Multiple promoters control expression of the *Yersinia enterocolitica* phage-shock-protein A (*pspA*) operon

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The widely conserved phage-shock-protein A (*pspA*) operon encodes an extracytoplasmic stress response system that is essential for virulence in *Yersinia enterocolitica*, and has been linked to other important phenotypes in *Escherichia coli*, *Salmonella enterica* and *Shigella flexneri*. Regulation of *pspA* operon expression is mediated through a promoter upstream of *pspA* that depends on sigma factor RpoN (σ^{54}) and the enhancer binding protein PspF. PspA, PspB and PspC, encoded within the *pspA* operon, also regulate expression by participating in a putative signal transduction pathway that probably serves to modulate PspF activity. All of this suggests that appropriate expression of the *pspA* operon is critical. Previous genetic analysis of the *Y. enterocolitica* *pspA* operon suggested that an additional level of complexity might be mediated by PspF/RpoN-independent expression of some *psp* genes. Here, an *rpoN* null mutation and interposon analysis were used to confirm that PspF/RpoN-independent gene expression does originate within the *psp* locus. Molecular genetic approaches were used to systematically analyse the two large non-coding regions within the *psp* locus. Primer extension, control region deletion and site-directed mutagenesis experiments led to the identification of RpoN-independent promoters both upstream and downstream of *pspA*. The precise location of the PspF/RpoN-dependent promoter upstream of *pspA* was also determined. The discovery of these RpoN-independent promoters reveals yet another level of transcriptional complexity for the *Y. enterocolitica* *pspA* operon that may function to allow low-level constitutive expression of *psp* genes and/or additional regulation under some conditions.

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INTRODUCTION

The phage-shock-protein system (Psp) may help the bacterial cell to survive during dissipation of the proton-motive force and is conserved in many Gram-negative bacteria (for a recent review see Darwin, 2005). It was originally identified in *Escherichia coli* K-12 (Brissette *et al.*, 1990), where it has been studied extensively. Expression of the *E. coli* *psp* regulon (*pspABCDE* and *pspG*) occurs in response to several stresses, including heat shock, osmotic shock, ethanol treatment, exposure to proton ionophores, and the overexpression of some cell envelope proteins, most notably secretins (for reviews see Darwin, 2005; Model *et al.*, 1997).

The homologous *psp* regulon of *Yersinia enterocolitica* (*pspABCDycjXF* and *pspG*) is predicted to have similar inducing signals. Recently, specific induction of *Y. enterocolitica*

pspA operon expression was observed following overexpression of secretins and some cytoplasmic membrane proteins, and upon disruption of the F_0F_1 -ATPase (Maxson & Darwin, 2004). The Psp system of *Y. enterocolitica* is also essential for virulence in a mouse model of infection (Darwin & Miller, 1999, 2001). This is probably because Psp is essential during production of the Ysc type III secretion system (Darwin & Miller, 2001). Apparently, the *Y. enterocolitica* Psp system must respond to a stress caused by mislocalization of the YscC secretin component of the type III secretion system (Darwin & Miller, 2001). Consequently, null mutations of some *psp* genes cause severe growth defects when *yscC* is overexpressed (Darwin & Miller, 2001; Green & Darwin, 2004; Maxson & Darwin, 2006).

Regulation of *E. coli* *psp* gene expression has been well studied, and much of the current understanding is probably applicable to the homologous *Y. enterocolitica* *psp* regulon. The *E. coli* *pspA* and *pspG* control regions each contain an RpoN (σ^{54})-dependent promoter, as well as binding sites for integration host factor (IHF) and PspF, a member of the enhancer binding protein family (Jovanovic *et al.*, 1996;

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Abbreviation: RACE, rapid amplification of cDNA ends.

