The Evolution of the Phage Shock Protein Response System: Interplay between Protein Function, Genomic Organization, and System Function

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Abstract

Sensing the environment and responding appropriately to it are key capabilities for the survival of an organism. All extant organisms must have evolved suitable sensors, signaling systems, and response mechanisms allowing them to survive under the conditions they are likely to encounter. Here, we investigate in detail the evolutionary history of one such system: The phage shock protein (Psp) stress response system is an important part of the stress response machinery in many bacteria, including Escherichia coli K12.

Here, we use a systematic analysis of the genes that make up and regulate the Psp system in E. coli in order to elucidate the evolutionary history of the system. We compare gene sharing, sequence evolution, and conservation of protein-coding as well as noncoding DNA sequences and link these to comparative analyses of genome/operon organization across 698 bacterial genomes. Finally, we evaluate experimentally the biological advantage/disadvantage of a simplified version of the Psp system under different oxygen-related environments.

Our results suggest that the Psp system evolved around a core response mechanism by gradually co-opting genes into the system to provide more nuanced sensory, signaling, and effector functionalities. We find that recruitment of new genes into the response machinery is closely linked to incorporation of these genes into a psp operon as is seen in E. coli, which contains the bulk of genes involved in the response. The organization of this operon allows for surprising levels of additional transcriptional control and flexibility. The results discussed here suggest that the components of such signaling systems will only be evolutionarily conserved if the overall functionality of the system can be maintained.

Key words: comparative genomics, bacteria, signaling system, phage shock stress response, signal transduction network.

Introduction

The ability to process and respond to environmental signals is an essential feature of living organisms. A change in the environment, for example, when a microbial pathogen enters its host, can have profound effects on the internal state and dynamics. Typically, stress response mechanisms are then initiated in order to adapt to the new surroundings, which may include changes in oxygen availability, ambient pH, osmolarity, salt concentration, nutrient availability, or attack from the innate and adaptive immune systems. Maintaining suitable response mechanisms is thus a necessary prerequisite for pathogens to establish successful infections and persistence inside susceptible hosts and more generally for survival in changing and often hostile environments. Here, we investigate how the molecular machinery underlying one such response was assembled and has evolved in bacteria.

In Escherichia coli, the phage shock protein (Psp) stress response system is responsible for repairing damage to the inner membrane of the cell (Kobayashi et al. 2007) and maintenance of the proton-motive force (pmf) across the inner membrane (reviewed in Model et al. 1997; Darwin 2005; Jovanovic et al. 2006). The Psp stress response is invoked, for example, during phage infection, secretin production, blockage of protein export or fatty acid/phospholipid biosynthesis, exposure to organic solvents, extreme heat or osmotic shock, high ambient pH, and action of protonophores, and has been characterized in great detail (reviewed in Model et al. 1997; Darwin 2005). The Psp system appears to be widely shared across sequenced Enterobacteria (and more generally gram-negative bacteria) (Darwin 2005; Huvet et al. 2009). The Psp system has also been implicated in the virulence of Salmonella, Shigella, and Yersinia (reviewed in Model et al. 1997; Darwin 2005, 2007; Rowley et al. 2006).

The response in E. coli is mediated by nine proteins that are organized into five units: the psp operon, containing the genes coding for PspA, PspB, PspC, PspD, and PspE; and four single-gene units for PspF, PspG, and the...
two-component system comprised of ArcA and ArcB (see fig. 1). The pspABCDE operon together with pspF and pspG form the Psp regulon. PspF negatively controls its own expression and positively controls the rest of the regulon by binding at the pspA and pspG promoters (Model et al. 1997; Lloyd et al. 2004; Darwin 2005); no other specific binding sites for the transcription factor PspF are known or have been predicted in the E. coli genome.

Although the physiological role of the system and the functions of all the proteins (PspD, PspE, and PspG) have not yet been fully elucidated, the outline regulatory processes have been well characterized in E. coli. Under nonstress conditions, PspA binds and inhibits PspF, forming a PspA–PspF inhibitor complex (Elderkin et al. 2002, 2005; Joly et al. 2009). Under stress conditions, activation of the membrane-bound proteins PspB and PspC, most likely via structural changes in these proteins, mediates the release of PspF from its negative regulation by disrupting the PspA–PspF inhibitory complex. This process initiates, via the binding of PspF to its target upstream DNA-binding sites, the transcriptional response to the stress conditions (Weiner et al. 1991; Gueguen et al. 2009). One main effector of the response appears to be the PspA multimer, which helps to reestablish pmf across the inner membrane (Kobayashi et al. 2007). The nonorthodox two-component signaling (TCS) system consisting of the phosphorelay kinase ArcB and its cognate receptor ArcA influences the strength and effectiveness of the transcriptional response to the inducing stress conditions in microaerobiosis (Jovanovic et al. 2009). However, the ArcB–ArcA system is also known to be involved directly in other stress response systems (Malpica et al. 2006) and so will be considered here in parallel to the genes making up the Psp regulon. Previous data-driven attempts in evolutionary systems biology have focused predominantly on the structure of such networks and how they have changed over (evolutionary) time (Luscombe et al. 2004; Babu et al. 2009). These systems-level evolutionary analyses often investigate the extent of and causes for statistical correlations between evolutionary properties of proteins and their position or role in protein interaction or metabolic networks (Agrafioti et al. 2005; Drummond et al. 2006; Lee et al. 2008; Feist et al. 2009). Here, we focus instead on the function of a particular stress-related signaling network. Adopting this perspective requires us to consider systems that have been experimentally characterized, ideally in several species. For many of the important stress response systems, including osmotic, hypoxia, limitation of combined nitrogen, pH, heat shock, and the phage shock stress response, we do have extensive functional data and, in an increasing number of cases, also mechanistic models with varying degrees of detail and sophistication (Hlavacek et al. 2006).

