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Review

Protecting from Envelope Stress: Variations on the Phage-Shock-Protein Theme

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During envelope stress, critical inner-membrane functions are preserved by the phage-shock-protein (Psp) system, a stress response that emerged from work with *Escherichia coli* and other Gram-negative bacteria. Reciprocal regulatory interactions and multiple effector functions are well documented in these organisms. Searches for the Psp system across phyla reveal conservation of only one protein, PspA. However, examination of Firmicutes and Actinobacteria reveals that PspA orthologs associate with non-orthologous regulatory and effector proteins retaining functions similar to those in Gram-negative counterparts. Conservation across phyla emphasizes the long-standing importance of the Psp system in prokaryotes, while inter- and intra-phyla variations within the system indicate adaptation to different cell envelope structures, bacterial lifestyles, and/or bacterial morphogenetic strategies.

Trends

Multigene Psp systems, initially described in Gram-negative bacilli, also exist in a large number of rod-shaped bacteria among Firmicutes and Actinobacteria.

A key effector of the Psp response, PspA, is conserved across bacterial phyla; conservation even extends to chloroplasts of higher plants.

Additional regulatory and effector proteins of the Psp response differ markedly among bacterial phyla.

The genomic organization of the Psp genes within Firmicutes and Actinobacteria is surprisingly variable.

Comparisons of variants of the multigene Psp system are expected to reveal mechanistic and evolutionary features of this important stress-response system.

Prelude: Mechanisms for Preserving Cell Envelope Homeostasis

Bacteria, whether infecting a living host or existing in a free-living state, have evolved adaptive responses to environmental change, host factors, and competition within their microenvironments. Maintaining cell envelope homeostasis is paramount to cell function: the cell envelope is the interface with the outside world, and it regulates the two-way movement of substances having various sizes and chemical composition. The envelope also houses danger-sensing systems, such as dedicated two-component systems and accessory sigma factors that activate global adaptive response pathways (for examples, [1–4]). One mechanism for maintaining envelope integrity is the phage-shock-protein (Psp) response, a multigene system involving a cascade of protein interactions that stabilize the cell membrane during times of stress [5–7].

The Psp response was discovered by Peter Model and his colleagues at the Rockefeller University in New York City. They noticed that infection of *Escherichia coli* cells by the filamentous phage f1 and production of the phage-encoded secretin (a pore-forming protein) caused massive production of a bacterial protein they called PspA [8]. The Psp response was subsequently found to include multiple, coregulated genes that express regulatory and effector functions [5–7]. Psp functioning has been linked to bacterial virulence and to multiple bacterial phenotypes, among which are susceptibility to membrane-perturbing antibiotics and the ability to form biofilms [4,5,9]. Thus, the Psp response is a key component of bacterial physiology; as such, a comprehensive understanding of the system may lead to novel targets for antimicrobial therapeutics.

Until recently the Psp system had been studied almost exclusively in Gram-negative enteric bacteria, such as *E. coli*, *Yersinia enterocolitica*, and *Salmonella enterica* serovar Typhimurium (reviewed in [5–7]). Nevertheless, phylogenetic analyses did show that orthologs of PspA, the namesake protein of the system, are ubiquitous among phyla in Eubacteria and Archaea; they

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are even found in the thylakoid membranes of *Cyanobacteria* and plant chloroplasts [5,6]. Thus, PspA is a conserved component of prokaryotic and photosynthetic plant cells, and the system appears to have ancient origins. Recent work has indicated that the Psp response varies considerably among bacterial phyla. For example, in *Bacillus subtilis*, a member of the Firmicutes, the antibiotic-stress-responsive Lia (lipid II cycle-interfering antibiotic) system expresses a PspA ortholog (LiaH) along with unique regulatory proteins and a protein that anchors LiaH to the membrane [10,11]. Moreover, *Mycobacterium tuberculosis*, a pathogenic member of Actinobacteria, possesses a conserved PspA element coupled to yet another set of unique regulatory and membrane-binding proteins [12]. Thus, the *pspA* determinant is evolutionarily conserved, but the neighboring genomic context is not.

For historical reasons we consider the Psp response of enteric bacteria to be the main 'theme' of this membrane-stabilizing system, while its counterparts in Firmicutes and Actinobacteria are taken as variations on the theme. Reviewing such 'Psp variations' might provide a context for comparative studies and reveal novel functional and evolutionary features. We (i) briefly review the key characteristics of the Psp system of enteric bacteria, *B. subtilis*, and *M. tuberculosis*, and (ii) analyze conservation and genomic context of the *pspA* determinant in Firmicutes and Actinobacteria. The results lead to hypotheses concerning mechanistic and evolutionary aspects of the Psp system. Due to our focus on bacterial cell envelope stress, we do not discuss Vipp1/IM30, the PspA ortholog associated with the thylakoid membrane systems of photosynthetic organisms; it is the subject of recent elegant biochemical studies [13,14] and recent reviews [15,16].

