higher degree of Psp system reduction, containing only homologues of PspA. In the Gram-positive bacterium *S. lividans*, the expression of a PspA homologue was induced upon membrane stress (Vrancken *et al.*, 2008). *Bacillus subtilis* contains a PspA homologue, LiaH, which responds to alkaline shock (pH 9) and envelope stress (Wiegert *et al.*, 2001; Jordan *et al.*, 2008). A PspA homologue was also found in the halophilic archaea *Haloferax volcanii*, where it was upregulated under high-salt (3.5 M NaCl) conditions (Bidle *et al.*, 2008). Furthermore, a PspA homologue, Vipp1 (vesicle-inducing protein in plastids 1), is essential in plants and *Synechocystis* for thylakoid membrane formation and photosynthesis (Westphal *et al.*, 2001; Aseeva *et al.*, 2007). Upon Vipp1 deletion, the structure of thylakoids in *Synechocystis* is impaired and the cells are unable to perform photosynthesis (Westphal *et al.*, 2001). Recently, it has been reported that Vipp1's effect on the photosynthetic activity of cells may be independent of its effect on thylakoid formation (Gao & Xu, 2009). Notably, Vipp1 can complement for the loss of PspA in an *E. coli pspA* mutant strain, suggesting that PspA function is conserved within Vipp1 and hence across bacteria, archaea and
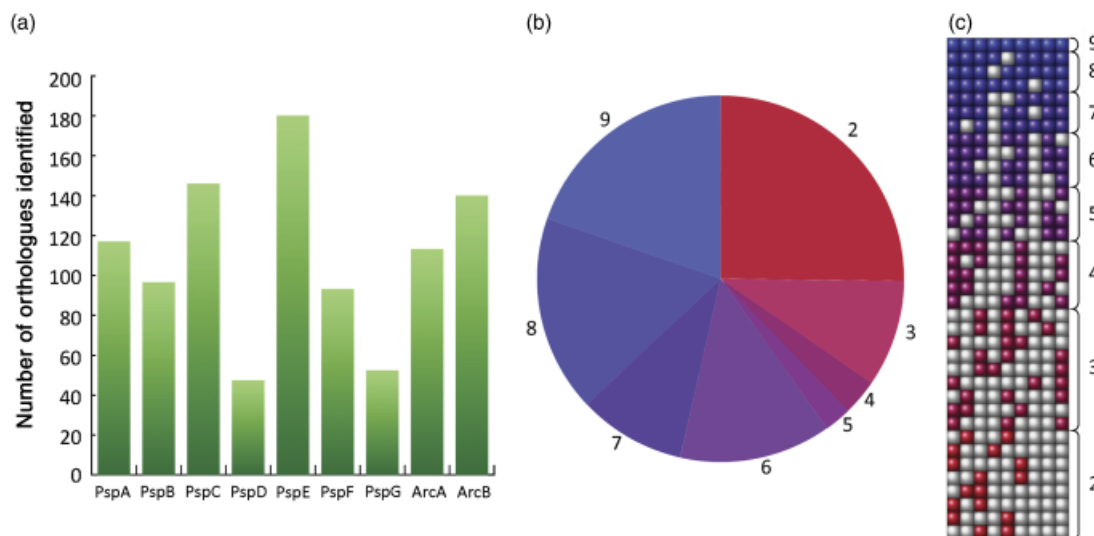
Fig. 11. Psp system gene orthologues: distribution patterns in 699 bacterial genomes. (a) Number of orthologues identified in the 699 genomes for each of the Psp proteins. (b) Proportion of genomes containing between 2 and 9 orthologues of the Psp and Arc proteins. One hundred and twenty-nine species contain more than one orthologue. The colour code ranges from red to blue, corresponding to species containing 2–9 orthologues. (c) A profile depicting the presence or absence of orthologues observed in species with more than one orthologue. The profiles are ordered from those with the largest number of orthologues (top) to those with lower numbers (bottom); the order is PspA to ArcB as in (a) colour coded as in (b).

eukaryotes (DeLisa *et al.*, 2004). Taken together, it appears that PspA may play a fundamental role in membrane physiology and energy conservation in all three domains of life.