Here, we perform a systematic and comprehensive analysis of the Psp system by 1) identifying orthologs in 698 fully sequenced bacterial genomes, 2) inferring the patterns of gain and loss of genes over the course of evolutionary history, 3) assessing the rates of sequence divergence and evolution, 4) testing for coevolution among the proteins making up the Psp stress response system, 5) comparing potential Psp stress response systems across different bacterial species, and 6) test experimentally the impact of a simplified Psp system in different oxygen-related environments.

Materials and Methods

Sequences and Annotations

The sequences and annotations for 698 bacterial species considered here were retrieved from the “Genome Assembly/Annotation Projects” data in the NCBI ftp (ftp://
ftp.ncbi.nih.gov/genomes/Bacteria/). For each species, we used the refSeq annotation library.

Ortholog Identification
A reciprocal best hit approach was used to identify orthologs (using Blastall 2.2.19, ftp://ftp.ncbi.nih.gov/blast/executables/LATEST/). Each protein of *E. coli K12* MG1655 was aligned against the full protein set of each of the 697 remaining bacterial species. The first hit of each Blast run was then aligned against the full set of *E. coli K12* MG1655 proteins. Two proteins were defined as potential orthologs if and only if the first hit of the second blast search corresponded to the protein used as a query in the first one (Moreno-Hagelsieb and Latimer 2008).

To minimize the number of false positive, those results were filtered: If the alignment involved less than 50% of the smallest protein and the sequence identity was lower than 30%, the protein pair was excluded from the set of potential orthologs.

In *E. coli APEC O1*, this procedure first did not discover orthologs for PspB and PspD, despite the remaining genes in the *psp* operon showing a genomic organization that was in agreement with the observed one in other *E. coli* species. This is due to erroneous annotation in the published genome sequence as a blastall search revealed homologs (with an almost perfect sequence identity compared with *E. coli K12* MG1655) to both genes at the expected positions. Following this observation, we decided to apply a blastall search for all *psp* genes in a subset of species used in this study close to *E. coli* to evaluate the proportion of potentially missing, or misannotated, genes. This approach revealed four potential missing genes to add to the two from *E. coli APEC O1*.

Phylogenetic Reconstruction
The species phylogeny was inferred using the 23S rRNA (Ludwig and Schleifer 1994). Usually, each species contains several copies of the 23S rRNA, and to apply a classic method of phylogeny inference, one sequence per organism was chosen at random. On this set of 23S rRNA sequence per organism, a multiple alignment was performed using MAFFT 6.611 (http://align.bmr.kyushu-u.ac.jp/mafft/software/) and used in PhyML 2.4.4 (http://atgc.lirmm.fr/phyml/) to infer the species tree. Different alignment and phylogeny inference procedures resulted in very similar trees. Of the 127 species of interest, just 125 species contain sequences annotated as 23S rRNA leading to a tree of 125 species and not 127.

Coinheritance of Gene Pairs
We used the software BayesTraits (http://www.evolution.reading.ac.uk/BayesTraits.html) to compute the likelihood of two evolutionary models: The first model corresponds to independent evolution of the two genes, whereas the second model assumes that their evolution is correlated (or anticorrelated); here, correlation (anticorrelation) means that the two genes are seen together more (less) often than we would expect given their distribution across the phylogeny. A standard likelihood test ratio was then used to test if the independent model could be rejected in the light of the results obtained for the correlated evolution model. Because of the large number of statistical tests involved, we applied a multiple-testing (false discovery rate [FDR]) correction.

Statistical Control for Ortholog Sharing
The system investigated here contains five genes that in *E. coli* reside in the same operon. Because of this, they are more likely to be inherited together and the operon structure thus forms a potentially confounding factor in all further evolutionary analyses. We therefore studied the genes in all other operons that (in *E. coli*) contain five genes. The operon information was obtained from RegulonDB (http://regulondb.ccg.unam.mx/index.jsp), and orthologs were identified along the same lines as described above. For each operon, the number of species containing 0, 1, 2, 3, 4, and 5 orthologs were then computed (from our set of species used in the detailed analysis of the Psp system’s evolution).

We also studied the profile of orthologs for randomly selected set of four orthologs. We extracted 1,000 sets of four proteins not included in operons (according to RegulonDB) and computed (in the species of interest) the number of species containing 0, 1, 2, 3, and 4 orthologs, respectively. Finally, we tested whether the observed number of species containing an ortholog for the four non–operon-based proteins of the Psp system is equivalent to the population of four randomly selected proteins from *E. coli*.

Identification of Extended Operons
The identification of potential extended versions of the *psp* operon was done using intergenic distance and the organization of genes surrounding each ortholog of the *psp* operon genes. Analysis of *E. coli* operons shows that genes in operons tend to be separated by intergenic segments that are shorter than 20 bp, but sometimes segments of 50 bp or more occur (Karimpour-Fard et al. 2008). Therefore, we have used 100 bp as a crude but conservative cutoff to determine whether two genes could be part of the same operon.

Computation of the dN/dS Ratio
The dN/dS ratio was computed with the “kaks” R function from the “seqinr” package (http://cran.r-project.org/web/packages/seqinr/index.html). Multiple alignments were obtained for all sets of orthologous proteins. For each sequence, the amino acids were then replaced by the DNA sequence, maintaining the codon organization. The dN/dS ratio was computed for each ortholog pair using the respective sequences from the multiple alignment. To obtain the percentage identity, the same approach was applied on the amino acid multiple alignment. The computation itself was performed using the “dist.alignment” function (matrix option set to “identity”) of “seqinr.”