The Theme: The Psp System of *E. coli* and Other Enterobacteria

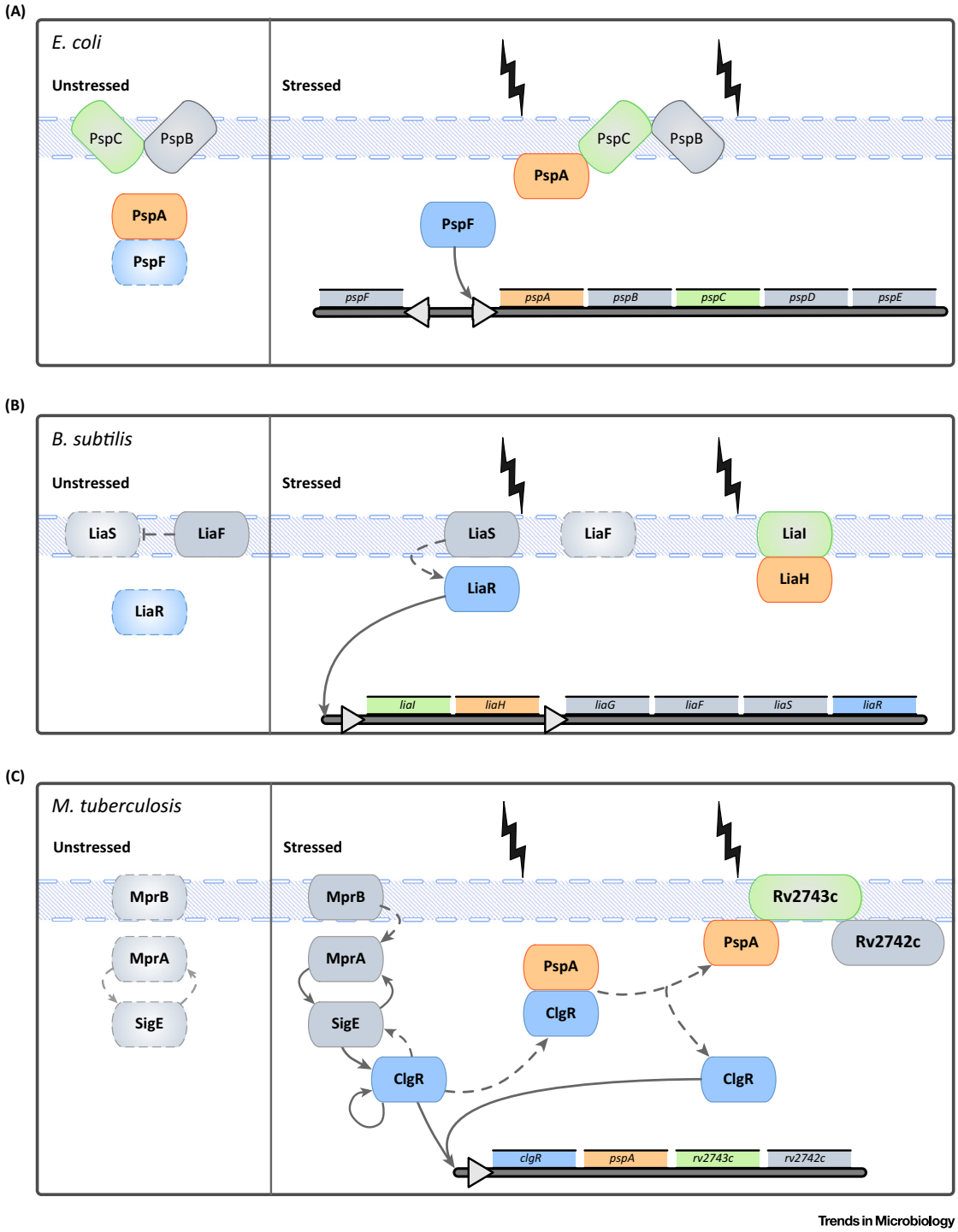
The Psp system of Gram-negative bacteria consists of a four-gene 'minimal' module composed of *pspF* and *pspABC* [5,6,17] (Figure 1A, Key Figure). PspF is an enhancer-binding protein of the AAA + ATPase family that activates the stress-responsive RpoN (σ^{54}) subunit of RNA polymerase [18]. The second locus, *pspABCDE* in *E. coli*, is adjacent to and divergently transcribed from *pspF*. *pspF* is constitutively expressed, while transcription of the *psp* operon depends on PspF and induction of σ^{54}. The *psp* regulon also includes the distantly located gene *pspG* [19,20]. The role of distal (and distant) genes in the regulon, such as *pspD* and *pspE* of *E. coli* and *pspG*, remains poorly defined (reviewed in [21]). The system responds to a variety of general stressors, such as heat, osmotic shock, ethanol, and uncouplers (reviewed in [21]). In addition, the *psp* regulon may be uniquely induced by the mis-association with the inner membrane of secretins, pore-forming proteins that typically associate with the outer membrane [20,22].

The Psp system involves multiple, stress-dependent interactions among its key components: the transcription factor PspF, the peripheral inner-membrane protein PspA, and the integral membrane proteins PspBC (most recently reviewed in [7]). A central characteristic of the system is the dual activity of PspA. In the absence of stress, PspA inhibits PspF activity through direct protein–protein interactions ('regulatory' PspA). In envelope-stressed cells, PspA releases PspF from inhibition. PspF then becomes competent to activate the σ^{54}-dependent transcription of the *psp* regulon. PspA establishes interactions with membrane-bound PspBC and also directly with the membrane (membrane-stabilizing, 'effector' PspA). Thus, the Psp response revolves around two stress-induced, partner-switching mechanisms: (i) PspA transitions from the regulatory PspA-F complex to the effector, membrane-bound state [23], and (ii) the C-terminal portion of PspC is released from the PspB interaction and can bind PspA [24,25] (Figure 1A).