Maintenance of the Psp system

Considering the genomic organization of the genes in the Psp system provides additional clues as to how the system is inherited (Fig. 12). A glance of all published bacterial genomes (on the order of 1000 at the time of writing) shows that the *psp* operon has grown over the course of evolutionary history by adding genes to the end of the operon: there is clear evidence that *pspD* and *pspE*, for example, were added after *pspBC* and *pspD* before *pspE* (Huvet *et al.*, 2009). The gene shared most widely across the species (here, we do not consider arcAB as this two-component system is also involved in other processes) is *pspE*. However, orthologues for *pspE* that are found outside of the operon show markedly reduced levels of sequence similarity to copies of *pspE* present inside the operon. This and other results (e.g. the σ^{70} promoter site upstream of *pspE* in several species including *E. coli*) suggest that incorporating a gene into the operon goes hand in hand with the protein product being recruited into the Psp response machinery.

Maintaining genes in a single operon has the advantage of retaining genes that need to act in concert in a single transcriptional unit. The *psp* operon shows how it is still possible to maintain transcriptional flexibility (through hairpin structures between *pspA* and *pspB*; overlap of *pspB* and *pspC*; and the σ^{70} promoter site between *pspD* and

pspE). Interestingly, *pspG* appears in a putative mini operon together with *pspA* and *pspC* homologues in the species *Aeromonas salmonicida* A449. This could suggest that *pspG*, which, in *E. coli*, is in a different genomic region, but part of the regulon, may be in much closer regulatory association with other *psp* genes.

Detailed phylogenetic analyses show that the genes belonging to the Psp system are inherited together in a nonrandom fashion. This probably indicates that they can only fulfil their functions in concert. This has two implications: first, the manner in which combinations of *psp* orthologues are found can be indicative of their relative importance and functional role in the response; second, if too few genes, or the wrong set of genes, are found in a species, then the functionality of the system can be severely compromised and an organism may just as well jettison the whole set of genes. The latter does seem to be the case if we look at the distribution of genes across the sequenced bacterial species: here, we find a tendency to either have (nearly) all or none of the *psp* genes.