Bacterial Strains and Growth Conditions
The wild-type bacterial strain used in this study was MG1655 (Lloyd et al. 2004). The bacterial strain MVA49
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(MG1655ΔpspBC ΔpspG::kan, KanR) was constructed by transducing a ΔpspG::kan (KanR) from MVA40 (Lloyd et al. 2004) into MG1655ΔpspBC (Lloyd et al. 2004). Strain MVA103 (MVA49 (pspA-lacZ), KanR AmpR) was constructed by transducing a φ(pspA-lacZ) (AmpR) from MC4100::psp3 (Dworkin et al. 2000) into MVA49. Transductions were carried out using the P1vir bacteriophage as described in Miller (1992). Strains were routinely grown in Luria–Bertani (LB) broth or on LB agar plates at 37 °C (Miller 1992). For aerobic growth, overnight cultures of cells were diluted 100-fold into 5 ml (or 20 ml for growth rate experiment) of LB in a universal tube with loose-fitting caps and shaken at 200 rpm. For microaerobic growth, overnight cultures of cells were diluted 100-fold into 5 ml (or 20 ml for growth rate experiment) of LB and shaken at 100 rpm. The cells were then taken for either measurements of cell growth rate at OD600, the wild-type Psp response. Cells were grown overnight at 37 °C in LB broth containing the appropriate antibiotic and then diluted 100-fold (initial OD$_{600nm}$ ~ 0.025) into the same medium (5 ml). Following incubation to OD$_{600nm}$ ~ 0.4, cultures were assayed for β-gal activity as described by Miller (1992). For anaerobically grown cells, β-gal activity was measured following overnight growth at 37 °C with no shaking (initial OD$_{600nm}$ ~ 0.010 and after incubation ~ 0.4). For all β-gal assays, mean values of six independent assays taken from technical duplicates of three independently grown cultures of each strain were used to calculate activity. The data are shown as mean values with standard deviation error bars.

**Western Blot Analysis**

For the western blot analysis, bacterial cells were collected (1 ml) at OD$_{600}$ ~ 0.4. Proteins were separated on 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes using a semidry transblot system (Bio-Rad). Western blotting was performed as described (Elderkin et al. 2002) using antibodies to PspA (1:1,000 dilution with anti-rabbit secondary antibody) and antibodies to pIV (a gift from M. Russel) (1:10,000 with anti-rabbit). The proteins were detected using ECL plus Western Blotting Detection Kit according to manufacturers’ guidelines (GE Healthcare). Images were captured in a FujiFilm—intelligent Dark Box by an image analyzer with a charge-coupled device camera (LAS-3000).

**Results**

**Maintenance of psp Genes**

In this study, we investigate the properties of the Psp system across all the bacterial species present in the NCBI database (ftp://ftp.ncbi.nih.gov/genomes/Bacteria/). We first determine the orthologs for the Psp system proteins (see Materials and Methods). The number of genomes containing an ortholog varies considerably between proteins, with a 4-fold difference between the maximum and minimum (fig. 2a, white bar). The most abundant ortholog sets are found, in decreasing order, for pspE, pspC, and arcB, whereas pspF, pspG, and pspD are the least frequently shared genes.

These results contain information from species that have only a single identifiable ortholog to one of the genes associated with the *E. coli* Psp system. Because our interest focuses on the system level and how the Psp stress response system has evolved over time (including through gain and loss of genes), we therefore restrict most of the remaining analyses to organisms containing at least two orthologs to the PspA–G proteins; ArcA and ArcB are known to be involved in several processes (Kim and Cho 2006; Malpica et al. 2006) and are therefore treated differently here, as we expect their involvement in several pathways to have also affected their evolutionary history. This filtering of species impacts differently on each ortholog set (fig. 2a—black bar). This does not affect the results for proteins PspD, F, and G as these are rarely found alone, whereas the number of species containing PspE and/or PspC drops quite considerably (by almost 50% for PspE), as these genes often

**β-Galactosidase (β-gal) Assays**

Activity from a single-copy chromosomal φ(pspA-lacZ) transcribesional fusion was assayed to gauge the level of psp expression. The φ(pspA-lacZ) transcriptionsional reporter fusion was introduced as a single copy into the chromosomal att site to retain the native pspA locus and permit

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occur on their own. This observation already suggests that in many species, cell membrane stress is dealt with by a very different system; it furthermore indicates that some orthologs of PspE and PspC can fulfill functions unrelated to their respective full roles in E. coli and other enterobacteria. The unorthodox TCS system ArcB/ArcA is maintained widely across this phylogenetic panel, underlining its important role in other stress response processes. Interestingly, however, detailed analysis shows that even TCSs can break apart over evolutionary time scales; for example, we find genomes where \textit{arcB} but not \textit{arcA} (and vice versa) orthologs can be identified.

In figure 2, we illustrate the distribution of the system’s constituent genes across all species (with at least one and two orthologs, respectively). The distribution of the numbers of orthologs present in the 127 species takes the shape of an asymmetrical “U,” where the most frequently observed results are for species to have two or all nine genes in their genomes; only a few species have four or five orthologs of the \textit{psp} genes. Over 70% of species with only two orthologs are “Firmicutes” (very few Firmicutes have more than two orthologs). For the “gamma-proteobacteria,” on the other hand (which include the \textit{Enterobacteria} such as \textit{E. coli}, as well as several other biomedically relevant pathogens, e.g., \textit{Salmonella}, \textit{Yersinia}, and \textit{Vibrio}), we find that almost the complete system is present in all the species (fig. 2b). A single “alpha-proteobacterium” is the only other species with six or more orthologs. The “U” shape observed in figure 2b is thus entirely due to the unequal distribution of genes among the different bacterial clades; for each individual clade (e.g., Firmicutes or alpha-proteobacteria), the corresponding distribution tends to be simpler; system composition (or mere presence) is therefore closely associated with the clade structure of the bacterial species considered here.

Moreover, and from a functional perspective probably more interestingly, we find that the manner in which orthologs are shared is not random (see supplementary fig. S1, Supplementary Material online); for example, approximately 65% of species with only two orthologs contain \textit{pspC} and \textit{pspE}. Conversely, a large proportion of species (15 of 22; \textit{P} value < 10\textsuperscript{-8}) containing eight orthologs lack only \textit{pspE}.