The Psp system is highly dynamic. Imaging of live *E. coli* cells supports a model in which, in the absence of stress, PspF is found in dynamic foci associated with the nucleoid and with the inner membrane at the cell poles [26]. In response to stress, PspF foci become less dynamic, and PspF is predominantly found at the nucleoid. Concurrently, PspA also undergoes multiple

Key Figure

The Psp Systems of *Escherichia coli* (A), *Bacillus subtilis* (B), and *Mycobacterium tuberculosis* (C)



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changes. Regulatory PspA is found predominantly as a hexamer and tends to associate with polar regions of the cell membrane, while effector PspA is a higher-order (36-subunit) multimer that binds to lateral regions of the membrane [26,27]. Further complexities exist. Recent work raises the possibility that PspF activity can be altered without dissociation of the PspA-F complex [28]. Moreover, PspA can associate with the membrane independently of PspBC, when expressed at high levels [23]. The independent association of PspA and PspBC with the cell membrane is consistent with the idea that PspA and PspBC may respond to different stress signals and/or relieve the membrane from damage caused by different stressors (reviewed in [7]). Indeed, PspA can block proton leakage of damaged membranes independently of PspBC, at least *in vitro* [29]. Likewise, PspBC may play a role in secretin-stress tolerance that is independent of its regulation of PspA function [30]. Thus, the Gram-negative Psp system appears to exert two independent effector functions that are expressed by proteins, PspA and PspBC, that also (directly or indirectly) regulate each other.

The signal(s) inducing the Psp response in Gram-negative bacteria remains unidentified. Neither dissipation of proton motive force nor redox state of the quinone pool have proven to be convincing candidates (reviewed in [7]). It was recently proposed, as is the case for other peripheral membrane proteins [31], that a feedback mechanism exists between biophysical properties of the membrane, such as stored curvature elastic (SCE) stress, and PspA [32]. SCE stress is the energy stored in the two monolayers of biological membranes when they are forced to flatten in the bilayer configuration [33]. As this stress increases, PspA binding increases, perhaps relieving SCE stress [32] and preventing unfavourable phase transitions of the membrane bilayer [33]. Changes in SCE stress (or consequent membrane alterations) have been proposed to be the unifying stress signal for Psp induction; from this perspective, the countering of dissipation of proton motive force by PspA might be secondary to its effect on SCE stress [32]. Since SCE stress is independent of membrane lipid composition, a relationship between PspA and SCE stress could explain why PspA function is conserved, and even exchangeable, among systems as diverse as *E. coli* and *Arabidopsis thaliana* chloroplasts [34]. Thus, a central role of SCE perturbations in the Psp response is plausible. However, since no method currently exists to measure SCE levels *in vivo*, it is unknown how changes in SCE would induce the *psp* operon and how the PspBC complex would participate in SCE homeostasis [7].

The Psp system affects the virulence of enteric bacteria (reviewed in [9]). For example, deletion of Psp functions renders *Y. enterocolitica* avirulent in a mouse model of infection, presumably due to the inability of *psp*-deficient mutants to counter the membrane-perturbing effects associated with induction of the Ysc type III secretion system during infection [35]. In another example, loss of virulence associated with *pspA* deletion in *Salmonella* spp. has been attributed to the inability to counter the limitation of divalent cations imposed by the metal-transport function of the host natural resistance-associated macrophage protein (NRAMP) [36]. This effect is presumably due to the dissipation of proton motive force associated with *pspA* inactivation [36]. With *E. coli*, *psp* defects lead to reduced biofilm formation and reduced generation of antibiotic-tolerant persister cells [37,38]. These two bacterial phenotypes may affect infection outcome; thus, little doubt exists that the Psp response is biologically important.

Figure 1. This figure highlights interactions and functional parallels among orthologous and non-orthologous proteins in the three Psp systems rather than mechanistic/localization properties that are reviewed elsewhere [7]. Each factor in the system is indicated by a box. For regulators, the boxes are marked with broken borders when the protein is inactive and with solid borders when the protein becomes active. In all three panels, the system-specific regulator is shown in blue, the PspA ortholog is shown in orange, while the cognate membrane-targeting protein is shown in green. All other proteins are in grey boxes. Lines connecting boxes are solid when indicating transcriptional effects and broken when indicating post-translational effects. Arrowhead, positive regulation; barhead, negative regulation. Stress is indicated by a lightning bolt.

Variation 1: The Two Psp Systems of *B. subtilis*

The genome of *B. subtilis* encodes two PspA paralogs: PspA and LiaH. The gene annotated as *pspA* is regulated by the accessory sigma factor σ^W, which directs transcription to genes involved in protecting the cell membrane from permeabilization by lantibiotics (antimicrobial peptides that are produced by Gram-positive bacteria [39]). The σ^W regulon includes *sppA*, which encodes a signal-peptide peptidase hypothesized to degrade lantibiotics, and the operons *yceCDEFGHI* and *yvlABCD* [40]. While the functions of these operons are largely unknown, two proteins, YceH and YvlC, lower susceptibility to the lantibiotic nisin. Interestingly, YvlC is an ortholog of *E. coli* PspC, but with about half the length and only ~30% amino acid sequence identity. The effects of inactivating *pspA* and *yvlC* on nisin resistance are additive, indicating that the two functions are independent, at least in part [40]. This result is consistent with PspA and PspC having independent effector functions in Gram-negative bacteria, as described above.