This coinheritance of the *psp* genes is in contrast to *arcAB*, which are found in separate regions of the circular *E. coli* genome. While these genes are often found together, we also observe a large number of cases where only one or the other are present (Fig. 12). This is probably a reflection of the flexibility of individual proteins in a two-component system to interact with constituent proteins of other systems. If in the same organism, this may, of course, give rise to crosstalk, but even this will depend on the environment and the corresponding physiological state of the cell.
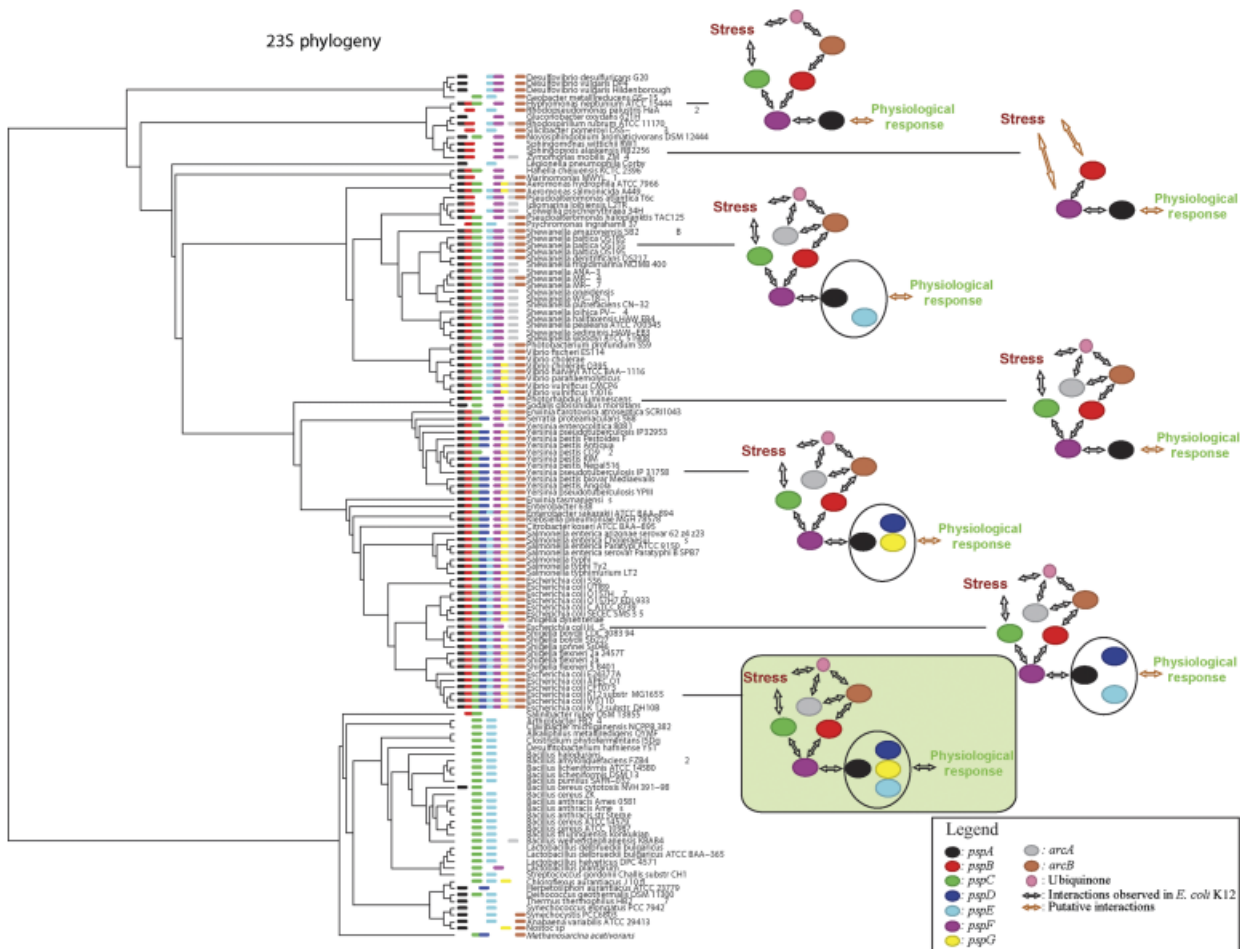
Fig. 12. Distribution and configuration profiles of Psp system orthologues. Listed are the results of all fully sequenced species on NCBI that had at least two clearly identifiable orthologues to *Escherichia coli* PspA–G proteins. The *Bacillus subtilis* LiaH strain did not yield two such proteins at the required level of stringency (a minimum of 30% sequence similarity across at least 50% of the sequence of the *E. coli* K12 version). Lists are organized based on their position in the phylogenetic tree. From left to right: (1) the phylogenetic tree inferred from the 23S rRNA gene sequences, (2) distribution profile of PspA to ArcB, (3) species names and (4) potential network organization of the Psp system for different observed sets of orthologues. The *E. coli* Psp system is highlighted in the green box. Note that in *B. subtilis*, LiaH is a homologue of PspA, but no other Psp homologues are present.

Prospects for modelling the Psp response

In light of the functional data on the Psp response and the levels of gene sharing across the prokaryotic phylogeny, it is tempting to identify a minimal Psp system. Using mathematical modelling of a biological system, we determine which systems could explain the biological data observed. The measurements of concentrations or absolute abundances at different time points are ideally suited for model inference and parameter estimation (Toni *et al.*, 2009; Toni & Stumpf, 2010). For the Psp response, however, we have only single time-point measurements for wild-type strains and different (overexpression or knockout) mutants. It is nevertheless possible to construct mathematical models that account for the bulk of the available observations. A minimal model accounting for the response to stress consists of

PspF, PspA, PspBC and the PspABCF complex, together with a flexible description of the Psp effector function (Toni *et al.*, 2009). Based on this model, we can describe sensing, transduction and initiation of the transcriptional response (once the PspF-negative control is released). A range of tools exist that enable us to model such systems even in the absence of detailed experimental data. Such methods, typically referred to as sensitivity or robustness analyses, allow to determine how flexible the response of such a mechanistic model is: whether the desired behaviour, for example, is only possible for a small range of parameters; then the model may not be sufficiently robust and we may need to improve the mathematical formulation of the model.