Correlated Evolution and Loss and Gain of Genes in the Psp System

We have tried to identify correlated evolution in the patterns of presence and absence for all pairs of genes in the Psp system. This would, for example, hint at the presence of one gene being necessary for another gene to fulfill its function (at least as far as relating to within the Psp response system) and hence to be maintained in the genome by natural selection (Hershberg et al. 2007).

We used the BayesTraits package to identify coevolving pairs of genes (Pagel 1994; Organ et al. 2009) (see Material and Methods). We find statistically significant evidence against the independent model only for the pair \textit{pspA–pspF} (\textit{P} value < 0.05; after correcting for multiple testing using FDR). Thus, all other gene pairings (interestingly also the two-component system \textit{arcB–arcA}) show no statistical evidence for having correlated patterns of presence/absence. This result does, however, hint that the \textit{pspA–pspF} subsystem may define a functional core of the phage shock response, consistent with the minimal Psp system found to be sufficient for stress response (Jovanovic et al. 2009).

Maintenance of the \textit{psp} Operon

Crucially, we find that the level of gene sharing across the species, including the somewhat special position held by \textit{pspE}, appears to be linked to, or reflected by, the emergence of the \textit{psp} operon (see fig. 1). This is confirmed by jointly considering together the phylogeny, genomic organization, and putative network architecture as illustrated in figure 3 (see supplementary fig. S3, Supplementary Material online).
FIG. 3. Profile of presence/absence and configuration of the Psp system orthologs. Representation of the Psp system orthologs obtained for the species of interest. The orthologs for each species are organized based on their position in the phylogenetic tree. From left to right: 1) Phylogenetic tree inferred from the 23S rRNA sequences, 2) presence/absence profile of the nine proteins of the Psp system from PspA to ArcB, 3) genomic organization of pspA to pspF orthologs according to the position of the first pspA gene nucleotide, and finally, 4) putative inferred network organization of the Psp system for different observed set of orthologs. The color code is identical to the one used in figure 1. The correspondence between the species name and number can be found in the supplementary table S1 (Supplementary Material online).
PspE appears to be shared more widely than many of the other Psp proteins, yet it exists in two different groups: One is highly conserved compared with the E. coli PspE sequence, whereas the other group is made up of a set of sequences that are considerably more divergent from their E. coli ortholog than the other psp gene orthologs from the same species. Sequence similarity with respect to the E. coli version does not decay progressively but presents a sharp drop in observed identity (see also Extended psp Operon and Gene Linkage section). This dichotomy correlates perfectly with the presence of pspE inside the psp operon and is not observed for the other proteins involved in the Psp response. For other proteins, we do observe a more gradual and slower decay in levels of observed sequence similarity and no such strong dependence on the genomic (operon) organization. This suggests that PspE’s role in the stress response is tightly coupled to being part of the operon. It is easy to imagine that recruitment of a gene into an existing operon, which through coordinated expression (directed by the use of common transcription factors), could result in a gain (or redirection) of function compared with the original gene.

As expected, the absence or presence of genes alone gives us only a partial picture of the evolutionary history of biological systems. But this analysis does suggest the synergistic effects of having complete systems (compared with E. coli): once a set of proteins working together in concert has been assembled in order to fulfill a function that affects the (Darwinian) fitness of an organism, then jettisoning one or a few of the genes from the genome may prove too detrimental for the organism.

As a result, we may expect to see this need to maintain a complete set of genes to be reflected at the operon level. In E. coli, the psp operon is 1 of 44 operons containing five genes (Pertea et al. 2009). The distribution of the number of orthologs found in other species is, in fact, fairly flat for the psp operon compared with the other 43 distributions (see supplementary fig. S2, Supplementary Material online).

Gain and loss of genes occur predominantly at the end of the operon; pspA, pspB, and pspC which are engaged in sensing stress, transducing the signal, and as an effector in the case of PspA (Model et al. 1997; Darwin 2005) (for Yersinia enterocolitica, PspB and PspC are also effectors; Mason and Darwin 2006b) are generally kept together in the species considered here. The less well functionally characterized genes (knock outs of pspD, pspE, as well as pspG, which resides outside the psp operon, exhibit no growth phenotypes and can maintain the pmf during stress) are absent from other species more often than pspABC and pspF. PspD may have an effector function that overlaps with that of PspA (Jovanovic et al. 2006). The PspA and PspD effector functions are distinct from the functions of PspG or PspE, which appear to be involved in modulating cellular metabolism under stress conditions (Adams et al. 2002; Jovanovic et al. 2006).

The observed pattern suggests that the Psp response system has become more nuanced and complex over time in E. coli. In particular, pspE appears to be a recent addition to the Psp response machinery, which in other bacteria fulfills a different role.

### Extended psp Operon and Gene Linkage

The analysis of the psp operon structure suggests that pspA–E may have not been the only genes making up the psp operon. Many approaches have been developed to predict operons; we used one of the most commonly used and simplest measures to identify potential extended versions of the psp operon (Brouwer et al. 2008) (see Material and Methods). Our objective here is just to identify genes potentially associated with the psp operon genes in the sequenced species and not explicitly predict operons ab initio.

This analysis reveals three interesting aspects of the psp operon’s evolutionary history (see supplementary fig. S6, Supplementary Material online). First, all the genes that belong to the psp operon in E. coli are potentially also part of other operon structures in at least one of the studied species. Second, several species contain psp operon structures with intergene fragments within the operon that are longer than 100 bp. Finally, some of the psp operon structures formed by pspAB, pspABC, or pspABCD are potentially extended by additional genes. In total, 32 of the 81 psp operon structures identified in this study contain at least one additional gene, ycjX. It had already been reported that ycjX and ycjF could be part of the psp operon structure in a number of bacterial species; however, no systematic analysis of the association between these two genes and psp operons had been performed up to now (Darwin 2005).

