Molecular Microbiology (1997) **24**(2), 255–261

MicroReview

The *Escherichia coli* phage-shock-protein (*psp*) operon

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Summary

The phage-shock-protein (*psp*) operon helps to ensure survival of *Escherichia coli* in late stationary phase at alkaline pH, and protects the cell against dissipation of its proton-motive force against challenge. It is strongly induced by filamentous phage pIV and its bacterial homologues, and by mutant porins that don't localize properly, as well as by a number of other stresses. Transcription of the operon is dependent on σ^{54} and a constitutively active, autogenously controlled activator. *psp*-operon expression is controlled by one negatively and several positively acting regulators, none of which is a DNA-binding protein. The major product of the operon, PspA, may also serve as a negative regulator of an unusual porin, OmpG.

Introduction

For many interesting *Escherichia coli* proteins (e.g. GroE, Rep, DnaJ, DnaK) there was a long lag between their discovery (which was often adventitious) and an appreciation of their role in bacterial physiology. The *E. coli* phage-shock-protein (*psp*) operon is still in this lag phase; it is reasonably well characterized in terms of the proteins it encodes, its regulation, and its induction, but the functions of its genes are not yet really understood. Increasingly, it has been suggested that it plays an important role in *E. coli* physiology. Thus, this review is intended to stimulate further interest and research in the function(s) of this operon.

The *psp* operon was discovered during the course of cloning filamentous-phage gene IV; overexpression of pIV, which is an outer membrane protein, from a runaway plasmid (Brissette *et al*., 1990) induced such abundant synthesis of PspA protein that it became a major fraction of the total cell protein. The operon contains several open reading frames (ORFs), at least four of which (PspA, B, C, and E) are expressed *in vivo* and *in vitro*; the product of the first gene, *pspA*, is the most abundant (Brissette *et al*., 1991). The operon is also expressed in response to a variety of environmental and intracellular stresses.

PspA expression is induced after filamentous-phage infection, as well as when gene IV is expressed from a plasmid (Brissette *et al*., 1990; Russel and Kazmierczak, 1993). Transcription of the operon is also induced by expression of pIV homologues from several different bacteria (Possot *et al*., 1992; Russel and Kazmierczak, 1993) or by overexpression of some outer membrane porins, particularly mutant porins defective in some aspect of integration into the outer membrane (Bosch and Tommassen, 1987; Carlson and Silhavy, 1993; Kleerebezem and Tommassen, 1993). Stresses, such as extreme heat shock or treatment with 10% ethanol and hyper- (but not hypo-) osmotic shock are also inducers (Brissette *et al*., 1990), but it is not clear whether these stimuli, many of which are lethal to the cell, elicit a normal induction response or short-circuit the normal controls on *psp* expression. The *psp* genes are also induced by media downshifts, and are induced very strongly by proton ionophores such as CCCP or other uncouplers (dinitrophenol or free fatty acids) (Weiner and Model, 1994), and by disruption of fatty acid biosynthesis, either by mutation or with the inhibitors diazaborine or cerulenin (Bergler *et al*., 1994). In addition, PspA protein is a major component of the limited protein synthesis that occurs during late stationary phase (Weiner and Model, 1994). Other than expression of gene IV or infection with filamentous phage, all the inducing stimuli also induce the σ^{32}-dependent heat-shock genes (*groE*, *dnaK*, *dnaJ*, *grpE* (Lindquist and Craig, 1988; Yura *et al*., 1993)), but *psp*-operon transcription does not require σ^{32} (Brissette *et al*., 1991) and hence none of the other components of the canonical heat-shock response; indeed, shift of a σ^{32ts} mutant to the non-permissive temperature intensifies the *psp* response, and for the stimuli that induce *psp* transiently, such as heat and exposure to ethanol, this prolongs the response (Brissette *et al*., 1990; 1991; Weiner *et al*., 1991). A participant in the canonical heat-shock response of *E. coli* is σ^{E}, which is responsible for transcription of σ^{32} at 50°C (Erickson and Gross, 1989) and is itself activated by overproduction of outer membrane proteins (Mecsas *et al*., 1993). Sigma E is not