Lloyd *et al.*, 2004; Weiner *et al.*, 1991, 1995). Induction of the *psp* regulon is completely dependent on PspF. Regulation is also mediated by several of the other Psp proteins. The peripheral cytoplasmic membrane protein PspA acts as a negative regulator, by directly interacting with PspF and inhibiting its activity (Dworkin *et al.*, 2000; Elderkin *et al.*, 2002, 2005). The integral cytoplasmic membrane proteins PspB and PspC act as positive regulators, presumably by interacting with PspA (Adams *et al.*, 2003) and possibly inhibiting its ability to interfere with PspF.

Previous analysis of *Y. enterocolitica* *pspA* expression, together with examination of the DNA sequence of its control region, indicated that it appeared to have a PspF/RpoN-dependent promoter (Darwin & Miller, 2001; Green & Darwin, 2004). Here, we confirm the presence of this promoter and determine its exact location. In addition, earlier genetic experiments raised the possibility of PspF- and, therefore, RpoN-independent expression of at least some of the genes within the *pspA* operon (Darwin & Miller, 2001). In this study, we strengthened this hypothesis by constructing an *rpoN* null mutation and using interposon analysis

of $\Phi(pspABC'-lacZY)$ operon fusion expression. We then identified RpoN-independent transcription initiation sites both upstream and immediately downstream of *pspA*. These promoters, which are predicted to be σ^{70} -dependent, offer a plausible explanation for the predicted low-level PspF/RpoN-independent expression of some *Y. enterocolitica* *psp* genes.

METHODS

Bacterial strains, plasmids and routine growth conditions.

Bacterial strains and plasmids used in this study are listed in Table 1. For routine plasmid manipulations the host strain was *E. coli* DH5 α . Plasmids with an R6K *ori* were maintained in *E. coli* CC118 λ pir, and conjugated into *Y. enterocolitica* from either *E. coli* S17-1 λ pir or *E. coli* SM10 λ pir. In most cases *E. coli* strains were grown at 37 °C, and *Y. enterocolitica* strains were grown at 26 °C or 37 °C as noted. All strains were grown in Luria–Bertani (LB) broth and on LB agar (Miller, 1972). Antibiotics were used as before (Maxson & Darwin, 2004).

Strain constructions. The λ red recombinase gene replacement system (Datsenko & Wanner, 2000), adapted for use in *Y. enterocolitica*