Orthologs for ycjX and ycjF are identified in almost all species also containing orthologs for at least pspA and pspB. In almost all the species studied, these two genes are adjacent. However, the physical organization of ycjX and ycjF in relation to the psp operon presents a large level of variability, especially in comparison with the psp operon genes or the ycjXF pair itself. For example, the species presenting an ortholog for pspA, pspB, and pspC but none for pspD present cases with ycjX and ycjF next to pspC, and pspB; and finally cases with ycjX and xcfF in different chromosomal locations. For the species presenting orthologs for pspABCD and pspABCDE, the same diverse set of profiles can be observed for these additional genes.

### Coordination with psp Genes Outside the psp Operon

A potential clue as to how pspG was recruited into the Psp response system is provided by Aeromonas salmonicida A449, where the pspG ortholog is found immediately adjacent to a sequence stretch containing paralogs to pspA and pspC; here, the distances between the coding sequences for the pspA and pspC paralogs and the pspC paralog and the pspG ortholog are 13 bp and 47 bp, respectively. This proximity together with experimental observations that the three genes are in E. coli coordinately regulated by pspF indicates that they could form their own operon. The orthologs for pspA and pspC that are detected using
best reciprocal Blast occur elsewhere in the genome of A. salmonicida A449 and are to either side of a clear \textit{pspB} ortholog (the published annotation of the genome, however, fails to identify the correct \textit{pspA} and \textit{pspC} orthologs). This establishes an extant transcriptional link between \textit{pspG} and \textit{pspAC}, which opens up the intriguing possibility that \textit{pspG} acquired its association with, and role in, the Psp response through historical association with an operon containing paralogs to \textit{pspA} and \textit{pspC}.

**Sequence Similarity and dN/dS**

Until now we have treated the orthologs, with the exception of PspE, as identical objects, which they are clearly not. The percentage sequence identities of the different orthologs were computed with respect to \textit{E. coli} and plotted based on the position of the species in the phylogenetic tree (phylogenetic relationships are based on the 23S rRNA sequences extracted for all the species) (fig. 4). As expected, the relative sequence identity is correlated with the evolutionary distance to \textit{E. coli}. In any given species, however, relative divergence to \textit{E. coli} can differ quite considerably between the different Psp and Arc orthologs. ArcA is the most conserved protein with, except for 4 cases of 82, a minimum of 60% amino acid sequence identity and is followed closely by the two pivotal \textit{psp} genes, PspA and PspF. The proteins with the largest number of orthologs (PspC and PspE) have the lowest levels of identity across the panel of sequenced bacterial species. In PspE, this is probably related to the potential alteration in its functionality once the gene is recruited into the psp operon.

Using standard evolutionary arguments, we can infer whether or not the evolution of a protein is in agreement with the behavior expected from neutrally evolving genes, by considering the ratio of nonsynonymous to synonymous substitution rates for all orthologs of the corresponding \textit{E. coli} genes (see fig. 5) (Yang and Bielawski 2000). The dN/dS ratios are mostly smaller than 1, which suggests the action of purifying selection. This is to be expected from genes, once acquired, which fulfill important functions for the organism and thus need to be maintained. Only for PspF do we observe large values of this ratio, which is indicative of adaptive evolution in a few species.

The link between sequence similarity and the dN/dS measure is illustrated further for PspF and PspE in figure 6 (the corresponding plots for the other protein sequences are shown in supplementary fig. S5, Supplementary Material online) where we show these two quantities for all pairwise species comparisons. For a large proportion of the gene pairs, the sequences are too divergent to allow the reliable calculation of a relevant dN/dS ratio, and the majority of pairs with a computable ratio involve closely related species (which concentrate around the diagonals.
in fig. 6). But pairs of species with high levels of sequence identity tend to show strong evidence for purifying selection and vice versa. The block structure in figure 6 clearly reflects the evolutionary relationships already apparent in the phylogeny in figure 3: at the sequence level, the clade structure is also clearly reflected.

Intergenic Regions
There is increasing evidence that the effects of selection extend well beyond the coding sequences (Hurst 2009; Paget and Helmann 2003)—as had previously been posited. The controlled and coordinated expression of genes is largely dependent on features in the noncoding sequence, predominantly the promoter and termination sites. We focus here on the regions separating pspF from the psp operon and the intergenic regions within the operon (supplementary fig. S4, Supplementary Material online).

The region between pspF and pspA contains the regulatory sequences that in E. coli control the repression of pspF expression and the activation of the genes in the psp operon (Weiner et al. 1991; Weiner et al. 1995; Jovanovic et al. 1996; 1997; 1999; Dworkin et al. 1997; Jovanovic and Model 1997). In E. coli, this region contains four annotated promoters; according to present experimental evidence (Jovanovic et al. 1996), three of these correspond to pspF and one to pspA. As expected for a promoter region, the overall sequences are highly divergent overall, but the sets of closely related species display similar sequence profiles. In the overall alignment, none of the three pspF promoters show a conserved profile beyond the enterobacteria. The multiple alignment presents a different result for the pspA/psp operon promoter. The signatures of the $\sigma^{54}$-dependent promoter (Wigneshweraraj et al. 2008), $\tau$-24 and part of the $\tau$-12 box, are practically identical in all the species considered here. A detailed analysis of the $\tau$-12 box reveals that the classic GC motif is replaced in almost all species by the unusual GT motif. All those results are congruent with the expected role of PspF as a transcription factor of pspA and the other genes of the psp operon in these species.

The above is, however, not the only regulatory sequence architecture found among the panel of species; in Yersinia, the upstream region of the psp operon, for example, contains an additional $\sigma^{70}$-dependent promoter, which adds additional complexity and flexibility to the transcription regulation in this system (Maxson and Darwin 2006a). However, in E. coli and Salmonella, the regulatory control of the operon (with the notable exception of pspE discussed in more detail below) is known to be entirely $\sigma^{54}$ dependent (Lloyd et al. 2004), as is also clear from our analysis.