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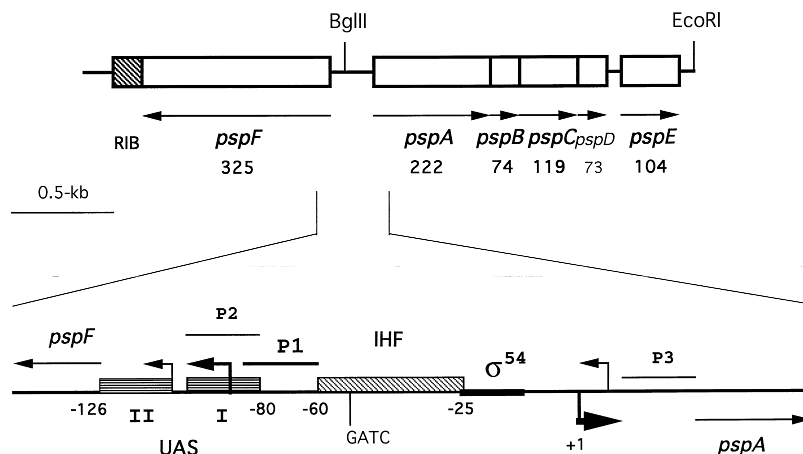

Fig. 1. The organization of the *pspF* gene, the *pspABCDE* operon and the *pspF–pspA–E* promoter-regulatory region.
Upper part. The *psp* cluster. White boxes represent genes; the hatched box represents RIB (reiterative *ihf* BIME (bacterial interspersed mosaic element)) element; numbers represent the number of amino acid residues encoded by relevant genes; see the text for details.
Lower part. The *pspF–pspA–E* promoter-regulatory region. UAS I and II (−89 to −126) are PspF-binding sites, upstream activating sequences; IHF 1 and 2 (−33 to −60) are IHF-binding sites (site 2 contains the methylation sequence, GATC, that remains unmethylated during the log-phase of *E. coli* growth); σ^{54} is the *pspA–E* σ^{54}-dependent promoter; P1–3 are *pspF* σ^{70} promoters (P1, major promoter; P2–3, minor promoters); the large rightward arrow +1 indicates the start of transcription of the *pspA–E* genes; leftward arrows indicate the *pspF* transcription start sites; see the text for details.

required for *psp*-operon induction, and expression of pIV does not activate σ^E or induce its synthesis (our unpublished results).

Phenotype of *psp* mutants

Bacteria in which the *psp* operon is disrupted grow and plaque filamentous (and a number of other) phages normally, and do not acquire auxotrophies. Nonetheless, the absence of the *psp* genes *does* have effects. *E. coli* lacking PspA–C survive much more poorly in stationary phase at alkaline pH (Weiner and Model, 1994), are somewhat more motile than *psp*$^+$ cells (our unpublished results), are slower to export proteins via the *sec* pathway (Kleerebezem *et al.*, 1996; Kleerebezem and Tommassen, 1993), and are less able to maintain their membrane potential when stressed (Kleerebezem *et al.*, 1996). Both the inducing stimuli and the nature of these phenotypes suggest that the *psp* genes constitute a stress-response operon which may respond to alterations in the energy charge of the cell. Because a nutritional downshift can itself induce the *psp* operon, it may well be that it responds to a decrease in the level of ATP or its rate of generation.

Description of the *psp* cluster

The *psp* cluster comprises two divergent transcription units (see Fig. 1). *pspA–E* transcription is driven by a σ^{54} promoter located upstream of the *pspA* gene, which leads to the synthesis of a 2 kb RNA from which at least four products are translated (PspA, B, C, and E) (Brissette *et al.*, 1991). None of these resembles other proteins in the database closely enough to provide strong, sequence-based clues to their function. The first (5′-proximal) 25.5 kDa protein, PspA, has three known homologues: PspB, a cold-shock protein from *Bacillus subtilis* (Graumann *et al.*, 1996), a protein (SYCSLRD) from *Synechocystis* (Kaneko *et al.*, 1995), and another protein (IM30) from pea chloroplast (Li *et al.*, 1994). The function of none of these homologous proteins is known. PspA contains a coiled-coil motif, and matches proteins such as spectrin and myosin by virtue of this motif (Weiner *et al.*, 1991). Antiserum against PspA reacts with a protein of approx. 25 kDa in extracts from *Salmonella typhimurium*, hence it is probably present in other Gram-negative bacteria (Weiner, 1993). PspA is a peripheral membrane protein, approx. 50% of which is associated with the inner membrane fraction from *E. coli* (Brissette *et al.*, 1990). PspB and C are both inner membrane proteins (Brissette *et al.*, 1991; Kleerebezem *et al.*, 1996), and PspE is periplasmic (Brissette *et al.*, 1991; Mielke and Russel, 1992). We have not observed synthesis of PspD, but this might be because it would be the same size as PspB. Recent experiments (our unpublished results) lead us to suspect that PspD may be required for induction by pIV.