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype/features	Source or reference
<i>Y. enterocolitica</i> strains*		
JB580v	Δ yenR (R ⁻ M ⁺) pYV ⁺	Kinder <i>et al.</i> (1993)
AJD432	Δ rpoN	This study
AJD950	Δ araGFB::[Φ (<i>pspABC'</i> – <i>lacZY</i>)]	This study
AJD952	Δ araGFB::[Φ (<i>pspA pspB</i> :: Ω -Sp <i>pspC'</i> – <i>lacZY</i>)]	This study
AJD953	Δ araGFB::[Φ (<i>pspA</i> :: Ω -Sp <i>pspBC'</i> – <i>lacZY</i>)]	This study
AJD957	Δ araGFB::[Φ (<i>pspA</i> – <i>lacZY</i>)]†	This study
AJD958	Δ araGFB::[Φ (<i>pspB</i> – <i>lacZY</i>)]†	This study
AJD960	Δ araGFB::[Φ (<i>pspABC'</i> – <i>lacZY</i>)] Δ rpoN	This study
AJD962	Δ araGFB::[Φ (<i>pspA pspB</i> :: Ω -Sp <i>pspC'</i> – <i>lacZY</i>)] Δ rpoN	This study
AJD963	Δ araGFB::[Φ (<i>pspA</i> :: Ω -Sp <i>pspBC'</i> – <i>lacZY</i>)] Δ rpoN	This study
AJD1057	Δ araGFB::[Φ (<i>pspA</i> – <i>lacZY</i>)] Δ rpoN†	This study
Plasmids		
pALTER-1	Tc ^r , pUC <i>ori</i>	Promega
pBR322	Ap ^r Tc ^r , pMB1 <i>ori</i>	Bolivar <i>et al.</i> (1977)
pSL1180	Ap ^r , super polylinker, pUC18 <i>ori</i>	Pharmacia
pVLT33	Km ^r , <i>tacp</i> expression vector, RSF1010 <i>ori</i>	de Lorenzo <i>et al.</i> (1993)
pVLT35	Sm ^r /Sp ^r , <i>tacp</i> expression vector, RSF1010 <i>ori</i>	de Lorenzo <i>et al.</i> (1993)
pAJD107	Ap ^r , super polylinker, pMB1 <i>ori</i>	Darwin & Miller (2001)
pAJD126	<i>tacp-yscC</i> ⁺ in pVLT35	Darwin & Miller (2001)
pAJD113	Cm ^r , <i>pspFpspABCDycjXF</i> ⁺ in pAJD107	This study
pAJD457	~2·1 kb <i>pspF'pspABC'</i> fragment in pBR322	This study
pAJD509	<i>tacp-yscC</i> ⁺ in pVLT33	This study
pAJD524	~0·7 kb <i>pspF'</i> – <i>pspA'</i> <i>KpnI</i> – <i>HindIII</i> fragment in pALTER-1	This study
pAJD525	~0·56 kb <i>pspA'pspB'</i> <i>HindIII</i> – <i>HpaI</i> fragment in pSL1180	This study
pAJD531	~0·56 kb <i>pspA'pspB'</i> <i>KpnI</i> – <i>HindIII</i> fragment in pALTER-1	This study
pAJD905	Cm ^r , R6K <i>ori</i> , <i>mob</i> ⁺ (RP4), <i>sacB1</i> ⁺ , <i>lacZY</i> operon fusion vector	Maxson & Darwin (2005)

*All *Y. enterocolitica* strains are derivatives of strain JB580v (pYV is a virulence plasmid).
†The Φ (*pspA*–*lacZ*) and Φ (*pspB*–*lacZ*) strains listed have the longest control region fragments, Δ 278 and Δ 270, respectively (see Fig. 2). Other derivatives were made with truncated control regions, or site-directed mutations, as listed in Tables 2 and 3.

(Maxson & Darwin, 2004), was used to construct an *rpoN* in-frame deletion mutation. Briefly, a $\Delta rpoN::kan$ mutation was constructed using Red recombinase-mediated allelic exchange. The kanamycin-resistance gene was then removed by FLP recombinase-mediated excision and the in-frame deletion mutation was confirmed by colony PCR and Southern hybridization analysis (data not shown). The *rpoN* null mutant was grown in the presence of 5 mM alanyl-glutamine to alleviate a minor growth defect in LB medium. The growth and regulatory phenotypes of *rpoN* deletion mutants could be fully complemented by a plasmid encoding RpoN (data not shown). Single-copy *lacZ* operon fusions were constructed, integrated into the *ara* locus and confirmed by colony PCR analysis as described previously (Maxson & Darwin, 2005). When necessary, strains were cured of the virulence plasmid as described by Darwin & Miller (2001).

Interposon analysis of $\Phi(pspABC'-lacZY)$. The *pspF' pspABC'* insert fragment of pAJD457 (Table 1) has a unique *Hind*III site within *pspA*, and a unique *Hpa*I site within *pspB*. These sites were used as the insertion point for the Ω -Sp cassette from plasmid pHP45 Ω (Fellay *et al.*, 1987). The Ω -Sp cassette was inserted into the *Hpa*I site of *pspB* as a blunt-ended *Sma*I fragment. To be consistent, the Ω -Sp cassette was also inserted into the *Hind*III site of *pspA* as a *Sma*I fragment. Therefore, pAJD457 was digested with *Hind*III and treated with DNA polymerase I large (Klenow) fragment and dNTPs, prior to insertion of the Ω -Sp cassette. For consistency, for both the *pspA* and *pspB* insertions, clones were chosen with the Ω -Sp in the same orientation. Following insertion of the Ω -Sp cassette, the *pspF' pspA::\Omega*-Sp *pspBpspC'* and *pspF' pspApspB::\Omega*-Sp *pspC'* fragments were cloned into pAJD905 (Table 1). Each operon fusion was integrated into the *ara* locus of *Y. enterocolitica* strains and confirmed by colony PCR analysis as described previously (Maxson & Darwin, 2005).