The control of pspE expression appears to be subtly different from the rest of the genes in the psp operon. In E. coli and Salmonella typhimurium, pspE has its own $\sigma^{70}$-dependent promoter site, which allows pspE to be expressed independently from the other genes. However, when under control of the operon $\sigma^{54}$ promoter, pspE is transcribed as part of the polycistronic psp operon mRNA. Importantly, the stress-induced transcription (under $\sigma^{54}$ control) of pspE will be markedly increased (5- to 10-fold) compared with the basal expression levels observed under normal growth conditions, corresponding to combined $\sigma^{70}/\sigma^{54}$-dependent transcription.

**Fig. 5.** Estimation of the selection pressure according to Escherichia coli K12. Histograms representing the dN/dS ratio estimates between the E. coli K12 sequences and all identified orthologs (see Materials and Methods).
The predominating features in the intergenic regions inside the psp operon are 1) the transcription termination site between pspA and pspB and 2) the boundary between pspB and pspC. The former results in stochastic dissociation between the transcriptional machinery and the chromosome, which results in markedly reduced transcription of pspB–E compared with pspA (at a ratio of roughly 1:4). Potentially PspBC acts on a PspA–F complex containing six subunits of PspA, offering some rationale for the differences in expression levels (Joly et al. 2009). The latter, however, appears to reflect a somewhat more subtle relationship between the two genes than is apparent from their respective amino acid sequences. In many species, the pspB and pspC sequences partially overlap; in E. coli, the stop and start codons of pspB and pspC overlap; in other species (such as Shewanellas ANA-3 or Shewanella baltica OS 185), the actual coding regions overlap up to approximately 10 nt. The resulting cotranslation will quite generally be helpful for protein complex formation, which in turn enables pairs or groups of proteins to fulfill any functions that depend on their joint action. Detailed analyses of all pspB–pspC junction regions furthermore reveals that in Sodalis glossinidius str. “morsitans,” a single gene is present in the region normally occupied by pspB and pspC (incorrectly annotated as an ortholog to pspB in the database); further inspection reveals that the predicted corresponding protein product is a chimera consisting of two domains, one belonging to PspB and one belonging to PspC.

Psp Response of the Reduced Psp Regulon

To address the question of how effective the Psp response will be in E. coli cells with a significantly reduced Psp regulon, we constructed a strain lacking pspBC and pspG genes (the probable first genes added to an hypothetical ancestral Psp system formed by pspA and pspF) and determined levels of psp induction and expression of PspA in the absence or presence of secretin pIV, the inducer of cell wall stress and under different oxygen-related environments (aerobic, microaerobic, and anaerobic growth conditions).

Importantly, the psp induction and expression of PspA in pspBCG triple mutant is markedly reduced under aerobic and microaerobicosis (fig. 7a and b). Because the same has been shown for induction of pspA in a pspBC mutant (Jovanovic et al. 2009), these new results support the view that the PspG protein does not contribute to the regulation of psp induction (Lloyd et al. 2004). Under anaerobiosis, the expression of PspA shows no difference between the wild-type and the triple mutant. Taken together, these results and the observation that pIV production in ΔpspF cells (occurring in the absence of Psp proteins) significantly impairs the cell growth (Jovanovic et al. 2006) suggest that expression of PspA (and perhaps PspD and PspE) is sufficient to maintain robust cell growth under psp-inducing ratio, the color is white. Finally, gray fields correspond to positions in the matrix for which no orthologs are present in the respective species or where the pair of orthologs is too divergent to allow for reliable estimates of the dN/dS ratio.
stress conditions (pIV production) at a level comparable with wild-type cells with the intact Psp regulon.

Based on these observations, we looked for the influence of the presence or absence of \( pspB, C, \) and \( G \) on cell growth. The results show that production of pIV reduces growth to similar extent in the wild-type and the \( pspBCG \) triple mutant cells under aerobic and microaerobic growth conditions compared with nonstressed cells (supplementary fig. S7, Supplementary Material online). This is in line with the observation that pIV production in cells lacking \( pspBC \) does not impair the growth in comparison with stressed wild-type cells (Jovanovic et al. 2006) and establishes that \( pspG \) does not contribute to the observed growth rates. Notably, in anaerobiosis, the \( pspBCG \) triple mutant cells show better growth (and so have a potential fitness advantage over wild-type cells) after 5-h growth under stress conditions (fig. 7c), confirming that the induction of the \( psp \) genes upon production of pIV does not depend on the presence of PspBC under anaerobic growth conditions and demonstrating a growth condition–dependent advantage of having the full complement of \( psp \) genes (Jovanovic et al. 2009). The statistical tests applied are described in the Bacterial Strains and Growth Conditions section of the Material and Methods.

PspG may be important for fine-tuning the metabolism toward anaerobic respiration under stress in aerobiosis and microaerobiosis (Jovanovic et al. 2006), indicating a growth condition–specific role for PspG in addition to the demonstrated conditional use of PspBC. We propose a potential increase of selective pressure upon wild-type cells compared with \( pspBCG \) triple mutant under stress growth conditions in anaerobiosis reflecting an advantage in aerobic growth of having PspBC and PspG. It should be noted that the results obtained with the \( pspBCG \) triple mutant are related to the \( E. coli \) version of the remaining \( psp \) genes, mainly \( pspA \) and \( pspF \). They could be different from their ancestral version. This could result in a difference of phenotype between an organism containing only \( pspA \) and \( pspF \) as they were in ancestral species (not containing \( pspBCDE \) and \( G \)) or as they are in \( E. coli \).