The gene encoding LiaH, the other PspA ortholog in the *B. subtilis* genome, lies within the *lialHGFSR* operon. The operon also encodes the integral membrane protein LiaI and the three-component regulatory system LiaFSR [41] (Figure 1B). In the absence of stress, a weak, constitutive promoter upstream of *liaG* (a gene of unknown function) maintains basal levels of the *liaGFSR* transcript [42]. Under these conditions, the activity of the sensor protein LiaS, which has a dual kinase–phosphatase activity, is inhibited by the membrane-bound LiaF, presumably via direct protein–protein interactions [42,43]. The cognate response regulator LiaR is not phosphorylated and remains inactive. However, when cells are exposed to envelope-perturbing antibiotics, such as bacitracin, vancomycin, nisin, or daptomycin, the inhibitory activity of LiaF ceases, and LiaS phosphorylates LiaR. Phosphorylated LiaR binds to the *liaI* promoter region and induces strong expression of the *lialHGFSR* operon [11,43] (Figure 1B). Excess production of LiaF relative to the LiaS sensor protein is required for the stress responsiveness of the system, but the biochemical basis of the LiaF inhibitory activity and the effect of stress conditions on physical interactions between LiaS and LiaF remain to be determined [43]. Regulation by a three-component system sets the Lia response of *B. subtilis* apart from its Gram-negative counterpart.

In the absence of stress, LiaH (the PspA ortholog) appears to be dispersed in the cytosol, while the integral membrane protein LiaI localizes in highly motile foci associated with the cell membrane. Following stress-mediated induction of the operon, the concentration of these two proteins increases, and LiaI recruits LiaH to numerous static foci on the cytoplasmic side of the cell membrane [10]. The role of LiaIH in the response to membrane-damaging antibiotics suggests that these proteins contribute to preserving membrane integrity, as is the case with the Psp response of Gram-negative bacteria [40,44]. Whether LiaH also exerts regulatory functions is unclear. A modest inhibitory effect of LiaH on the *liaI* promoter has been observed in stressed cultures [42], and positive autoregulation of LiaIH has also been postulated [45]. Thus, LiaH lacks the prominent regulatory function exerted by PspA over the cognate transcription factor PspF in Gram-negative organisms.

The two PspA paralogs of *B. subtilis* exhibit functional similarities. Deletion analysis of *pspA*, *liaIH*, and *yvlABCD*, singly and in combination, indicates that the three loci contribute independently to nisin resistance; moreover, *liaH* and *pspA* partially complement each other [40]. Thus *liaH* and *pspA*, which differ in genomic context and gene regulation, may encode at least partially overlapping functions related to protecting the bacterial envelope from antibiotic-induced damage.

Variation 2: The Psp System of *M. tuberculosis*

While the genomes of all sequenced mycobacteria (with the exception of *Mycobacterium leprae*) carry *pspA*, no orthologs of the previously identified *psp* genes map next to mycobacterial *pspA*.

In *M. tuberculosis, pspA* is encoded as part of a four-gene operon comprising the transcriptional regulator *clgR*, *pspA*, and two genes of unknown function, *rv2743c* and *rv2742c* (*M. tuberculosis* genes are numbered according to Cole *et al.* [46]) (Figure 1C). This four-gene operon, which is regulated by the envelope-stress-responsive accessory sigma factor σ^E and by ClgR itself (Figure 1C), has regulatory and functional properties reminiscent of the Psp system of enteric bacteria. When *M. tuberculosis* is subjected to surface stress or treatment with uncouplers, expression of the four-gene operon is induced. Expression profiles of ClgR target genes and protein–protein interaction analyses [12] suggest that three sets of events occur in stressed cells: (i) the four-gene operon is induced, (ii) PspA forms a complex with ClgR and inhibits ClgR activity, and (iii) PspA releases ClgR and associates with the Rv2743c/Rv2742c protein complex; then ClgR resumes its transcriptional regulatory activity (Figure 1C). It also appears that the multiprotein complex of PspA, Rv2743c and Rv2742c is targeted to the cell membrane, since the integral membrane protein Rv2743c can establish direct contacts with both PspA and Rv2742c [12]. Indeed, subcellular fractionation studies of *M. tuberculosis* identify both PspA- and Rv2742c-derived peptides in the cell membrane fraction [47], despite the absence of transmembrane domains or lipid-anchoring motifs in these proteins. Thus, as in the Gram-negative system, PspA of *M. tuberculosis* inhibits the activity of the cognate regulator and associates with an integral membrane protein, presumably through a partner-switching mechanism.

