Stress-induced expression of the *Escherichia coli* phage shock protein operon is dependent on σ^{54} and modulated by positive and negative feedback mechanisms

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The phage shock protein (*psp*) operon of *Escherichia coli* is strongly induced in response to heat, ethanol, osmotic shock, and infection by filamentous bacteriophages. The operon contains at least four genes—*pspA*, *pspB*, *pspC*, and *pspE*—and is regulated at the transcriptional level. We report here that *psp* expression is controlled by a network of positive and negative regulatory factors and that transcription in response to all inducing agents is directed by the σ -factor σ^{54} . Negative regulation is mediated by both PspA and the σ^{32} -dependent heat shock proteins. The PspB and PspC proteins cooperatively activate expression, possibly by antagonizing the PspA-controlled repression. The strength of this activation is determined primarily by the concentration of PspC, whereas PspB enhances but is not absolutely essential for PspC-dependent expression. PspC is predicted to contain a leucine zipper, a motif responsible for the dimerization of many eukaryotic transcriptional activators. PspB and PspC, though not necessary for *psp* expression during heat shock, are required for the strong *psp* response to phage infection, osmotic shock, and ethanol treatment. The *psp* operon thus represents a third category of transcriptional control mechanisms, in addition to the σ^{32} - and σ^E -dependent systems, for genes induced by heat and other stresses.

[Key Words: Phage shock protein; stress response; heat shock; σ^{54} ; filamentous bacteriophage; leucine zipper]

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Exposure to certain adverse environmental conditions, such as high temperature, causes all organisms to coordinately and vigorously induce the synthesis of a specific set of proteins called the heat shock proteins (HSPs; for reviews, see Lindquist and Craig 1988; Georgopoulos et al. 1990; Gross et al. 1990). This phenomenon, the heat shock response, is the product of perhaps the best conserved and most universal genetic network. Similarities in this response between prokaryotes and eukaryotes include the sequences of certain HSPs (e.g., the 90-, 70-, and 60-kD HSP families), the treatments that stimulate the response (e.g., heat, ethanol, heavy metal ions), and the large, rapid increases in heat shock gene transcription that follow environmental challenge. In *Escherichia coli*, previous work in several laboratories identified at least two mechanisms of transcriptional control for heat shock gene expression. Most of the detected heat shock genes (~17) are positively regulated by the σ -factor σ^{32} (*rpoH*; Neidhardt and VanBogelen 1981; Yamamori and

Yura 1982; Grossman et al. 1984). RNA polymerase (E), containing a recently discovered second σ -factor, σ^E (σ^{24} ; Erickson and Gross 1989; Wang and Kaguni 1989), transcribes at least two heat shock genes, one of which is *rpoH*. Unlike σ^{32} -controlled transcription, which can be strongly induced by shifts to temperatures that do not limit the cell growth rate (Neidhardt et al. 1984), σ^E -directed transcription of *rpoH* reaches high rates only at extreme or lethal temperatures (Erickson et al. 1987; Erickson and Gross 1989).

The phage shock protein (*psp*) operon consists of at least four genes (*pspA*, *pspB*, *pspC*, and *pspE*) and is induced by heat, ethanol, osmotic shock, and infection by the filamentous bacteriophage f1 (Brissette et al. 1991). Induction by f1, a single-stranded DNA phage, is due specifically to the phage gene IV protein (Brissette et al. 1990), an integral membrane protein that is required for virus production but is not part of the phage particle (Pratt et al. 1966; Brissette and Russel 1990); gene IV protein is the only *psp*-inducing stimulus that does not also induce the HSPs (Brissette et al. 1990). Simultaneous exposure of bacteria to two *psp*-inducing treatments produces an additive effect on *psp* expression, and

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the presence of phage shock proteins in the cell as a result of a previous inducing treatment does not prevent subsequent *psp* expression during a later treatment. The operon is controlled principally at the level of transcription, as are the other heat shock genes, and stress-induced transcription gives rise to two mRNAs—one covering the entire operon and one specific for *pspA* (Brissette et al. 1991). The rate of synthesis of PspA increases at least 50-fold during exposure to extreme conditions (Brissette et al. 1990). Induction in response to stress is independent of σ^{32} , but *psp* transcription during heat shock is prolonged in an *rpoH* mutant, suggesting that a product (or products) of the σ^{32} -controlled heat shock system acts to suppress *psp* expression (Brissette et al. 1990, 1991). The strength of *psp* induction depends directly on the magnitude of the applied stress, and similar to the σ^E -dependent transcription of *rpoH*, *psp* expression reaches its highest rates under growth-restricting or lethal conditions.

In our initial studies, the *psp* operon and its control elements were cloned onto a multicopy plasmid, and exonuclease deletions were introduced from both the 5' and 3' ends of the coding sequence (Brissette et al. 1991). These deletion constructs were assayed for expression of the *psp* genes, and the results suggested that the operon encodes both positive and negative regulatory factors. We now report that *psp* expression is repressed by PspA, and that PspB and PspC cooperatively activate the *psp* promoter, most likely by counteracting this PspA-mediated repression. The PspB and PspC proteins induce expression of the operon during f1 infection, ethanol treatment, and osmotic shock, but *psp* induction by high temperature occurs through a PspB- and PspC-independent mechanism. We show further that all stress-induced transcription of the operon is directed by the alternative σ -factor σ^{54} . σ^{54} is thus the third minor *E. coli* σ -factor found to participate in the regulation of the heat shock response.

Results

The psp operon is controlled by a σ^{54} -dependent promoter

The stress-induced promoter of the operon was mapped to the 253-bp segment immediately upstream from *pspA* (Brissette et al. 1991; Fig. 1). This region does not contain any matches to the consensus sequences for σ^E (Erickson and Gross 1989), σ^F (Arnosti and Chamberlin 1989), or