The initial studies (Toni & Stumpf, 2010) of the Psp response have shown that the response of a minimal model appears to be robust to details of the model, i.e. it is

straightforward to ensure that *in silico* models of the Psp response exhibit the desired behaviour.

More generally, however, it is of interest when such models produce unexpected outputs. For the minimal Psp model we observe, for example, that depending on the severity of membrane damage, we expect to observe oscillatory behaviour in some of the molecular species, in particular, the effector PspA oligomers. Here, for low levels of damage, the response may 'overshoot'. Experimental validations of these results still remain, but clearly, we can envisage extensions to these simple models, such as inclusion of the transcriptional delays, which may affect such oscillations. Without *in silico* analysis, however, the interplay between different processes and their feedback upon each other may feasibly be missed or would at least be hard to capture. Here, the interaction between modelling and experiment holds great promise in the future in order to design maximally informative experiments that capture the complexity of biological systems (but also any underlying simplifying principles).

Stress responses of the cell envelope

Escherichia coli has evolved various systems to counteract cell envelope stress. To date, the best-characterized cell envelope stress responses comprise the partially overlapping σ^E response, the Cpx system and Psp response (Darwin, 2005b, 2007; Ruiz & Silhavy, 2005; Rowley *et al.*, 2006).

Maxson & Darwin (2004) showed that in *Y. enterocolitica* Psp mainly responds to stresses that do not activate the σ^E or Cpx system. Additionally, deletion of *cpxA* in *E. coli* exerted no effect on Psp response induction (Jovanovic *et al.*, 2006). However, a *Salmonella* mutant lacking RpoE (σ^E) exhibited increased *psp* expression, and a double knockout of *rpoE* and *pspA* resulted in impaired survival in the stationary phase upon CCCP treatment (Becker *et al.*, 2005). Recall that CCCP is a proton ionophore in the IM that causes an unregulated influx of H^+ ions into the cytoplasm, thereby discharging the PMF. The authors proposed therefore a compensatory role of Psp response in the absence of a functional σ^E response (Becker *et al.*, 2005). Jovanovic *et al.* (2006) suggested that the Psp system can, under certain conditions, act in concert with the σ^E and the Cpx system.

The two-component Cpx system is induced by alterations in the cell envelope caused for example through cell adhesion, P pili synthesis, misfolded envelope proteins, overproduction of the lipoprotein NlpE and alkaline pH (Danese *et al.*, 1995; Nakayama & Watanabe, 1995; Jones *et al.*, 1997; Raivio & Silhavy, 1997; Danese & Silhavy, 1998; Raivio *et al.*, 1999, 2000; Ruiz & Silhavy, 2005; Rowley *et al.*, 2006). Stress conditions are sensed by the sensor kinase CpxA, which then activates the response regulator CpxR, thereby modulating the gene expression of the Cpx regulon. Upregulated genes include proteins involved in, for example, protein secretion (*secA*) and folding (*dsbA*), lipid biosynthesis (*psd*) or virulence and adherence (*pap*), while genes involved in chemotaxis and motility (*aer*, *motAB*, *cheAW*) or cell division (*minCDE*) are downregulated (Rowley *et al.*, 2006). From this transcriptomic profile, it has been suggested that the Cpx system plays a role in the early stages of infection and in the switch from a mobile to a sessile life in a biofilm, where the Psp response is induced (Dorel *et al.*, 2006).