Our understanding of the role played by the Psp system in mycobacterial biology is still incomplete. Inactivation of *clgR* results in increased susceptibility to surface stressors, and a *clgR* deletion mutant contains reduced levels of ATP following surface stress, consistent with the maintenance of proton motive force seen with the Gram-negative system. Moreover, mutants deficient in *clgR* or *rv2743c-rv2742c* are unable to grow inside macrophages, suggesting a positive effect of the Psp system on mycobacterial virulence [12]. A role for PspA in lipid storage and nonreplicating persistence of *M. tuberculosis* has also been proposed, since PspA is associated with lipid inclusions that accumulate in dormant mycobacteria [48]. Similar observations have been made with *Rhodococcus*, where PspA was found in the lipid droplet proteome [49]. Thus, the mycobacterial Psp system likely exhibits envelope-preserving functions similar to those seen with members of other phyla. Moreover, the Psp system may have adopted additional functions uniquely suited to actinobacterial physiology.

The Structure of the Theme and Variations

The three Psp systems sketched above (Figure 1) share two key features. The most obvious is the universal presence of PspA, which is known or likely to be a peripheral inner-membrane protein that prevents dissipation of proton motive force. Another conserved feature is the presence of at least one integral membrane protein that targets PspA to the plasma membrane. While the membrane-anchoring function appears to be conserved, the particular protein(s) expressing the function is not.

A major distinction among the three bacterial systems depicted in Figure 1 concerns regulation (Box 1). The PspA proteins of *E. coli* and *M. tuberculosis* can establish direct interactions with the cognate transcriptional regulators and integral membrane proteins. Thus, it is likely that the regulatory, partner-switching mechanism described with PspA of Gram-negative bacteria also occurs in *M. tuberculosis*, but with non-orthologous partners. By contrast, the Lia system of *B. subtilis* has evolved a regulatory mechanism in which the switch element presumably lies in the relative balance between phosphatase and kinase activities of the sensor protein LiaS. The *pspA* gene of *B. subtilis* may exemplify yet another regulatory mechanism that involves direct regulation by an accessory sigma factor. In this case, *pspA* lacks the neighboring genes known to contribute to the Psp response. Whether novel, coregulated partner functions exist for this *pspA* paralog is currently unknown.

Box 1. Transcriptional Regulation of Known Psp Systems

In enterobacteria, transcription of the *psp* operon is induced by PspF, a regulator encoded by a gene adjacent to and divergently transcribed from the *psp* operon. PspF activates transcription from RpoN (σ^{54}) promoters such as that regulating the *psp* operon. In the absence of stress, PspA binds to PspF and prevents PspF interaction with RpoN. During stress, recruitment of PspA to the cell membrane (directly and/or via PspB/PspC) results in release of PspF from PspA-mediated inhibition, PspF interaction with RpoN, and induction of the Psp response (reviewed in [5–7]).

In *Mycobacterium tuberculosis*, the Psp response is expressed by the *clgR-pspA-rv2743c-rv2742c* operon. PspA binds to the positive transcriptional regulator ClgR, and prevents ClgR from binding to DNA. Following exposure to stress, release of ClgR from PspA-mediated inhibition requires functional Rv2743c/Rv2742c. It is presumed that the Rv2743c/Rv2742c complex targets PspA to the membrane [12]. Despite the regulatory parallels between enterobacteria and *M. tuberculosis*, no protein sequence/structure commonalities exist between ClgR and PspF, or between PspBC and Rv2743c/Rv2742c.

In *Bacillus subtilis*, *liaH* (encoding the PspA ortholog), a member of the *liaIHGFSR* operon, is regulated by the three-component regulatory system LiaFSR. A weak, constitutive promoter upstream of *liaG* guarantees expression of the last four genes in unstressed cells. The membrane-bound, inhibitory protein LiaF locks the sensor LiaS, a dual histidine kinase/phosphatase, in its phosphatase configuration. During stress, the LiaS kinase activity is relieved from inhibition, and the response regulator LiaR is phosphorylated. Phosphorylated LiaR binds upstream of *liaI*, inducing expression of two multicistronic transcripts, *liaIH* and *liaIHGFRS* [11,42,43].

Of the three systems summarized above, the system found in *B. subtilis* utilizes a unique regulatory paradigm, in which positive regulation of the *lia* operon is provided by a response regulator. Stress responsiveness is provided by effects of LiaF on LiaS activity and, consequently, on LiaR phosphorylation and activation. Despite coregulation, the products of *liaFSR* are produced at different levels (LiaF > LiaS > LiaR), which are critical for proper stress response [43].