σ^{32} (Cowing et al. 1985) but does possess several potential σ^{70} promoters (Hawley and McClure 1983) and one good match to the consensus sequence for σ^{54} (Hunt and Magasanik 1985). To identify the *psp* promoter, the mRNA start sites for transcripts induced by heat, ethanol, and f1 infection were mapped by primer extension and RNase protection assays. The primer extension analysis (data not shown) indicated that the mRNAs induced by these three agents share the same promoter with an initiation site located 41 bp upstream from *pspA*. (Each extension reaction terminated at one major site and two adjacent minor sites.) This putative RNA start maps to the initiation point predicted for the σ^{54} promoter sequence. To rule out the possibility that this 5' terminus resulted from RNA processing or the premature termination of the primer extension reactions, RNase protection assays (Fig. 2A) were performed on total bacterial RNA in which unprocessed transcripts were capped with [α - 32 P]GTP and vaccinia virus guanylyl transferase. Only RNAs with 5' triphosphates or diphosphates can receive a GMP cap, forming $G^5-(^{32}P)pp^5$ termini; RNA fragments protected by unlabeled antisense probes will not be visualized unless capped. The capped RNA was hybridized to antisense probes that either covered the 5'-terminal region of the *psp* mRNA (−55 to +202, where +1 is the putative initiation site; lane 1) or included only sequences internal to the *pspA* gene (+80 to +202; lane 3). The first probe should protect a capped 202-base fragment, whereas the second probe should yield an uncapped 122-base RNA. A probe complementary to the *rpoH* promoter region was used as a positive control for the protection of capped message (lane 2). Control assays using uncapped total RNA and radiolabeled antisense probes were also performed (lanes 4–6), enabling all protected RNAs to be visualized. The 202-base protected fragment in lane 1 confirms that the 5' terminus identified by primer extension is correct and shows further that this terminus does not result from processing. The probe internal to *pspA* (lane 3), as expected, did not reveal a 122-base fragment but did yield a smaller amount of the 202-base RNA. This protected fragment results from an in vitro transcription reaction in which a small amount of the anti-sense RNA did not terminate at +80 because of the incomplete digestion of the DNA template.

In parallel with the RNA mapping, a null mutant in *rpoN*, the gene encoding σ^{54} , and its wild-type parent were exposed to heat shock, ethanol, osmotic shock, or f1 infection, and proteins were pulse-labeled with

Figure 1. Schematic diagram of the *psp* operon. The predicted number of amino acids in each phage shock protein is indicated beneath the corresponding gene. Relevant restriction sites are marked. Nucleotide numbers are the same as those of the complete operon sequence in Brissette et al. (1991). Open reading frame 4 (Orf 4) is tentatively designated pspD. (P) Stress-induced promoter.

Figure 2. Identification of the *psp* promoter. (A) Ribonuclease protection assays. RNA was isolated from L1 bacteria 5 min after a temperature shift from 37 to 50°C. (Lanes 1–3) Capped bacterial RNA protected by unlabeled antisense riboprobes; (lanes 4–6) unlabeled bacterial RNA protected with ³²P-labeled riboprobes. The antisense probes were as follows: (Lanes 1,4) pΔ3' 9 linearized with *Bgl*III (complementary to the *psp* promoter region and the 5' terminus of *pspA*); (lanes 2,5) pJLB27 restricted with *Xba*I (complementary to the *rpoH* promoter region); (lanes 3,6) pΔ3' 9 digested with *Bsm*I (complementary to sequences within the *pspA* gene only). (B) PspA synthesis in a σ^{54} mutant. Bacteria were grown at 37°C, subjected to various *psp*-inducing treatments, and pulse-labeled with [³⁵S]methionine. ³⁵S-Labeled proteins were immunoprecipitated with anti-PspA serum and electrophoresed on a 15% SDS–polyacrylamide gel. (Lanes 1–5) Strain K561 ($rpoN^+$); (lanes 6–10) strain L57 ($rpoN^-$). The bacteria were treated as follows: (lanes 1,6) no treatment; (lanes 2,7) 48°C, 5 min; (lanes 3,8) 0.6 M NaCl, 20 min; (lanes 4,9) 8% ethanol, 30 min; (lanes 5,10) f1 infection, 30 min. (C) Sequence of the *psp* promoter. The σ^{54} recognition sequence is underlined. (●) The major transcription start site; (○) two adjacent minor sites.

[³⁵S]methionine (Fig. 2B). Immunoprecipitations with anti-PspA serum demonstrate that *psp* expression in response to stress is abolished by the *rpoN* mutation and, therefore, that this expression is controlled by RNA polymerase containing σ^{54} .

PspB and PspC activate psp operon expression

Earlier deletion studies suggested that PspC activates expression of the operon (Brissette et al. 1991). To confirm this regulatory function for PspC, we deleted *pspC* from the chromosome by homologous recombination and replaced it with the gene conferring kanamycin resistance. The deletion of *pspC* was confirmed by Southern blot and immunoprecipitations using anti-PspC serum (data not shown). The strain was then assayed for *psp* expression by immunoprecipitation of ³⁵S-labeled PspA. The Δ *pspC* strain did not induce the remaining *psp* genes during f1 infection (Fig. 3A; cf. lanes 4,9) and displayed a reduced *psp* response to osmotic shock (0.6 M NaCl; lanes 3,8) and ethanol treatment (lanes 5,10). Surprisingly, the phage shock proteins were still strongly expressed in response to high temperature (lane 7). To demonstrate that the changes in *psp* inducibility did not result from a mutation outside the operon or a polar effect of the kanamycin-resistance gene, *pspC* was restored to the Δ *pspC* strain on a plasmid under the control of the *lac* promoter (pJLB25). As shown in Figure 3, B and C (lanes 7,8), induction of the operon in response to f1 infection is regained in cells carrying the *pspC* expression construct pJLB25, even in the absence of IPTG. Furthermore, the addition of IPTG to pJLB25-containing cells that are not phage infected (or undergoing any other stressful treatment) strongly induces the chromosomal *psp* operon (Fig. 3B,C, lane 6); the amount of PspC pro-

duced without IPTG is not sufficient for *psp* operon expression in the absence of f1 infection. The overexpression of PspC results in such strong PspA induction that PspA is easily visualized on SDS–polyacrylamide gels of total bacterial protein (Fig. 3B; lanes 6–8). In phage-infected cells, the strong synthesis of f1 proteins results in a decline in the amount of [³⁵S]methionine incorporated into *E. coli* proteins (Fig. 3B, lanes 3,4,7,8), which most likely explains why bacteria treated with IPTG alone produce slightly more PspA than those undergoing simultaneous f1 infection and IPTG treatment (Fig. 3C, lanes 6,8). The results demonstrate that PspC possesses a positive autoregulatory function and that this protein is required for *psp* promoter expression in response to f1 infection. The responses to osmotic shock and ethanol are partially dependent on PspC, and expression during heat shock is PspC independent.

We have found that the sensitivity of the *psp* response to osmotic shock varies from strain to strain. For example, the strain L1 induces the phage shock proteins more vigorously than K38 and its derivatives at lower salt concentrations (0.3 M NaCl). The deletion of *pspC* from L1 (creating L32) completely abolishes *psp* expression in response to the addition of 0.3 M NaCl (unpubl.).