**Discussion**

When considering the evolutionary process that has given rise to the complex molecular signaling machinery of, for example, stress response mechanisms, it is important to consider the need of bacteria (and other organisms) to interact with the changing environment. This, of course, experts selection pressure on the components making up the system (Pinney et al. 2007; Ratmann et al. 2009). In addition to selection, other factors such as genetic drift also affect the evolution of biological systems (Lynch 2007a, 2007b). It has been argued that nonadaptive processes can give rise to systems that exhibit comparable levels of complexity to those observed in biological organisms (Lynch 2007a). In

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**Fig. 7.** Psp response of a \( \Delta pspBC \Delta pspG \) mutant. (a) Effect of continual secretin production on the psp induction of \( \Delta pspBC \Delta pspG \) mutant under different growth conditions. The expression of \( pspA–\lambda \text{lacZ} \) chromosomal transcriptional fusion was measured (β-gal activity, see Materials and Methods) in wild-type (WT; MVA44 [Jovanovic et al. 2006]) and \( \Delta pspBC \Delta pspG \) (pspBCG; MVA103) strains in the absence (−pIV) or presence (+pIV) of pIV secretin under aerobic, microaerobic, or anaerobic growth conditions. (b) Expression of PspA in a \( \Delta pspBC \Delta pspG \) mutant producing pIV secretin under different growth conditions. The samples from all strains used in (a) were collected at \( OD_{600} \approx 0.4 \), and expression of PspA protein was determined using western blot and antibodies against PspA (α–PspA). The expression of pIV in the corresponding samples was detected using western blot and antibodies against pIV (α–pIV). (c) Effect of continual secretin production on the growth of \( \Delta pspBC \Delta pspG \) mutant strain under anaerobic growth conditions. The plasmid \( pGJ4 \) with leaky expression of pIV secretin was transferred into wild-type (WT; MG1655) and \( \Delta pspBC \Delta pspG \) (pspBCG; MVA49) strains (genotypes indicated on the right-hand side). The strains with or without \( pGJ4 \) were grown under anaerobic growth conditions as described in the Materials and Methods and optical density was measured at hourly intervals. The data are from a single experiment in which all strains were tested simultaneously (the experiment was carried out on three separate occasions to ensure reproducibility—see supplementary fig. S7, Supplementary Material online).
the absence of population-level data, we must therefore be careful in interpreting the results of evolutionary analyses.

Patterns of coinheritance suggest strongly that the constituent genes do not evolve independently when considered as a functional system (here, details of the system’s function do not matter in the first instance); if we think of the proteins as useful tools, then an organism may just as well jettison the whole toolbox once too many individual tools have been lost. There are, however, subtle differences that require a more nuanced description. These are presumably due to the intricacies of the molecular machinery underlying the Psp response, which are determined by the multiple roles played by several genes; first of all, the TCS consisting of ArcA and ArcB is also involved in different processes (Malpica et al. 2006). Second, PspA plays a pivotal role in receiving the signal and releasing the negative control upon PspF once the stress has been sensed and transduced by a combination of the PspB and PspC sensors (reviewed in Model et al. 1997; Darwin 2005); furthermore, PspA acts as an effector involved in repairing cell wall damage and contributes to reestablishing pmf (Kleerebezem et al. 1996; Jovanovic et al. 2006; Kobayashi et al. 2007; Engl et al. 2009). PspB has an additional role in mediating quantitative effects of the Psp response through interactions with ArcB, integrating the ArcAB two-component system into the Psp response machinery (Jovanovic et al. 2009). In fact, a mechanistic description only the effects of pspA, pspB/C (jointly, rather than independently), and pspF may suffice to capture the qualitative response.

Interestingly, the level of correlated evolution does not appear to matter when considering only pairs of genes: in such an analysis only PspA and PspF show statistical evidence for correlated evolution. However, when considering the whole system, then system-level correlated evolution—that is, beyond the level of pairs of genes—becomes apparent.

At the sequence level, there is clear evidence that all the genes have been under purifying selection; this evidence becomes overwhelming when assessed not across the whole phylogeny but on a clade-by-clade basis. In fact, using the wealth of the available information in one fell swoop may easily mask these signatures at the sequence level; here, evolutionary distance (or differences in ecological niches occupied by different species) could play the role of a confounding factor or hidden variable (Thorne and Stumpf 2007; Chuang et al. 2009).

Our results highlight the crucial role, which the operon organization has played in the evolutionary history of the Psp response system. A range of plausible mechanisms have been put forward to explain operon organization and evolution: 1) operons may form to ensure that proteins with related function or involved in the same process are maintained as a coherent unit; 2) being under shared transcriptional control maintains balanced mRNA expression levels and stochiometries; 3) the order in which genes are transcribed and polyribosomal mRNA is translated could ensure that proteins needed first or upstream of the others are made available in the right order; and finally, 4) the (somewhat controversial) “selfish operon” model assumes that the colocalization/coexpression may not be an advantage to the organism containing the operon but rather directly to the operon genes (Fondi et al. 2009).

All of our results clearly support the notion that the operon grew in the head-to-tail direction from the 3’ end of pspF from the pspF pspA divergent transcription unit. Importantly, the physical organization of the genes in the operon is not simply a function of where they occur in the pathway/network (see fig. 1c). Rather, in light of the complete set of results discussed above, the most likely scenario is that the system has evolved around a core cell wall-stress response consisting of only PspA and PspF, which by themselves are known to be able to induce a functional stress response under specific conditions (Jovanovic et al. 2009). These genes form a minimal system that can elicit a response where PspA is the sensor, regulator, and effector and PspF the regulated gene activator. Addition of PspB and PspC (or perhaps a chimeric protein combining domains of both) would then have fine-tuned the initiation and modulation of the response, which agrees well with what is known about their function in E. coli (Weiner et al. 1991; Gueguen et al. 2009) and the experimental results presented above. Such a configuration retains the essential control function at the transcriptional level for PspF. Finally, PspD and PspE must then have been added, leading to the operon structure now observed in E. coli K12 and other species.