PspA, B, C, and perhaps D, all have a role in regulating

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Induction of the *psp* operon

f1 (pIV)
Other stimuli
overproduction of PspC
Ethanol (10%)
PspB PspC (PspD)
overproduction of PspF
sigma-32 regulon
Osmotic shock (0.75 M NaCl; 0.5 M Sucrose)
***pspABCDE* induction**
PspF
PspA
Heat shock (48-50 °C)
PspB PspC (PspD)
PspA

Fig. 2. Induction and regulation of the *psp* operon. Inducing stimuli and cellular factors influence PspA, B, C, (D) and E production (inducing stimuli or positive regulation are indicated by arrows; negative regulation is indicated by perpendicular bars; mechanism unknown is indicated by a dashed arrow; see the text for details).

transcription of the *pspA–E* operon. PspA acts as a negative regulator, while PspB and C act in concert as positive regulators, by relieving the repression imposed by PspA (Brissette *et al.*, 1991; Weiner *et al.*, 1991; 1995) (see Fig. 2). The absence of PspA results in constitutive, high-level expression which is independent of the presence of PspB, C or D (Weiner *et al.*, 1991). In the presence of intact PspA, PspB and PspC are absolutely required for induction by phage infection or by f1 pIV (Weiner *et al.*, 1991), but are only partially required for induction by some of the other inducing stimuli (Weiner *et al.*, 1991) (Fig. 2). Induction of the *psp* chromosomal copy by heat is independent of PspB, C and D (Weiner *et al.*, 1991). The sequences of PspB, C and D all predict that they contain leucine zipper or leucine zipper-like structures; they are not, however, DNA-binding proteins. Since PspA resembles a coiled-coil protein, it is likely that the inducing signal is transduced through protein–protein interactions by using these potentially interacting domains. PspE, the last protein of the operon, does not appear to have a role in controlling expression of the operon; its function is unknown, and indeed experiments have failed to demonstrate a functional link between this protein and the rest of the *psp* proteins (our unpublished results). The DNA sequence predicts a *pspE* signal sequence, and this signal sequence is functional, as determined by PspE–PhoA fusions (Mielke and Russel, 1992). The level of *pspE* mRNA is not appreciably affected by induction because there is a separate, presumably σ^{70}-dependent, promoter which drives transcription of an RNA encoding only *pspE*. Upon heat induction, expression from this promoter is decreased, while transcription of the 2 kb polycistronic RNA is increased; these two effects counteract one another leaving the level of *pspE* RNA more or less unchanged (Weiner *et al.*, 1991)

Transcription from almost all σ^{54} promoters requires the participation of an activator (Collado-Vides *et al.*, 1991; Kustu *et al.*, 1989; Morett and Segovia, 1993; Popham *et al.*, 1989), and this is also true for the *pspA–E* operon. The *pspF* gene that encodes the activator is closely linked to the *pspA–E* operon, and is divergently transcribed from it via σ^{70} promoters (Jovanovic *et al.*, 1996) (see Fig. 1). In contrast to the other genes of the *psp* cluster, *pspF* is related to many others in the database, and its product, PspF, is a member of the enhancer-binding-protein family of activators (Jovanovic *et al.*, 1996). Most other such activators consist of an N-terminal domain that serves a regulatory function, a central catalytic domain required for activation which interacts with σ^{54}-containing RNA polymerase, and a C-terminal domain that contains the helix-turn-helix motif that constitutes a DNA-binding domain (Albright *et al.*, 1989; Collado-Vides *et al.*, 1991; Morett and Segovia, 1993; Parkinson and Kofoid, 1992; Shingler, 1996; Xiao *et al.*, 1994). PspF lacks an N-terminal domain and is constitutively active *in vivo* and *in vitro*, consistent with the observation that deletion of the *pspA* gene results in high-level, constitutive expression from the *psp* promoter. The constitutive activity of PspF is in accord with results from other laboratories, which have shown that deletion of the N-terminal part of some other σ^{54} activators leaves them constitutively active (Grimm *et al.*, 1995; Gu *et al.*, 1994; Huala *et al.*, 1992; Lee *et al.*, 1994; Perez-Martin and de Lorenzo, 1996; Ronson, 1988; Xiao *et al.*, 1994).