The induction of *psp* synthesis by high temperature in the Δ *pspC* strain contrasts with earlier results with the cloned operon. In our previous expression studies, exonuclease deletions starting at the 3' end of the coding sequences of the operon eliminated the heat inducibility of the plasmid-borne *psp* genes when the deletions extended into *pspC* (Brissette et al. 1991). We have repeated our earlier experiments and, in addition, placed *pspC* on a plasmid in *trans* with one such 3'-deletion construct that carries only *pspA*, *pspB*, and the upstream control elements. Expression of the cloned *psp* promoter during heat shock was restored by the *trans* copy of *pspC* in a

Figure 3. PspC activates *psp* expression. (A) Bacteria were grown at 37°C, exposed to various *psp*-inducing treatments, and pulse-labeled with [³⁵S]methionine. ³⁵S-Labeled proteins were immunoprecipitated with anti-PspA serum and analyzed by SDS-PAGE. (Lanes 1–5) Strain K561 (*psp*⁺); (lanes 6–10) strain J136 (Δ *pspC*). Treatments were as follows: (lanes 1,6) No treatment; (lanes 2,7) 48°C, 5 min; (lanes 3,8) 0.6 M NaCl, 20 min; (lanes 4,9) f1 infection, 30 min; (lanes 5,10) 8% ethanol, 30 min. (B) Total ³⁵S-labeled protein from J136 (Δ *pspC*) containing either pGL101B (lanes 1–4) or pJLB25 (lanes 5–8; *pspC* under *lac* control). (Lanes 1,5) No treatment; (lanes 2,6) 2 mM IPTG, 30 min; (lanes 3,7) f1 infection, 30 min; (lanes 4,8) f1 infection; 2 mM IPTG, 30 min. The arrow indicates the f1 gene V and coat proteins. (C) Immunoprecipitations with anti-PspA serum of the samples shown in B. Lanes in C correspond to those in B.

recA mutant (data not shown), showing that the plasmid-borne copies of the operon require PspC for induction by heat.

A series of experiments similar to those described for *pspC* were performed on the *pspB* gene. *pspB* was deleted from the chromosome and replaced with the kanamycin-resistance gene by the same method used to construct the Δ *pspC* strain. Again, the deletion mutant was checked by Southern blot. Assays for *psp* expression in the absence of PspB (Fig. 4A) show that like PspC, PspB is required for induction of the operon by f1 (cf. lanes 4,8) but is not needed for heat shock expression (lanes 2,6). Phage shock protein synthesis following osmotic shock (Fig. 4A,B) or ethanol treatment (data not shown) is reduced in the absence of PspB, again similar to the results obtained in the Δ *pspC* strain.

The *pspB* gene was restored to the *pspB* null mutant under *lac* control on a plasmid (pL1). The expression of *pspB* from pL1 prevented efficient infection by f1 and f2 phage (f2 is an RNA phage not related to f1 but, which like f1 infects through the F-pilus), suggesting that the overproduction of PspB caused a pilus defect; thus, bacteria carrying pL1 were assayed for the restoration of full *psp* inducibility using osmotic shock as the test treatment. As shown in Figure 4B, the expression of PspB from the plasmid results in a greatly elevated response of the chromosomal operon to the addition of high salt (cf. lanes 7 and 8 with lanes 11 and 12). The production of PspB from the plasmid is high even without IPTG (lane 1), and this PspB synthesis stimulates chromosomal *psp* expression weakly in the absence of any stress (lane 9). Increasing PspB production (lane 2) further by adding IPTG to unstressed pL1-containing cells, however, does not result in the strong induction of the chromosome-encoded *psp* genes (lane 10). In fact, *psp* operon expres-

sion declines to some extent when compared to pL1-containing cells not treated with IPTG.

The ability of pL1 (*pspB* expression construct) to restore the high level *psp* response to osmotic shock shows that the decreased *psp* expression observed in the Δ *pspB* strain is a result of the loss of PspB and not a polar effect of the Kan^R insertion. In further support of this, the production of PspB from pL1 in the Δ *pspC* strain does not induce the chromosomal operon (L. Weiner, unpubl.). Thus, PspB cannot activate expression in the absence of PspC, and PspC must be synthesized in the Δ *pspB* strain.

The results of the experiments shown in Figure 4 demonstrate that PspB, like PspC, activates *psp* expression, and that PspB is required for bacteria to mount a normal *psp* response during f1 infection, osmotic shock, and ethanol treatment; the pathway for induction during heat shock is again distinct and does not utilize PspB. In contrast with the findings for PspC, increases in PspB concentration above a certain level do not yield corresponding increases in *psp* expression and may be inhibitory. The weak PspA synthesis induced by plasmid-encoded PspB cannot be visualized on total protein gels. In contrast, as shown in Figure 3B, the overproduction of PspC results in *psp* overexpression and suggests a direct, constant relationship between the amount of PspC in the cell and the amount of *psp* transcription.

We also examined the effect of PspB on the expression of the operon cloned on a plasmid. The deletion of *pspB* from the cloned operon eliminates the heat inducibility of the plasmid-borne genes, and the placement of *pspB* on a second plasmid in *trans* restores the induction by heat shock (data not shown). Thus, for the response to high temperature, the cloned operon requires two activating factors—PspB and PspC—which the single, chromosomal copy does not need.

Figure 4. PspB positively regulates the *psp* genes. (A) Bacteria grown at 37°C were pulse-labeled with [35 S]methionine following exposure to *psp*-inducing treatments. 35 S-labeled proteins were immunoprecipitated with anti-PspA serum and analyzed by SDS-PAGE. (Lanes 1–4) Strain K561 (*psp*⁺); (lanes 5–8) strain L65 (Δ *pspB*). Treatments were as follows: (lanes 1,5) No treatment; (lanes 2,6) 48°C, 5 min; (lanes 3,7) 0.6 M NaCl, 20 min; (lanes 4,8) f1 infection, 30 min. (B) L65 containing either pGL101B or pL1 (*pspB* under the *lac* promoter) was pulse-labeled with [35 S]methionine following IPTG addition (2 mM; 30 min), osmotic shock (0.6 M NaCl; 20 min), or both treatments. 35 S-labeled proteins were immunoprecipitated with either anti-PspB (lanes 1–4) or anti-PspA (lanes 5–12) serum and electrophoresed. (B) A composite of two exposures of the same autoradiogram; lanes 1–4 were exposed for 15 hr; lanes 5–12 were exposed for 72 hr. The experiment is summarized by the chart below:

Lane	1	2	3	4	5	6	7	8	9	10	11	12
Antiserum	B	B	B	B	A	A	A	A	A	A	A	A
Plasmid gene	B	B	B	B	–	–	–	–	B	B	B	B
IPTG	–	+	–	+	–	+	–	+	–	+	–	+
NaCl	–	–	+	+	–	–	+	+	–	–	+	+

PspB enhances PspC-dependent gene expression

The Δ *pspB* strain (L65) and its wild-type parent (K561) were transformed with plasmids carrying either *pspC* (pJLB25) or *pspB* and *pspC* (pLW33), under *lac* control. The strains were assayed again for chromosomal *psp* expression with and without IPTG by immunoprecipitating 35 S-labeled PspA. The level of PspC synthesis in each strain was determined to be the same by immunoprecipitation of 35 S-labeled PspC (data not shown). As shown in Figure 5, the production of PspC alone in the Δ *pspB* strain induces PspA (lane 8), although this *pspA* expression is significantly lower than that induced by PspC synthesis in the wild-type strain (lane 6). The expression of *pspB* and *pspC* from pLW33 in either K561 (*psp*⁺) or L65 (Δ *pspB*; lanes 2,4) yields the same level of chromosomal *psp* expression as the PspC production from pJLB25 in K561 (where the only source of PspB is the chromosome).

These results show that PspB is not absolutely required for the induction of the *psp* genes via the PspC-dependent pathway, as PspC synthesis activates *psp* expression in L65 (Δ *pspB*). PspC-controlled expression is weak in the absence of PspB, however, and L65 is restored to full *psp* inducibility by pLW33, showing that the differences between L65 and K561 do not arise from

an effect of the Kan^R gene insertion on the surrounding genes. PspB thus cooperates with PspC in the induction of the operon and appears to enhance the positive regulatory activity of PspC. The coexpression of *pspB* and *pspC* from a multicopy plasmid does not result in a greater stimulation of the chromosomal *psp* genes than the expression of only *pspC* from the same vector in wild-type cells; that is, the effects of coexpressing *pspB* and *pspC* are not additive. The single-copy, chromosomal *pspB* gene provides sufficient PspB to cooperate with the overproduced PspC, suggesting that stoichiometric concentrations of PspB and PspC are not required for optimal *psp* induction. One possible interpretation of this data is that PspB catalytically activates PspC, perhaps by modifying or extending the half-life of PspC; PspC was shown to be modified when produced in heat-shocked cells (Brissette et al. 1991). The half-life of PspC, however, was determined in pulse-chase experiments to be the same whether PspC is produced in the presence or absence of PspB (data not shown). Immunoblots with anti-PspC serum also did not detect a difference in the migration of PspC from the Δ *pspB* strain, implying that PspB is not involved in PspC modification.

PspA negatively regulates the psp genes

In our previous studies of the operon cloned on a plasmid, a 3'-exonuclease deletion that removed the 3' end of the *pspA* gene and all downstream sequences (p Δ 3' 8) resulted in the strong production of a truncated PspA protein at both 37°C and 50°C (Brissette et al. 1991). This constitutive *pspA* expression suggested that a negative regulatory factor or sequence element had been deleted. To identify the component mediating repression, the complete *pspA* gene and its upstream promoter elements were cloned onto a plasmid (pLW2). Carboxy-terminal mutations in PspA were then created by either deleting the 3' end of *pspA* (pLW9) or introducing a frameshift after codon 168 (pLW27). The frameshift mutation results in a substantial change in the carboxy-terminal se-

Figure 5. PspB enhances PspC-directed expression of the phage shock proteins. K561 (*psp*⁺) and L65 (Δ *pspB*) bacteria containing either pJLB25 (*pspC* under the *lac* promoter) or pLW33 (*pspB* and *pspC* under *lac* control) were pulse-labeled with [35 S]methionine before and after the addition of 2 mM IPTG (30 min). 35 S-labeled proteins were immunoprecipitated with anti-PspA serum and electrophoresed. (Odd-numbered lanes) No IPTG; (even-numbered lanes) + IPTG. (Lanes 1,2) K561, pLW33; (lanes 3,4) L65, pLW33; (lanes 5,6) K561, pJLB25; (lanes 7,8) L65, pJLB25. The figure is summarized by the chart below:

Lane	1	2	3	4	5	6	7	8
Chromosomal <i>pspB</i>	+	+	–	–	+	+	–	–
Plasmid genes	BC	BC	BC	BC	C	C	C	C
IPTG	–	+	–	+	–	+	–	+

quence of the PspA protein without significantly altering the DNA or mRNA sequences. The 3'-deletion plasmid pLW9 should produce a truncated PspA protein of 179 amino acids, and the frameshift construct pLW27 should give rise to a 197-amino acid PspA; wild-type PspA is 222 amino acids (Fig. 1). These plasmids were transformed into bacterial strain HB101 and assayed for *pspA* expression before and after a shift to high temperature. As shown in Figure 6A, pLW2 weakly expresses *pspA* at 37°C and 50°C (lanes 3,8; as stated earlier, the plasmid-borne operon requires *pspB* and *pspC* in high copy number for the response to heat shock; lanes 2,7), but both pLW9 (3' deletion) and pLW27 (frameshift) display strong, constitutive PspA production (lanes 4,5,9,10). The high-level expression of *pspA* comes from the *psp* promoter, for this expression is abolished by a mutation in *rpoN* (data not shown). These data strongly suggest that the component effecting *psp* repression is the PspA protein. At 50°C, the cells containing the frameshift construct pLW27 synthesize both the predicted 197-amino-acid PspA and a second protein of ~25 kD that reacts with the anti-PspA serum (lane 9). This larger protein is not wild-type PspA expressed from the chromosomal operon, for it is produced when pLW27 is assayed in the Δ *pspA* strain L2 (data not shown). The protein most likely comes from the plasmid and represents translation through the opal stop codon that terminates the frame-shifted *pspA* gene. The frequency of translational readthrough is higher for opal codons than for either amber or ochre (Sambrook et al. 1967; Horiuchi et al. 1971; Moore et al. 1971; Weiner and Weber 1971), and this leakiness in termination is apparently increased at high

temperature. The next stop codon 37 nucleotides downstream from the opal codon is an ochre, and termination here would give rise to a frameshifted PspA of 210 amino acids.