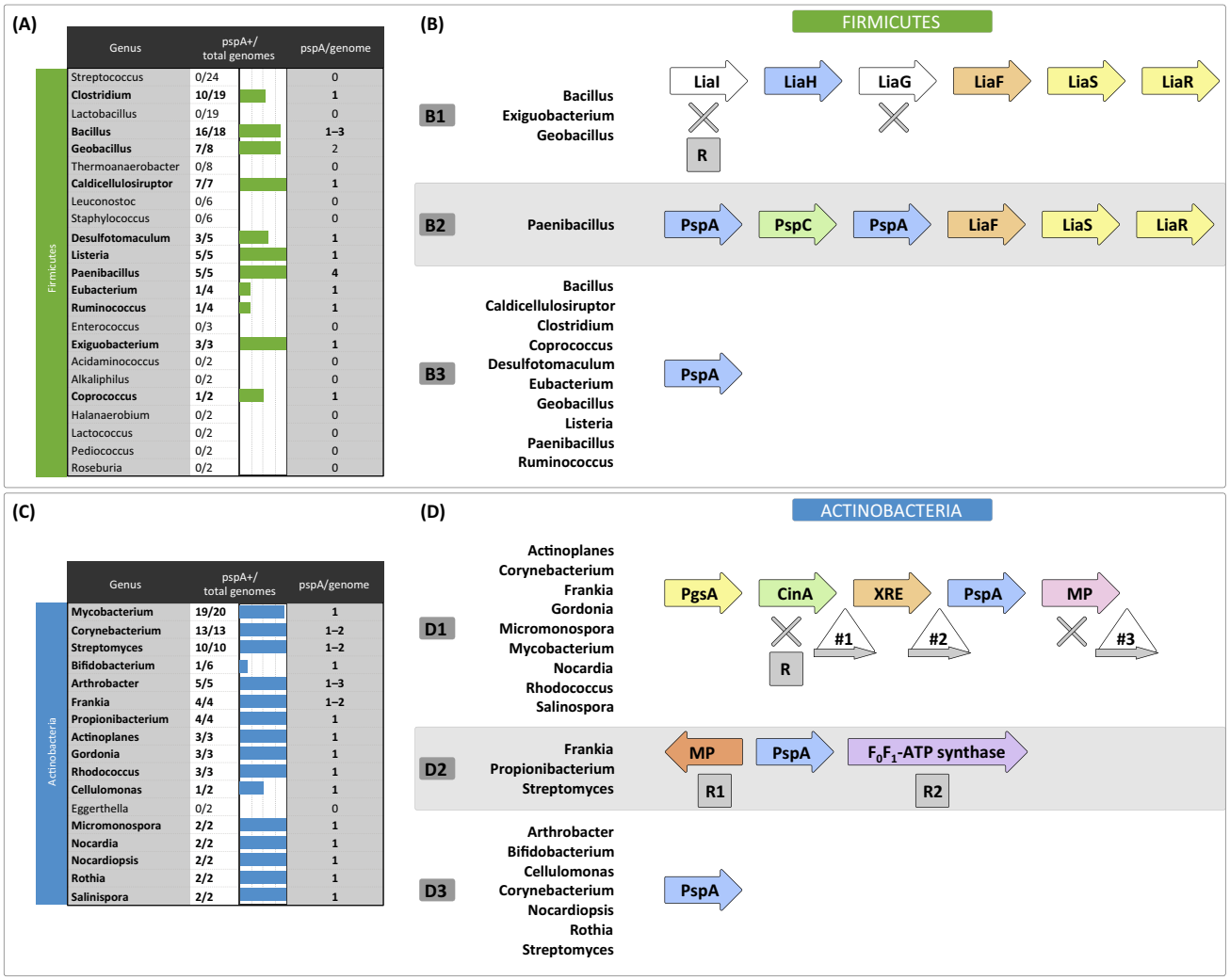
Common aspects notwithstanding, important dissimilarities exist between the Psp systems of enterobacteria and *M. tuberculosis*. While *pspF* is independently transcribed from a constitutive promoter, *clgR* is the first gene of the *psp* operon, and its promoter is subjected to dual stress-responsive regulation: from ClgR itself and from the accessory sigma factor σ^{E}, which is in turn induced by the surface stress-responsive two-component system MprAB. Moreover, the inhibitory regulator–PspA complex forms prior to stress in enterobacteria but only after stress in *M. tuberculosis*.

The Psp systems of *B. subtilis* and *M. tuberculosis* represent a profound departure from the theme defined by enterobacteria, and they differ from each other. Thus, a closer look at the configuration of the Psp system beyond Gram-negative bacteria is warranted. Below we present an analysis of Firmicutes and Actinobacteria.

Six Variations: The PspA System in Firmicutes and Actinobacteria

Three Variations in Firmicutes

In the phylum Firmicutes, not all genera contain *pspA* (approximately half of those shown in Figure 2A are *pspA*-positive). All *pspA*-containing genomes in the class Clostridia belong to the family Clostridiaceae and contain a single copy of the gene (including *Clostridium*, *Caldicellulosiruptor*, *Desulfotomaculum*, *Eubacterium*, *Ruminococcus*, and *Coprococcus*) (Figure 2A). In contrast, the class Bacilli includes families containing a single *pspA* copy (Listeriaceae) or one or more copies (Bacillaceae and Paenibacillaceae) per genome (Figure 2A). Three configurations were discerned in terms of *pspA* genomic context (Figure 2B). The first, exemplified by the Lia system of *B. subtilis*, is found only in Bacillaceae and Paenibacillaceae (Figure 2B B1). Absence of *liaG* and/or *liaI*, or replacement of *liaI* with a low-homology ortholog (30% amino acid sequence identity), define secondary variants of this configuration. A second configuration is found in *Paenibacillus* spp., where the *liaIH* module is replaced with two copies of *pspA* separated by a gene annotated as *pspC* (encoding a protein with ~20% amino acid sequence identity with *E. coli* PspC) (Figure 2B B2). Both of these configurations are characterized by the presence of an integral membrane protein (LiaI, or LiaI orthologs, or PspC orthologs). The PspA-membrane-targeting function appears to be conserved, although the protein exerting that function is not conserved. A third configuration is represented by an 'orphan' version of *pspA*, that is, *pspA* is located in a genomic region that lacks known determinants of the Lia/Psp