Regulatory elements of the *psp* operon

As shown in Fig. 1, the region between the *pspA–E* operon and the *pspF* gene contains a σ^{54} promoter (Weiner *et al.*, 1991), binding sites for PspF (upstream activating sequence (UAS) I and UAS II) (Weiner *et al.*, 1995; our unpublished results) and integration host factor (IHF) (Weiner *et al.*, 1995), and oppositely orientated σ^{70} promoters responsible for transcription of *pspF*.

Normal induction and high-level expression of the *psp* operon depends on these sites and their cognate binding proteins. Transcription of *pspA–E* is completely dependent on σ^{54} RNA polymerase (Weiner *et al.*, 1991) and

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PspF (Jovanovic *et al*., 1996), and is stimulated by IHF (Weiner *et al*., 1995). As has been shown for other σ^{54} promoters (Charlton *et al*., 1993; Collado-Vides *et al*., 1991; Freundlich *et al*., 1992; Goosen and van de Putte, 1995; Hoover *et al*., 1990; Kustu *et al*., 1989), IHF bends the DNA and facilitates the interaction between PspF bound to its UASs and σ^{54} RNA polymerase bound to the promoter. The effect of IHF depends on the inducing stimulus; in an IHF null mutant (*himA himB)* the reduction in expression of PspA induced by pIV is pronounced, while induction by hyper-osmotic shock is almost unaffected (Weiner *et al*., 1995). Deletion of the PspF-binding sites (UASs) markedly diminishes *psp* transcription, but not as completely as the absence of the protein. Like the absence of IHF, deletion of the *psp* UASs has a relatively modest effect on induction by hyper-osmotic shock. *In vitro*, *psp* transcription from a supercoiled template is much less dependent on IHF and on the presence of the UASs than is transcription from a linear template (our unpublished results). Since osmotic shock affects DNA topology (Higgins *et al*., 1988; Ni Bhriain *et al*., 1989), increased negative supercoiling may substitute for the IHF and UAS requirement in the *psp* intergenic region.

Figure 1 shows that there are three (σ^{70}) promoters for *pspF* (Jovanovic *et al*., 1996). The PspF binding sites (UASs) partially overlap two of the promoters as well as the transcriptional start sites and ribosome-binding sites of the *pspF* gene. As might be predicted from such an arrangement, PspF expression is autogenously controlled (our unpublished results). The amount of PspF is approx. 150 copies per cell, and is not changed by induction of the *pspA–E* operon (our unpublished results). In cells encoding a *pspF* mutant in which the region specifying the helix-turn-helix DNA-binding motif has been deleted, the level of the mutant protein (PspFΔHTH) is approx. 20-times higher than that of the wild-type protein, reflecting the loss of autogenous control (our unpublished results). Even with this higher level of truncated protein, the expression of the *pspA–E* operon is as low as when the *pspF* gene is deleted (Jovanovic *et al*., 1996). However, overproduction of either the wild-type or the DNA-binding mutant protein can overcome the negative regulation by PspA and induce operon expression, presumably by direct interaction with σ^{54} RNA polymerase (Jovanovic *et al*., 1996).

Regulation of the *psp* operon is complex, not least because different inducing stimuli require different *psp* gene products (see Fig. 2). Because the operon as a whole is under negative control by PspA (Weiner *et al*., 1991), induction can be thought of as overcoming the negative regulatory role of the PspA protein. Simple overproduction of the positive regulator (PspC) (Weiner *et al*., 1991) or the activator (PspF) (Jovanovic *et al*., 1996) is sufficient to induce *psp* expression. Overproduction of PspA, on the other hand, is sufficient to block the induction that normally results from filamentous-phage infection (J. Rakonjac and P. Model, unpublished results). We have recently found that purified PspF specifically activates *psp* transcription by σ^{54} RNA polymerase *in vitro*, and that addition of purified PspA abolishes this activation (our unpublished results). Since only PspF is a DNA-binding protein, *psp*-operon regulation must proceed via protein–protein interactions. Formally, but not necessarily mechanistically, PspF and PspA resemble NifA and NifL, in that PspF and NifA are constitutively active and transcription is inhibited by PspA and NifL.