The σ^E response is induced by temperature fluctuations, exposure to ethanol or detergents, osmotic and oxidative stresses as well as during biofilm formation, stationary-phase growth and the overproduction of OM proteins (Raivio *et al.*, 1999; Ruiz & Silhavy, 2005; Rowley *et al.*, 2006). It has been proposed that these conditions cause misfolding of OM proteins, resulting in the activation of the alternative σ factor σ^E via regulated intramembrane proteolysis (Alba & Gross, 2004; Ehrmann & Clausen, 2004; Ades, 2008). σ^E then directs the transcription of the σ^E regulon genes. Dartigalongue *et al.* (2001) identified 20 promoters regulated by σ^E. The gene products are mostly involved in the folding and degradation of proteins in the cell envelope; examples include DsbC, SurA, FkpA and DegP (Lipinska *et al.*, 1988; Erickson & Gross, 1989; Danese & Silhavy, 1997; Dartigalongue *et al.*, 2001).

After several single-system studies conducted in isolation, overlapping responses to a number of extracytoplasmic stresses have been reported (see Transcriptome studies) (Darwin, 2005a; Andersen *et al.*, 2006; Bury-Mone *et al.*, 2009). The coordinated action of Cpx, σ^E and Psp under several stresses (e.g. when *glnS* is non functional) seems to be important for cell survival (Becker *et al.*, 2005).

Perspectives

The interactions occurring between Psp proteins provide an example of the use of a membrane-bound system processing information (the stress signal or signals), which is then relayed onto a cytoplasmic DNA-bound transcription complex. How the pieces of the Psp system work together remains largely unknown. It is possible that the pairwise interactions observed between Psp proteins reflect capacities to form a multiprotein complex, and this, or its subcomplexes, may exhibit particular IM localizations and movements. In the absence of any post translational modification occurring on Psp proteins, protein–protein interactions may dominate signalling (and as noted earlier, the presence of functionally important coiled-coil and leucine zipper motifs in Psp proteins argues for this). The dynamic formation of signalling complexes and the protein self-association observed for PspA may well generate pockets of locally concentrated signal processing complexes. Whether or not Psp signalling complexes are preassembled or whether their

assembly itself is a part of the switching event remains to be determined. One advantage of an assembled signalling complex lies in its ability to block cross-talk from indiscriminate other factors. The activation of *psp* expression via the Cpx system only in the absence of PspBC may be providing evidence in favour of a preassembled complex (G. Jovanovic, C. Engl & M. Buck, unpublished data). Such preassembled complexes also confer an advantage in being able to readily receive activating signals. Modelling the regulation of the Psp sytem by taking into account the amounts of individual complexes, their particular states and the dependencies for interconverting between states will be necessary in order to generate useful hypotheses that allow further rational experimental design.

Numerous examples now exist demonstrating that the bacterial cell is highly organized in a spatial sense. Polar localized proteins provide classic examples, and the bacterial chromosome is known to have a particular orientation within the cell. Sequences of the replication origin can be found at the cell poles. The PspA and PspG proteins show polar localizations, but how this arises is not yet known. The curvature and lipid composition of the membrane at the pole and at the junction of the lateral and polar membrane may play a role in retaining such complexes. How the polar complexes initially arise may involve some distributed self-association events in the membrane; these in themselves may be favoured by well-separated (to reduce local competition) nucleating lower-order complexes capturing their partner proteins. Such distributions may 'drive' complexes to be formed far apart (at the poles) and subsequently stabilized by membrane curvature (Shapiro *et al.*, 2009).

What might drive changes in the localization of Psp proteins is of interest, in particular because the gene activation event requiring PspF occurs on the chromosome. The PspA–PspF inhibitory complex may or may not: (1) exist at the IM, (2) exist in the cytoplasm, (3) dissociate and (4) change configuration to allow regulated PspF control by PspBCA. Many of these possibilities can be addressed using cell imaging and specific classes of variants in PspA and PspF that show altered membrane binding and or hetero-oligomeric associations.

Finally, the details of the action of PspF should be revealed using methods such as FRET to report changes in DNA and protein configurations as a function of the PspF nucleotide-bound state. Placing these observations into a structural framework arrived at using high-resolution protein X-ray crystallography and medium-resolution cryoEM should yield a detailed mechanistic model.

Acknowledgements

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