Trends in Microbiology

Figure 2. Structure of *psp* Loci in Firmicutes and Actinobacteria. A total of 348 fully-sequenced, representative/reference genomes of the phyla Firmicutes and Actinobacteria in the PATRIC database [68] (one genome per species) were selected for analysis. The presence of genes encoding PspA orthologs (within ± 50% of the query protein length) was determined using five iterations of PSI-BLAST and an E-value cutoff of 1E-5 based on primary amino acid sequence (which corresponds to ~27% sequence similarity). Multiple PspA query proteins were used (*E. coli* PspA, *B. subtilis* PspA and LiaH, and *M. tuberculosis* PspA) to reduce species-specific bias. Only genera having two or more genomes analysed are shown in the figure. (A, C) List of genomes analysed for the presence of genes coding PspA orthologs (loosely indicated as *pspA*) in *Firmicutes* (A) and *Actinobacteria* (C). For each genus, the first column shows the number of *pspA*-containing genomes (*pspA*$^+$) versus the total number of analysed genomes, the second column is a graphic depiction (expressed as %) of the data in the first column, while the third column shows the number of *pspA* genes per genome. (B, D) structure of *psp* loci in Firmicutes (B) and Actinobacteria (D). Arrow-shaped boxes indicate conserved genes, while the name inside the box indicates either the gene or the protein encoded by the gene. An X below a gene box indicates absence of the corresponding gene in some genomes; a boxed R indicates that the corresponding gene is replaced with another gene in some genomes (R has a numbered subscript when more that one gene replacement was identified); numbered, small arrows at the base of a triangle indicate insertion of one or more genes in the corresponding position in some genomes. Abbreviation: MP, membrane protein.

response. This third configuration characterizes (i) all genomes of Listeriaceae and Clostridia and (ii) the second *pspA* copy in Bacillaceae and Paenibacillaceae (Figure 2B B3).

Three Variations in Actinobacteria

In the phylum Actinobacteria, all genera in the class Actinobacteria have at least one copy of *pspA*; *Eggerthella*, which lacks *pspA*, belongs to the class Coriobacteria (Figure 2C). In this phylum, the number of *pspA* determinants per genome appears to be associated with genus

rather than with higher-order taxonomic classifications. Of the three genera most represented in reference genome lists, most species in *Mycobacterium* (19/20) and *Streptomyces* (8/10) carry a single copy of *pspA*, while most species of *Corynebacterium* (12/13) carry two copies. Three configurations of *pspA* genomic context are seen in Actinobacteria (Figure 2D). One is represented by the Psp system of *M. tuberculosis*. In this configuration (Figure 2D D1), *pspA* maps between an upstream gene for a transcriptional regulator of the XRE_HTH family of DNA binding proteins (ClgR) and a downstream gene encoding an integral membrane protein. Interestingly, in all nine genera exhibiting this configuration, the three-gene module is preceded by the same two genes, *pgsA* (encoding a phosphatidylglycerophosphate synthase) and *cinA* (encoding a molybdopterin-binding competence-inducible protein), indicating conservation of genomic context. Secondary variants of this first configuration exist. In *Frankia* spp., intervening genes are found adjacent to the XRE-family regulator gene (either upstream or downstream; insertions #1 and #2 in Figure 2B B1). Species in the *M. tuberculosis* complex uniquely contain an additional, fourth gene in the ClgR-regulated operon (insertion #3 in Figure 2B B1). This fourth gene, of unknown function, is required for full functioning of the Psp system in *M. tuberculosis* [12] (Figure 1C). The second configuration among Actinobacteria is characterized by the presence of a gene encoding an F_0F_1-ATP synthase downstream of *pspA*. In this configuration, the gene upstream from *pspA* usually encodes an integral membrane protein (Figure 2D D2). The presence of an adjacent ATP synthase gene is intriguing, given that a function attributed to PspA is to prevent dissipation of proton motive force, and that disruption of genes encoding subunits of the F_0F_1-ATP synthase induces the Psp response in *Y. enterocolitica, S. enterica* serovar Typhimurium, and *E. coli* [29,50,51]. Indeed, in *Propionibacterium* spp., genes encoding components of the electron transport chain map downstream from the configuration shown in Figure 2D D2, indicating proximity of *pspA* to genes involved in generation of proton motive force and ATP synthesis. As seen in Firmicutes, a third configuration is characterized by an orphan copy of *pspA* (Figure 2D D3). The orphan *pspA* may constitute either the only copy in the genome or the second copy within genomes carrying the configurations shown in Figure 2D D1–D2.

Counterpoint: Paralogs of *pspA*

The presence of multiple *pspA* copies in the genomes of many species may be explained in several ways. First, *pspA* paralogs may express additive functions. For example, a *liaH pspA* double mutant of *B. subtilis* exhibits more severe defects than either single mutant [40]. Second, regulation of different *pspA* loci by factors responding to different stressors might result in expression of the PspA membrane-stabilizing function under a range of stress conditions. This idea currently lacks experimental support. Third, since increased production of PspA circumvents the requirement for membrane-targeting partners in *Y. enterocolitica* [23], production of PspA from multiple genes may result in protein levels that render PspA partitioning to the membrane independent of membrane-targeting partners. Functional characterization of the individual paralogs and identification of the genes that regulate the orphan *pspA* copies are necessary to test the above scenarios.