Induction of the *psp* operon

The most potent stimulus of the chromosomal copy of the operon is filamentous phage pIV or one of its homologues, such as PulD of *Klebsiella oxytoca* (Brissette *et al*., 1990; Possot *et al*., 1992; Russel and Kazmierczak, 1993). This induction is completely dependent on the presence of intact *pspB* and *pspC* genes (Fig. 2): in the absence of either, induction fails. At the other extreme, heat induction (50°C) of the chromosomal copy of the operon, while not as potent as induction by pIV, is independent of PspB, C or D. Other inducing stimuli, including 10% ethanol and hyper-osmotic shock, fall between these two extremes; induction by these agents is stimulated by, but not entirely dependent on, PspB and C (Weiner *et al*., 1991). (When the entire operon is carried on a high-copy-number plasmid, the requirement for PpsB and C is more stringent, presumably because the cell contains more PspA even before induction.) A further distinction between pIV induction and these latter agents is that heat, ethanol and hyper-osmotic shock stimulate a transient response, peaking at about 5 min after the stimulus is presented, while the induction stimulated by pIV and its homologues continues as long as these proteins are synthesized (Brissette *et al*., 1990). Even for pIV and other outer membrane inducers, however, the relevant parameter may be the synthesis rather than the mere presence of these proteins, because if inducer-protein synthesis is turned off, synthesis of PspA protein declines faster than can be accounted for by dilution of the inducing protein by cell growth (Brissette *et al*., 1990).

In trying to understand the induction of the operon and its function, we have concentrated on the stimulation by pIV (or its homologues) because this is the only inducer that is not deleterious to cell growth at moderate pIV levels, does not simultaneously induce the σ^{32}-dependent heat-shock regulon, and requires all the positively acting *psp*-regulatory proteins. pIV is an outer membrane protein, one of two non-structural proteins required for filamentous-phage assembly and secretion (Russel, 1994b). The pIV homologues that induce the *psp* operon are also

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outer membrane proteins, and are required by both type II (general secretory) and type III (contact-dependent) (Wengelnik *et al*., 1996) protein-secretion systems. These systems secrete a variety of virulence factors to the exterior of Gram-negative organisms, some into the external milieu, and some into the cytoplasm of eukaryotic target cells, both animal and vegetable (Russel, 1994b). Induction of the *psp* operon is not a simple response to disruption of the outer membrane, because cells expressing wild-type pIV do not leak their periplasmic contents and are not unusually susceptible to drugs or detergents that are normally prevented by the outer membrane from damaging the cell (Russel *et al*., 1997). On the contrary, filamentous phage pIII, which does make the outer membrane very permeable, does not induce the *psp* operon (Brissette *et al*., 1990). Kleerebezem and Tommassen (1993) reported that non-specific jamming of the *sec*-dependent export machinery induced a *psp* response, but neither we (Brissette *et al*., 1990) nor Carlson and Silhavy (1993) have found that to be the case. Mutant pIV proteins retained in the cytoplasm because they have missing or defective signal sequences do not induce the *psp* operon (Brissette *et al*., 1990). Wild-type pIV is found in the outer membrane as a homomultimer; mutants which cannot multimerize also do not induce *psp* expression (Russel, 1994a). These observations led us to propose that only properly localized pIV could induce the *psp* response (Russel, 1994a), an inference which is probably wrong. Pugsley and co-workers (d'Enfert and Pugsley, 1989) reported that PulS, an outer membrane lipoprotein, is required for pullulanase secretion, and more recently demonstrated that PulS is needed to properly insert the pIV homologue, PulD, into the outer membrane (Hardie *et al*., 1996). In the absence of PulS, PulD is unstable, but it is the failure to localize in the outer membrane, rather than the instability, that is related to *psp* induction. (Hardie *et al*., 1996). When expressed at high levels in *E. coli*, PulD itself is a potent inducer of the *psp* operon, but when PulS is supplied, the *psp* response is eliminated (Hardie *et al*., 1996). A chimaeric protein in which the last two amino acids of pIV are replaced by 65 amino acids from the C-terminus of PulD, is functional for filamentous-phage assembly only if PulS is also supplied (Daefler *et al.*, 1997). In the absence of PulS, the chimaeric protein induces a strong *psp* response, but when PulS is present there is virtually no PspA induction (Daefler *et al.*, 1997).