To confirm that PspA negatively regulates the operon, we tested PspA for the ability to repress the *psp* promoter in *trans*. We placed the wild-type *pspA* gene on a plasmid under *lac* control (pJLB24) and transformed this construct into bacteria containing the truncated *pspA* and its promoter on a compatible plasmid (pJLB26; the *pspA* mutant of pLW9 transferred to pACYC184). The bacterial strain used in this experiment contains a chromosomal deletion of the *pspA*, *pspB*, and *pspC* genes and was constructed with the same gene replacement technique employed previously. This strain was chosen to better understand the transcriptional mechanism responsible for the constitutive expression of the mutant *pspA* genes. The mutant PspA proteins are produced at high levels in HB101 despite the absence of *pspB* and *pspC* from the plasmid. HB101 possesses a chromosomal copy of the *psp* operon, and the question arises as to whether PspB and PspC derived from the chromosome are required for expression of the PspA mutants.

As shown in Figure 6B, the carboxy-terminal deletion mutant of PspA is constitutively produced in a strain lacking PspB and PspC (lanes 5,6). The production of wild-type PspA in *trans* with the mutant represses synthesis of the truncated PspA (lanes 7,8). The expression of *pspA* from the *lac* promoter without IPTG is sufficient to effect repression. Low levels of the truncated PspA protein are visible (lanes 7,8) upon long exposures of these immunoprecipitations. These results demonstrate that PspA negatively regulates the operon and that PspB and PspC are not required to activate expression when PspA is mutated. The constitutive production of the truncated PspA strongly suggests that PspA activity prevents the operon from being highly expressed under normal conditions.

The truncated and frameshifted PspA proteins are constitutively synthesized in HB101 despite the presence of the wild-type *pspA* gene in the chromosome. The inability of the chromosome-derived PspA to repress the plasmid constructs suggests that either the multiple copies of the plasmid-borne *psp* promoter overwhelm and titrate out a repressor or that the mutant *pspA* genes negatively complement wild-type *pspA*. To address these possibilities, we fused the *psp* promoter region to the *lacZYA* genes on a high-copy plasmid and transformed this construct (pLW38), as well as its parent plasmid carrying the *lac* genes without their promoter and operator (pSKS107; Shapira et al. 1983), into either L1 or its Δ *pspA* sibling L2. We reasoned that if the *pspA* mutants negatively complement *pspA*⁺, then the *psp-lac* fusion should be strongly expressed only in the Δ *pspA* strain L2. The L1 transformants containing either pSKS107 or pLW38 and the L2 transformants carrying pSKS107, grew well on rich media, produced a faint blue color on plates with 35 μ g/ml of X-gal, and synthesized low levels of β -galactosidase (as determined by analyzing ³⁵S-labeled proteins on SDS-polyacrylamide gels; data not shown).

Figure 6. PspA represses *psp* expression. (A) Bacterial strain HB101 containing various plasmid constructs was pulse-labeled with [³⁵S]methionine before (lanes 1–5) and 5 min after (lanes 6–10) a shift from 37°C to 50°C. ³⁵S-labeled proteins were immunoprecipitated with anti-PspA serum and analyzed by SDS-PAGE. (Lanes 1,6) pBS; (lanes 2,7) pPS-1 (the complete *psp* operon on pBS); (lanes 3,8) pLW2 (wild-type *pspA* and its promoter on pBS); (lanes 4,9) pLW27 (pLW2 with a frameshift in *pspA*); (lanes 5,10) pLW9 (pLW2 with a 3' deletion of *pspA*). (B) J134 (Δ *pspA-pspC*) bacteria, each containing two different plasmids in *trans*, were pulse-labeled with [³⁵S]methionine before (odd-numbered lanes) and 30 min after (even-numbered lanes) the addition of 2 mM IPTG. ³⁵S-labeled proteins were again immunoprecipitated with anti-PspA serum and electrophoresed. (Lanes 1,2) pGL101B, pACYC184; (lanes 3,4) pJLB24 (*pspA* under the *lac* promoter), pACYC184; (lanes 5,6) pGL101B, pJLB26 (*pspA* 3' deletion); (lanes 7,8) pJLB24, pJLB26. B is summarized by the chart below (the truncated *pspA* is designated *pspA*^{*}):

Lane	1	2	3	4	5	6	7	8
<i>pspA</i> ⁺	–	–	+	+	–	–	+	+
<i>pspA</i> [*]	–	–	–	–	+	+	+	+
IPTG	–	+	–	+	–	+	–	+

In contrast, L2 bacteria transformed with pLW38 grew very slowly on rich media, and produced star-shaped or papillated colonies. These cells proved difficult to manipulate in experiments designed to measure β -galactosidase production. We therefore titered transformant colonies of approximately equal size from both strains (as soon as the colonies appeared, without intervening storage) for the number of viable cells containing a plasmid and expressing ampicillin resistance. All colonies, including L2 transformed with pLW38, contained 6×10^8 to 9×10^8 total viable cells. The ratio of Amp^R to total cells was ~ 1 for L1 carrying either pSKS107 or pLW38, and L2 containing pSKS107. In the case of L2 transformed with pLW38, the ratio of Amp^R to total viable cells varied between 2×10^{-4} and 3×10^{-3} . These experiments suggest that the mutation of *pspA* in L2 results in the strong induction of the *psp-lac* fusion construct to levels that are extremely harmful and growth restricting. The presence of the wild-type chromosomal copy of *pspA* in L1 bacteria apparently limits the *psp*-controlled expression of the *lac* genes. Thus, the strong, constitutive production of the truncated and frameshifted PspA proteins most likely results from negative complementation and not the titration of a repressor.

Discussion

Our results demonstrate that expression of the *psp* operon is controlled by a network of positive and negative regulatory factors (Fig. 7). *psp* expression is suppressed during balanced growth under normal conditions and is only transiently induced by heat and osmotic shock. (In contrast with the response to these stresses, *psp* expression remains high as long as gene IV protein is produced.) Continuous exposure to high temperatures or salt leads to a gradual decline in Psp synthesis to approximately pre-shock levels [Brissette et al. 1990]. Repression is therefore established under various circumstances and may not be governed by a single mechanism. Both *pspA* and *rpoH* (σ^{32}) mutations disrupt the negative regulation of the *psp* genes. Mutations in *pspA* result in high rates

of expression from the *psp* promoter in the absence of any harmful environmental conditions. This constitutive Psp expression is suppressed by wild-type PspA produced in *trans* from a heterologous promoter. PspA thus participates in a negative feedback loop, most likely preventing Psp synthesis during balanced growth and perhaps determining the duration of *psp* induction.

The synthesis of carboxy-terminal PspA mutants from a plasmid appears to interfere with the function of wild-type PspA derived from the chromosome. This interference, termed negative complementation, has been found in other cases (such as certain *lac* repressor mutants; Beckwith 1987) to result from the formation of nonfunctional wild-type-mutant protein complexes. The PspA protein possesses the heptad repeats characteristic of proteins that form coiled-coil structures [Brissette et al. 1991]; therefore, on the basis of its sequence, PspA would be predicted to form a complex with either itself or another protein. The regions containing the coiled-coil heptad repeats are retained by the truncated and frameshifted PspA proteins.