β -Galactosidase assays. The effect of YscC production on the expression of $\Phi(pspABC'-lacZ)$ and $\Phi(pspA-lacZ)$ operon fusions was determined as described previously (Darwin & Miller, 2001). Briefly, saturated cultures were diluted into 5 ml LB broth containing appropriate antibiotics, in 18 mm diameter test tubes, so that the OD₆₀₀ was approximately 0.04. Cultures were grown on a roller drum at 37 °C for 2 h. Then 0.2 mM IPTG (final concentration) was added to induce *yscC* expression. Growth at 37 °C continued for a further 2 h prior to harvest for enzyme assays.

To monitor expression of the $\Phi(pspB-lacZ)$ operon fusion, saturated cultures were diluted into 5 ml LB broth, in 18 mm diameter test tubes, so that the OD₆₀₀ was approximately 0.06. The cultures were grown on a roller drum at 26 °C for 4 h, prior to harvest for enzyme assays.

β -Galactosidase enzyme activity was determined at room temperature (approx. 22 °C) in permeabilized cells as described by Maloy *et al.* (1996). Activities are expressed in arbitrary units, which were determined according to the formula of Miller (1972). Individual cultures were assayed in duplicate, and values were averaged from at least three independent cultures. Data were rounded to two significant figures.

RNA isolation and primer extension analysis. Total RNA was isolated from *Y. enterocolitica* *rpoN*⁺ or $\Delta rpoN$ strains containing the multicopy *psp* locus plasmid pAJD113 and the *tacp-yscC* expression plasmid pAJD126. Cultures were grown as described above for the β -galactosidase assay experiments. RNA was isolated with the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. End-labelling of oligonucleotides and primer extension reactions were done with the Primer Extension System-AMV Reverse Transcriptase (Promega). For analysis of the region upstream from *pspA* the primer 5'-CTGTGGATCTTCAGCTTTATCCAG-3' corresponds to *pspA* codons 20 to 26 in the template strand. For the region downstream from *pspA* the primer 5'-CGTGCATCGTCAGTTAACTGC-GAT-3' corresponds to *pspB* codons 45 to 52 in the template strand.

The primers were labelled at the 5' end with [γ -³²P]ATP and used in extension reactions containing 5 μ g RNA. To generate size markers the same primers were used in DNA sequencing reactions of the pAJD113 template using the *fmol* DNA Cycle Sequencing System (Promega). Samples were resolved by denaturing 8% polyacrylamide-urea electrophoresis and visualized by autoradiography.

Promoter deletion analysis. Truncated control region fragments were generated by PCR using a common downstream primer and upstream primers that annealed at various distances upstream. The fragments were cloned into the *lacZ* operon fusion plasmid pAJD905 and the DNA sequences were confirmed. The operon fusions were integrated into the *ara* locus and confirmed by colony PCR analysis as described previously (Maxson & Darwin, 2005).