Concluding Remarks

Mechanistic studies of the multigene Psp configurations among representative members of various bacterial phyla, together with an analysis of the genome context of the *pspA* determinant in Firmicutes and Actinobacteria, yield several insights. First, with its combination of a conserved determinant (*pspA*) plus non-orthologous proteins expressing additional conserved functions (transcription factors and integral membrane proteins), the multigene Psp system constitutes an example of diverse evolutionary solutions to a common requirement (preservation of membrane function during stress). In this view, the structure of the integral membrane proteins in the system may reflect profound differences in the inner membrane among bacterial genera [52,53]. These differences may include the chemical nature of integral and/or non-integral components of the

Outstanding Questions

Can the inter- and intra-phyla variations of the Psp system be used as a case study for how evolutionary variation fits conservation of a critical function – in this case, envelope preservation – with different bacterial lifestyles or cellular structures?

Why is the presence of PspA strongly associated with the rod-like shape? Does this association reflect particular shape-maintenance requirements of cylindrical cells and/or stress susceptibilities of their cell envelope?

In Gram-negative bacteria, PspA and PspBC express two different effector functions, and they also regulate each other (directly or indirectly) by partner-switching. *M. tuberculosis* also has a pair of proteins, PspA and Rv2743c, that perform reciprocal regulation. Does the Psp system of *M. tuberculosis* (and other Actinobacteria sharing the same module) also express two effector functions?

What is the role of *pspA* paralogs? How are the orphan variants of *pspA* regulated? Do they require a membrane-targeting partner?

The Psp module of the *M. tuberculosis* complex carries two genes downstream from *pspA*, while nontuberculous mycobacteria and other actinobacteria only have one. What is the role of the gene that is present only in tuberculous mycobacteria?

membrane (lipids and proteins) and/or the membrane's biophysical properties. Likewise, the variations in the gene-regulatory functions in the Psp system likely reflect differences in regulon structure of envelope-related functions in different phyla. For example, the σ^{54} regulon of *E. coli* and related bacteria, which includes the *psp* operon, appears to be associated with cell envelope biogenesis and surface structures [54]. Additionally, the σ^{W} regulon of *B. subtilis*, which regulates *pspA*, and the MprAB-σ^{E} network of *M. tuberculosis*, which regulates the *clgR-psp* operon, control envelope-stress responses of their respective organisms [3,55]. Second, the intra-phylum variations of the multigene Psp configurations observed in Firmicutes and Actinobacteria may reflect different life styles. Indeed, the phylum Actinobacteria exhibits remarkable biodiversity among its members [56]. Third, comparing Psp configurations across genera may also lead to identification of chromosomal rearrangements. For example, *Streptomyces* spp. carries both *pspA* (Figure 2C) and *clgR* [57], but it lacks the actinobacterial configuration in which the two genes are adjacent (Figure 2D D1). Thus, studying the structure of Psp variations might shed light on genome remodeling events. Fourth, many species were found to carry two or more copies of the *pspA* determinant. These additional copies typically lack the neighbouring genomic organization of any of the known multigene configurations. These orphan *pspA* configurations may reveal additional variations of the Psp theme (novel regulators and/or novel membrane-targeting partners). Alternatively, they may show that PspA functions independently from (coregulated) integral-membrane proteins. In other cases, they may even reveal that the presence of a PspA homolog is not accompanied by a Psp-like stress response. For example, the YjfJ paralog found in *E. coli* lacks structural domains that are critical for the known PspA activities (interactions with other Psp proteins and the inner membrane, and the ability to oligomerize) [58]. We expect comparative studies across Psp configurations to reveal regulatory and functional properties of the system and each of its components more effectively than the investigation of individual systems (see Outstanding Questions).

In Gram-negative species, Firmicutes and Actinobacteria, the multigene Psp system is predominantly found in rod-shaped cells (typically bacilli and, in a few cases, coccobacilli or elongated cells). An association between Psp function and bacterial rod-like shape is supported by reports of direct and indirect interactions of PspA with the cytoskeleton proteins MreB and RodZ of *E. coli* [59,60], which are implicated in maintaining the rod-like cell shape of this microorganism [61–63]. Moreover, since cell shape results from the reciprocal influence of mechanical forces and chemical interactions [64,65], the above-mentioned link between PspA binding to the membrane and mechanical properties of the membrane [32] further suggests that Psp function, maintenance of membrane integrity, and the bacterial rod-like shape may be connected. Finally, we note that the cytoskeletal, actin-like proteins of *E. coli*, *B. subtilis*, and Actinobacteria differ from each other [66,67], raising the intriguing possibility that, across bacterial phyla, variations in the multigene Psp system may have evolved in concert with variations occurring in particular classes of cytoskeletal proteins. Thus, hitherto unrecognized relationships may exist between Psp function and bacterial morphogenesis. Investigating these relationships may open a new chapter in the fast-evolving studies on the chemistry and biophysics of the making of cells.

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Supplemental Information

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