Expression of the *psp* genes is prolonged in an *rpoH* mutant during heat shock [Brissette et al. 1991], showing that induction of the σ^{32} -controlled regulon is required to turn off the *psp* response. The HSPs DnaK [Tilly et al. 1983], DnaJ [Sell et al. 1990], and GrpE [Gross et al. 1990] are known to negatively regulate the σ^{32} -dependent system, but we do not know yet whether these three heat shock proteins participate in the *psp* shutoff mechanism. Chromosomal deletions of the *psp* genes do not yield any obvious effects on bacterial growth or viability, and we have suggested previously that this lack of a phenotype could result from the existence of other bacterial genes with similar or overlapping functions [Brissette et al. 1991]. The ability of both PspA and the σ^{32} -directed HSPs to negatively regulate the *psp* operon provides some evidence for this proposed functional overlap.

The PspB and PspC proteins cooperatively activate *psp* expression, forming a positive feedback loop. The deletion of either *pspB* or *pspC* eliminates the strong response of the chromosomal operon to f1 infection, osmotic shock, and ethanol. These expression phenotypes are complemented by *pspB* or *pspC* restored to their respective deletion strains on a plasmid. The synthesis of either PspB or PspC from a plasmid under normal conditions stimulates the chromosomal *psp* genes, showing that environmental stress is not required to generate active PspB and PspC when these proteins are produced in high quantity.

The plasmid expression studies also reveal differences in the effects of PspB and PspC on the level of *psp* activation. The overproduction of PspC from a plasmid results in the overexpression of the chromosomal operon, whereas the overproduction of PspB yields only weak chromosomal *psp* expression. Thus, the level of phage shock protein synthesis is more directly dependent on the concentration of PspC than PspB. PspC, when overexpressed, does not require PspB to perform its activating function, as PspC produced from a plasmid stimulates the chromosomal operon in cells lacking PspB. PspB does

Figure 7. Summary of *psp* regulation. Positive and negative control pathways are indicated. The roles of PspB and PspC in the responses to various treatments are shown.

contribute to the apparent efficiency of PspC function, however, as the presence of PspB significantly enhances PspC-directed expression. In the reciprocal experiment, PspB expressed from a plasmid was found to require PspC to induce the chromosomal *psp* genes.

The level of *psp* expression appears dependent on the interplay of PspA, PspB, and PspC. Mutations in *pspA* are sufficient to cause strong, constitutive *psp* expression under normal growth conditions. The PspB and PspC proteins are not required to induce or maintain expression in the absence of functional PspA. Because *pspA* mutations abolish any need for PspB and PspC, the regulatory roles of PspB and PspC may be to antagonize the repression mediated by PspA. Hence, PspB and PspC may activate *psp* expression by counteracting the negative feedback mechanism controlled by PspA.

In contrast to the chromosomal *psp* genes, the plasmid-borne operon requires PspB and PspC for induction during heat shock. One possible explanation for these results is that the chromosome and plasmid differ in topology and that topological features of the *psp* promoter play a significant role in its regulation. We thus examined *psp* expression following treatment with novobiocin, which inhibits DNA gyrase and thereby prevents the formation of negative supercoils (Gellert et al. 1976). In heat-shocked, novobiocin-treated cells, induction of the cloned operon remains dependent on PspB and PspC, and chromosomal activation stays PspC independent (L. Weiner, unpubl.). Similarly, novobiocin does not prevent *psp* expression in wild-type bacteria during osmotic shock, even though hypertonic stress increases negative supercoiling, and this supercoiling was shown to regulate the osmoresponsive gene *proU* (Higgins et al. 1988). We suggest, then, the alternative explanation that the plasmid-borne operon requires PspB and PspC as a result of the high levels of PspA that accumulate in the plasmid-containing cells. As shown in Figure 6A, bacteria containing the high-copy pLW2 (which carries only the *pspA* gene and its promoter) constitutively synthesize a significant amount of PspA at 37°C. These relatively high concentrations of PspA (compared to the normal bacterial condition) may lead to a requirement during heat shock for the apparently antirepressing activities of PspB and PspC.

PspB and PspC enable the *psp* operon to respond to certain changes in its environment and thus connect the operon to its surroundings. Studies of other regulatory pathways have found that many bacterial systems employ a common mechanism for processing and reacting to environmental information (for reviews, see Gross et al. 1989; Stock et al. 1990). In these pathways, a kinase (frequently called the sensor) responds to an intracellular signal by phosphorylating a regulatory protein, often a DNA-binding transcription factor. The kinases share a homologous domain at their carboxyl termini, and the regulator proteins possess similar amino-terminal domains.

pspB and *pspC* do not belong to either the kinase or regulator gene families (Brissette et al. 1991) but could function nonetheless as a two-component signal trans-

duction system with generally analogous properties. PspC, like proteins in the regulator class, is the more direct determinant of the level of gene expression. Many regulator proteins control gene expression by binding to promoter elements, and a subset of these factors contains the helix–turn–helix motif, an evolutionarily conserved structure for binding DNA (Pabo and Sauer 1984). We do not know yet whether PspC binds to DNA, and this protein does not contain the helix–turn–helix motif. PspC is predicted to possess a leucine zipper (Brissette et al. 1991), a structure present in many eukaryotic transcriptional activators and involved in protein dimerization through the formation of a coiled coil (Landschultz et al. 1988; Hu et al. 1990 and references therein). The leucine zipper of PspC contains six leucine heptad repeats and a valine heptad in phase with the leucines. In many factors, the leucine zipper is adjacent to a basic domain that is associated with DNA binding (Hope and Struhl 1986; Kouzarides and Ziff 1988; Landschultz et al. 1988), but this domain is not present in PspC.

PspB is not required for PspC function but enhances PspC-dependent gene expression. Similar regulatory phenotypes have been reported for EnvZ (Villarejo and Case 1984; Mizuno and Mizushima 1987) and GlnL (NtrB; McFarland et al. 1981; Chen et al. 1982), members of the kinase or sensor family, in that both proteins stimulate but are not essential for the activity of a DNA-binding transcription factor. Like the enzymatic sensor proteins, PspB could interact with PspC catalytically, because stoichiometric concentrations of PspB and PspC are not required to optimize *psp* expression. However, we have not observed a difference in PspC modification or stability in *pspB* mutant bacteria. We do not rule out the possibility that PspB modifies PspC in a way not detected in our gel system.