The search for genes that are potentially part of the psp operon but not observed in E. coli revealed that few species might contain extra genes within their respective psp operons. In all species studied and presenting an alternative version of the psp operon, the same set of genes were observed (ycjX and ycjF). However, the genomic organization reveals that these extra genes show a much weaker level of physical association to the psp operon than do the psp genes. Although we cannot exclude the possibility of a functional link between ycjXF and the Psp system, we found that almost each sub psp operon (with the exception of pspAB) could be observed without ycjXF in close vicinity. The phylogenetic profile of genomic organization suggests that events of physical association or dissociation occurred independently.

According to RegulonDB (Gama-Castro et al. 2008), ycjX and ycjF are part of a separate transcriptional unit, verified experimentally in E. coli, composed of three genes (ycjX, ycjF, and tyrR). The profile of ortholog presence/absence, within the 127 species studied here in detail, reveals that these three genes have almost identical profiles. At a genomic level, almost all ycjX are next to ycjF and a large proportion of them are next to tyrR. ycjX is reported to be associated to a σ^7 promoter. This promoter is annotated as a regulator in the heat-shock response system (Nonaka et al. 2006). The Psp system, although regulated by a different promoter, is also activated during heat-shock stress. This represents a potential functional link between the two systems and might explain the observed association between them. However, the Psp system is also
known, at least in *E. coli*, to respond to a large variety of other stresses providing situations during which one system will be needed and not the other, thereby reducing the potential need for operon-like expression regulation and structure. Interestingly, early analyses of *psp* transcription suggest that product of the σ^32^-controlled heat-shock system could act to suppress *psp* expression (Weiner et al. 1991). These results indicate that in *E. coli*, the *psp* operon and *ycjX* and *F* are expressed under different conditions and thus are perhaps less likely to interact functionally. A detailed analysis of the promoter region of the different *ycjX* and *F* orthologs could help us to understand the potential relationship between these genes and the Psp system.

Our present functional knowledge of PspD, PspE, and PspG is insufficient at the moment, but we conjecture that their role may be primarily as effectors because a contribution to regulation can be ruled out (Model et al. 1997; Jones et al. 2003; Lloyd et al. 2004). A subtle effect of PspE may already be expected from the observation that it is shared widely across the bacterial species considered here, especially in species lacking any of the other *psp* genes. The present evidence implies that its role in the Psp response occurred concomitantly with its recruitment into the *psp* operon. Interestingly, the physical linkage between an ortholog of *pspG* and a paralog of *pspA* in *A. salmonicida* A449 suggests that recruitment of *pspG* into the Psp response was also initiated indirectly, perhaps through coexpression due to shared transcriptional regulation. There is also recent evidence that, in *E. coli*, PspC, besides with PspA, directly interacts physically with PspG (Jovanovic et al. 2010), supportive of a particular relationship between PspA, C, and G within the Psp system.

The ArcB/ArcA two-component system is involved in several biological signaling and stress response processes, and when and how it was recruited to contribute to the Psp response cannot be inferred from our analysis, but again, the role here is to modulate the strength of response without being essential to it (Jovanovic et al. 2006, 2009).

If functional orthologs can be identified, then we may be able, with due care, to transfer our knowledge of system-level processes between model and nonmodel organisms. But what happens if we have some species for which only a subset of relevant orthologs can be identified? This could either 1) be evidence that the system can operate without these genes through a (potentially) simpler mechanism; 2) falsely imply the absence of the orthologous genes when they are actually present in the nonmodel genomes but cannot be identified using standard methods; or 3) indicate that in these organisms other proteins have been co-opted to fulfill the missing roles, which have apparently unrelated functions in the model organism, for example, *E. coli* (Berg and Lässig 2006; Lee et al. 2008; Alexander et al. 2009; Xia et al. 2009; Lewis et al. 2010).

Building onto an existing (however rudimentary) stress response system (e.g., *pspA* and *pspF*) seems like a probable evolutionary strategy, where increasing sophistication and honing of the response by adopting additional proteins as response regulators and effectors results in an apparent increase in the complexity of the system. Such an attractive model receives additional support from the analysis of other enterobacterial Psp response systems. Although *pspABC* and *pspF* are generally maintained, other proteins seem to have been incorporated in the *psp* operon and have replaced *pspD* and *pspE* in, for example, *Yersinia enterocolitica* or *Idiomarina loihiensis* (Darwin 2005). Interestingly, our experimental analysis show that such reduced systems may in fact sometimes perform better than the full system under certain environmental conditions.

The coordinated operation of a system such as the Psp response is maintained by a variety of mechanisms: Having all the required genes in the same operon is the most obvious means of achieving this; having a defined regulon characterized by shared promoters is another—perhaps less restrictive—option to ensure well ordered responses; finally, physical protein–protein interactions can mediate flexible and spatiotemporally well-defined coherent responses. Our detailed analyses have shown the different ways in which the Psp response was assembled over evolutionary time courses and is maintained today in *E. coli* (and related species). These include: overlapping reading frames that increase or decrease coexpression of neighboring genes depending on how this affects the binding of RNA polymerase to DNA; proteins that may form chimeras or break up into separate entities, depending on the need to maintain coherence and physical linkage between protein domains; additional within-operon promoter sites that provide transcriptional flexibility, allowing for additional flexibility when new proteins are recruited into operons; equally, transcription termination (hairpin loops) sites inside the operon can fulfill the same role by adjusting the relative abundance of mRNA of different genes inside the same operon. This last mechanism can, for example, ensure that different proteins are produced in the required relative abundances without the need for further posttranslational modifications.

A final note of caution may be in place: The selection pressure experienced by bacteria (or more generally any organism) is much more diverse than anything probed under laboratory conditions. Functional analyses may therefore be presenting us with too simple a picture as far as the complexities of signaling and regulatory responses to environmental stress are concerned. In particular, studying stress response systems in isolation may be oversimplifying matters, especially if cross-talk is the rule rather than an exception, which we would hazard to guess.

**Supplementary Material**

Supplementary material, table S1, and figures S1–S8 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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