In view of these observations, we propose that it is the *process* of inserting pIV or its homologues into the outer membrane that somehow triggers the *psp* induction. This hypothesis can account for a number of other puzzling observations. Carlson and Silhavy (1993) reported that a *lamB* mutation that slows signal-peptide cleavage and outer membrane insertion of this maltoporin makes cells expressing the gene very sick, and is a potent *psp*-operon inducer. Kleerebezem and Tommassen (1993) noted that certain mutations in the *phoE* gene, which encodes the phosphate porin, are also strong inducers of the *psp* response, while wild-type PhoE induces this response only if massively overexpressed. What these proteins may have in common is the capacity to insert into the outer membrane, but they are hindered in some way (as is, presumably, PulD in the absence of PulS), such that the insertion is slow, inefficient, or at an aberrant location. This would also account for the observation that induction of *psp*-operon expression falls quickly once pIV synthesis is halted, although pIV is stable.

It is not clear what signal might be generated by the process of insertion into the outer membrane. Pugsley and co-workers (Hardie *et al*., 1996) have suggested that PulS acts as a chaperone for PulD insertion. We have no evidence for a similar, pIV-specific gene. A screen of *E. coli* genes required for filamentous-phage assembly identified thioredoxin and two as yet uncharacterized genes that affect lipopolysacharide biosynthesis, but no gene that could be considered to be a *pulS* homologue; however, these negative results cannot be considered as conclusive.

There is no PulS homologue in the current *E. coli* sequence database. Our working hypothesis is that f1 pIV can insert into the outer membrane without such a chaperone, but less effectively than does PulD in the presence of PulS. We further suggest that the process of insertion leads to a stress that is recognized as an inducing stimulus by the *psp* system. This hypothesis can be extended to account for the observation that mutations which slow the insertion process, but still leave the protein competent to multimerize/insert, will induce the *psp* operon rather strongly, while mutations which prevent protein export, or which completely block multimerization, will not do so. A variant on this hypothesis would suggest that all of these proteins have some capacity to insert into the inner membrane (presumably from the periplasmic side) and to breach its permeability barrier. Proteins like PulS would then inhibit this inner membrane damage, either by facilitating insertion into the outer membrane, or by acting directly.

Function of the *psp* operon

The phenotypes that have been reported for cells in which the operon is deleted are as follows: lowered survival at alkaline pH in stationary phase (Weiner and Model, 1994), slower protein translocation (Kleerebezem *et al*., 1996; Kleerebezem and Tommassen, 1993), greater motility, and the loss of membrane potential when cells are subjected to a specific, proton-motive force (pmf)-depleting stress (Kleerebezem *et al*., 1996). Overexpression of *pspA* diminishes motility (our unpublished results), and

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this is more marked than the increase in motility observed in the absence of the *psp* operon. As has also been suggested by Kleerebezem *et al.* (1996), these phenotypes are consistent with a role for PspA in somehow preventing loss of protons from the cytoplasm, and we tentatively propose that to be its function. We do not know yet whether PspA has a direct role in this proposed function, or whether it acts as a regulator of the transcription of other genes as well as of its own synthesis. The complexity of the regulatory circuit, and the observation that large amounts of PspA (but not PspB, C or D) are produced from the chromosomal copy of the gene upon strong stimulation, leads us to believe that the protein acts directly in preventing loss of protons from the cytoplasm, in addition to being a regulator. On the other hand, B. Fajardo and R. Misra (personal communication) have recently found that a *cog* mutation, which results in the expression of OmpG, a porin not normally expressed in *E. coli* (Misra and Benson, 1989), can be suppressed by overexpression of PspA. This, together with the map position of *cog*, suggests that the *cog* mutation is in *pspA*. Here, PspA is most probably acting in a regulatory, rather than direct, fashion. Determination of the exact biochemical role of the *psp* proteins will require more effort, but it is fairly clear that the *psp* operon participates in the fine-tuning of *E. coli* homeostasis under stress conditions.

Acknowledgements

We thank Marjorie Russel and Jasna Rakonjac for critical reading of the manuscript. Work in this laboratory is supported, in part, by NSF Grant MCB-9316625. G.J. is supported by a fellowship from the Beckman Fund for Biomedical Research. J.D. held a predoctoral fellowship from the National Science Foundation, and was also supported by Training Grant CA 09763 from the National Institutes of Health.

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