The *psp* operon is transcribed in response to stress by RNA polymerase containing the alternative σ -factor σ^{54} . σ^{54} is thus the third minor *E. coli* σ -factor, in addition to σ^{32} and σ^E , which controls heat shock gene expression and participates in the heat shock response. The *psp* genes are induced most strongly under extreme or lethal conditions, and this pattern of gene expression resembles transcription controlled by σ^E (Erickson et al. 1987; Erickson and Gross 1989). The evolution of a regulatory mechanism for the *psp* operon that is independent of σ^E , as well as σ^{32} , suggests that Psp synthesis may be needed under circumstances that do not induce the σ^E - or σ^{32} -directed systems. At present, the only known *psp*-inducing stimulus that does not induce other HSPs is the fl gene IV protein, a membrane protein of unknown biochemical function required for phage secretion and morphogenesis (Brissette and Russel 1990). The *Klebsiella pneumoniae* protein PulD, a homolog of the gene IV protein required for pullulanase secretion (d'Enfert et al. 1989), was recently shown to also induce the *E. coli* *psp* genes (M. Russel, pers. comm.).

σ^{54} transcribes bacterial genes with a diverse array of functions and is unique among σ -factors in its biochemistry. Like PspC, it is predicted to possess hydrophobic heptad repeats capable of forming a zipper-like structure

(Sasse-Dwight and Gralla 1990). All known σ^{54} -dependent genes require the binding of an activator protein to upstream sequences for transcription to proceed (Kustu et al. 1989). In at least two cases, these upstream sequences are similar to eukaryotic enhancers in that they can activate transcription irrespective of their distance and orientation from the promoter (Reitzer and Magasanik 1986; Birkman and Bock 1989). The *psp* promoter possesses at least one notable difference from other σ^{54} -dependent promoters: Although σ^{54} recognition sequences contain highly conserved GG and GC doublets 10 bp apart (Kustu et al. 1989), the *psp* promoter lacks the GC sequence and utilizes GT instead.

At least three distinct regulatory pathways are now shown to govern transcription of the bacterial heat shock genes. These three pathways interconnect, as the σ^{32} -controlled system down-regulates *psp* expression, and the gene for σ^{32} is transcribed by σ^E . We think it likely that *psp* activation involves at least one additional transcription factor not yet identified. We have shown that a σ^{54} -directed mechanism for inducing the *psp* genes exists that does not utilize PspB and PspC; and, as stated, all previously studied σ^{54} -dependent promoters require an activating factor for σ^{54} initiation. It is also possible that PspA, PspB, and PspC regulate not only their own production but the synthesis of proteins not encoded by the operon.

The complexity in the regulation of bacterial heat shock transcription may be conserved in eukaryotes. Initial studies of eukaryotic heat shock gene expression identified a DNA sequence, called the heat shock element (HSE), which mediates the response to temperature increases and is present in the promoter regions of heat shock genes from many species (Bienz and Pelham 1987). In yeast, the HSE is bound by heat shock transcription factor (HSTF), the product of a single-copy gene (Sorger and Pelham 1988; Wiederrecht et al. 1988). Recently, a yeast heat shock gene was identified that lacks an HSE in its control region and appears to be independent of HSTF (Kobayashi and McEntee 1990). In tomato plants, three different genes have been cloned that encode proteins capable of binding to the HSE (Scharf et al. 1990). Thus, it is likely that in both prokaryotes and eukaryotes, transcriptional control of the heat shock response will involve the coordination of multiple regulatory pathways.

Materials and methods

Bacterial strains and phages

Bacterial strains used in this study, all derivatives of *E. coli* K12, are listed in Table 1. The f1 and P1_{vir} bacteriophage are from our laboratory collection. Transductions were performed according to Miller (1972), and transductants were selected for resistance to the appropriate antibiotic or growth without uracil (*pyrF*⁺ bacterial). Transformations were performed by using either the CaCl₂ procedure (Maniatis et al. 1982) or protocol 3 of Hanahan (1985).

Plasmids

Restriction enzymes were purchased from New England Bio-

Table 1. Bacterial strains

Strain	Relevant genotype	Source
K38	HfrC λ^+ <i>relA1 spoT1</i> <i>T₂^R(ompF627,fadL701)</i>	this laboratory
K561	K38 <i>lacI^q</i>	this laboratory
HB101 F ⁺	<i>hsdS20 (r_B⁻m_B⁻) F⁺::Tn5</i>	this laboratory
JC7623	<i>F⁻recB21 recC22 sbcB15</i>	A.J. Clark
YA149	HfrH <i>pyrF40 relA1 spoT1</i>	CGSC 4500
YMC18	<i>rpoN::Tn10</i>	B. Magasanik
L1	YA149 <i>pyrF⁺</i>	this laboratory
L2	L1 Δ <i>pspA::kan</i>	this laboratory
L12	JC7623 Δ <i>pspC::kan</i>	this laboratory
L14	JC7623 Δ <i>pspA-C::kan</i>	this laboratory
L30	JC7623 Δ <i>pspA::kan</i>	this laboratory
L32	L1 Δ <i>pspC::kan</i>	this laboratory
L57	K561 <i>rpoN::Tn10</i>	this laboratory
L63	JC7623 Δ <i>pspB::kan</i>	this laboratory
L65	K561 Δ <i>pspB::kan</i>	this laboratory
J134	K561 Δ <i>pspA-C::kan</i>	this laboratory
J136	K561 Δ <i>pspC::kan</i>	this laboratory

labs. T4 DNA ligase and the Klenow fragment of DNA polymerase I were from BRL. pBS SK/+ was from Stratagene. Plasmid DNA was purified according to Maniatis et al. (1982).

The expression vector pGL101B (Guarente et al. 1980; Fulford and Model 1988) was used to place the *psp* genes under the control of the *lac* UV5 promoter-operator; the *psp* genes were always cloned into the *Bam*HI site of the vector. pJLB24 was constructed by cloning the 0.85-kb *Bgl*II-*Hinc*II fragment carrying the *pspA* gene into pGL101B. *pspC* was placed under *lac* control (pJLB25) by using the 0.55-kb *Xmn*I-*Pvu*II fragment of pLW23 (Brissette et al. 1991). pLW23 consists of the *psp* operon on pBS with the operon sequences downstream of *pspC* deleted by exonuclease digestion. The 0.95-kb *Dde*I-*Nae*I fragment of pLW23 containing *pspB* and *pspC* was ligated to pGL101B to create pLW33. The *pspB* gene was cloned into pGL101B (pL1) as described previously (Brissette et al. 1991).

pLW2 carries the complete *pspA* gene and all control sequences of the *psp* promoter. This plasmid was constructed by ligating the 1.7-kb *Hinc*II fragment of pPS-1 (Brissette et al. 1991) into the *Hinc*II site of pBS. The 3'-terminal region of the *pspA* gene was deleted from pLW2 by digesting with *Bst*BI and *Xho*I, blunt-ending with the Klenow fragment of DNA polymerase I, and religating. This construct, pLW9, lacks all *psp* sequences downstream of nucleotide 1002 (*pspA* codon 168; for the complete *psp* operon sequence, see Brissette et al. 1991) and is similar to p Δ 3' 8 (Brissette et al. 1991), in which all sequences downstream of nucleotide 1018 were removed by exonuclease digestion. pJLB26 consists of the *pspA* 3'-deletion mutant cloned onto pACYC184 (Chang and Cohen 1978) and was constructed by ligating the 1.5-kb *Bam*HI-*Kpn*I fragment of pLW9 to pACYC184 digested with *Bam*HI and *Hinc*II. A frameshift mutation (pLW27) was introduced into the *pspA* gene on pLW2 by digesting with *Bst*BI, end-filling with the DNA polymerase I Klenow fragment, and religating. There are two tandemly repeated *Bst*BI sites starting at nucleotide 999; therefore, the construction of pLW27 resulted in the removal of nucleotides 1003-1009 and no nucleotide insertions.

The plasmids used to construct chromosomal null mutants replace *psp* sequences with the Kan^R cassette of pSKS101 (Shapira et al. 1983); the Kan^R gene was removed from pSKS101 with either *Eco*RI or *Bam*HI. pLW6 consists of the Kan^R cassette ligated to pPS-3 (Brissette et al. 1991) restricted with *Sac*II.

pLW18 was constructed by cloning the 0.65-kb *Sna*BI–*Sma*I fragment of pPS-3 into the *Sac*I site of pLW6. pLW18 therefore consists of pPS-3 (the complete *psp* operon on pBS) with the DNA segment between the *Sac*II and *Sna*BI sites deleted and replaced with the Kan^R gene. pLW26 was constructed by deleting the 1.38-kb *Bgl*II–*Sna*BI segment of pPS-3 and replacing it with the Kan^R cassette. pLW35 consists of the 1.0-kb *Xmn*I–*Eco*RV fragment of pPS-1 cloned into the *Kpn*I site of pLW2. pLW36 is pLW35 with the Kan^R cassette ligated into the *Xho*I site; the Kan^R gene thus replaces the sequences between the *Hinc*II and *Xmn*I sites in *pspB*.

pLW38 was constructed by isolating the 0.6-kb *Bsm*I fragment of pLW27 (containing the *psp* promoter and the first 12 codons of *pspA*), generating a blunt end with T4 DNA polymerase and ligating the fragment to pSKS107 (Shapira et al. 1983) restricted with *Sma*I. The plasmid pJET41 (provided by C. Gross and J. Erickson) was described previously (Erickson and Gross 1989). pJLB27 consists of the *Sph*I–*Sal*I fragment of pJET41 ligated to pBS digested with *Eco*RV and *Sal*I.

Deletion of the chromosomal *psp* genes

Bacterial *psp* null mutants were generated by the single-step homologous recombination method of Winans et al. (1985). The plasmids pLW36, pLW18, and pLW26, on which various *psp* genes are replaced with the Kan^R gene, were linearized and transformed into JC7623, a *recB recC sbcB* mutant. Transformants resistant to kanamycin (30 µg/ml) were shown by Southern blot and immunoprecipitation of ³⁵S-labeled proteins to have undergone gene replacement. The null strains are designated L63 (*ΔpspB::kan*; Kan^R replaces codons 10–62), L12 (*ΔpspC::kan*; Kan^R replaces codons 20–113), and L14 (*ΔpspA–pspC::kan*; Kan^R replaces the region from 96 bp upstream of the *pspA* start codon to codon 113 of *pspC*). Transcription of the Kan^R gene in these three strains is in the same direction as *psp* transcription. Northern blots have shown that this Kan^R cassette does not contain a transcriptional terminator and that transcripts initiated at the *psp* promoter proceed through the Kan^R gene to the end of the operon (T. Ripmaster and J. Brissette, unpubl.). Construction of the *ΔpspA* strain L30 was described previously (Brissette et al. 1991).

Analysis of *psp* expression

Bacteria were grown in DO salts (57.4 mM K₂HPO₄, 16.7 mM NaNH₄HPO₄, 9.5 mM citric acid, 0.8 mM MgSO₄; Vogel and Bonner 1956) supplemented with 0.4% glucose, 5 µg/ml of thiamine, and 19 amino acids (0.2 mg/ml each; no methionine). Samples of 2 × 10⁷ to 4 × 10⁷ cells were pulse-labeled for 60 sec with 20 µCi of [³⁵S]methionine (New England Nuclear; 1000 Ci/mmol), precipitated with cold trichloroacetic acid (5%), and resuspended in 25 µl of 4% SDS. Aliquots of the ³⁵S-labeled proteins were immunoprecipitated as described (Davis et al. 1985).

Identification of the *psp* transcription start site

RNA was isolated as described (Von Gabain et al. 1983) from bacteria at a density of 3 × 10⁸ cells/ml before and after a temperature shift, f1 infection, or treatment with 10% ethanol. Primer extension reactions were performed according to Treisman et al. (1982). The primer consisted of a 16-mer (JABR6) complementary to the 5'-terminal region of *pspA* (nucleotides 513–528) and was end-labeled with [^γ-³²P]ATP (Amersham) and T4 polynucleotide kinase (Pharmacia) as described previously (Maniatis et al. 1982). Ribonuclease protection assays were performed according to Melton et al. (1984) with RNase A (Sigma;

40 µg/ml, final concentration) and T1 (BRL; 1000 U/ml, final concentration). Anti-sense RNA probes were synthesized by using the Stratagene riboprobe system. The DNA templates for the riboprobe synthetic reactions were pJLB27 linearized with *Xba*I, or pΔ3'9 (Brissette et al. 1991) restricted with either *Bgl*II or *Bsm*I. Total bacterial RNA was capped as described (Adams et al. 1989) by using [^α-³²P]GTP (3000 Ci/mmol; Amersham) and vaccinia virus guanylyl transferase (BRL). The products of the primer extension and RNase protection assays were electrophoresed on 6% polyacrylamide/7.5 M urea gels.

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