Site-directed mutagenesis. The Altered Sites II *in vitro* Mutagenesis System (Promega) was used. The starting point for these constructions was plasmid pAJD457, which has a *pspF' pspABC'* insert (Table 1). For mutagenesis of the region upstream of *pspA*, a 0.7 kb *pspF'-pspA'* *Kpn*I-*Hind*III fragment of pAJD457 was cloned into pALTER-1. For mutagenesis of the region downstream from *pspA*, a 0.56 kb '*pspApspB'* *Hind*III-*Hpa*I fragment was cloned into pSL1180 (Table 1), and then transferred to pALTER-1 as a *Hind*III-*Kpn*I fragment. The oligonucleotides for mutagenesis were designed according to the manufacturer's instructions, and incorporated mismatches as indicated in Fig. 2. After mutagenesis, the DNA sequence of the insert fragment was determined to confirm the desired mutations, and to ensure that no spurious mutations had been introduced. Mutagenic fragments were then cloned into the *lacZ* operon fusion plasmid pAJD905 and the operon fusions were integrated into the *ara* locus and confirmed by colony PCR analysis exactly as described previously (Maxson & Darwin, 2005). Colony PCR and DNA sequence analysis was used to verify the presence of each site-directed mutation on the chromosome.

RESULTS

Interposon analysis indicates a complex *pspA* operon structure

A *Y. enterocolitica* *pspC* null mutant has a growth defect when the YscC secretin is overproduced and mislocalized (Darwin & Miller, 2001). *pspABCDycjXF* operon expression depends on the enhancer binding protein PspF, which is predicted to activate an RpoN-dependent promoter upstream of *pspA* (Green & Darwin, 2004). However, a *pspF* null mutation causes a less severe YscC-induced growth defect than a *pspC* null mutation, which led to a prediction of some PspF-independent expression of *pspC* (Darwin & Miller, 2001). Consistent with this, we have found that an *rpoN* null mutant also has a less severe YscC-induced growth defect than a *pspC* null mutant and that the YscC-related growth phenotypes of *pspF* and *rpoN* null strains are indistinguishable (data not shown). A likely location for an RpoN/PspF-independent promoter driving some *pspC* expression was thought to be the 161 bp non-coding region separating *pspA* and *pspB* (Darwin & Miller, 2001). We used insertional mutagenesis with the Ω interposon (a strong transcriptional terminator cassette; Fellay *et al.*, 1987) to begin to address this hypothesis.

Merodiploid strains were constructed with a $\Phi(pspABC'-lacZ)$ operon fusion integrated into the *ara* locus (Maxson &

Darwin, 2005) and an intact *pspF-pspABCDycjXF* locus. Therefore, polar effects of Ω -Sp interposon insertions on *lacZ* expression were determined in strains that retained intact copies of all *psp* genes. Derivatives were constructed with an Ω -Sp transcriptional terminator cassette inserted into the *pspA* or *pspB* genes of the Φ (*pspABC'-lacZ*) fusion and β -galactosidase activities were determined in the presence or absence of YscC overexpression (Fig. 1). The hypothesis predicts that the Ω -Sp insertion in *pspA* should abolish *lacZ* expression from the RpoN-dependent *pspA* promoter. The Ω -Sp insertion in *pspB* should abolish *lacZ* expression from both the RpoN-dependent *pspA* promoter, and the putative promoter in the *pspA-pspB* intergenic region.

In an *rpoN*⁺ strain, Φ (*pspABC'-lacZ*) expression was increased by approximately 50 % when YscC was produced (Fig. 1). As expected, the *pspA::* Ω -Sp insertion reduced Φ (*pspABC'-lacZ*) expression and abolished YscC-dependent induction. The *pspB::* Ω -Sp insertion reduced expression to a greater extent. These data support the prediction that some *lacZ* expression originates from downstream of the Ω -Sp insertion site within *pspA*.

When the operon fusions were analysed in an *rpoN* null mutant the interpretation became more complex. An *rpoN* null mutation abolished YscC-dependent induction of Φ (*pspABC'-lacZ*) expression, which is consistent with the predicted RpoN-dependence of the *pspA* promoter. However, the effect of the Δ *rpoN* mutation on Φ (*pspABC'-lacZ*) was much less than that of the *pspA::* Ω -Sp insertion in the *rpoN*⁺ strain (Fig. 1). Furthermore, even in the *rpoN* null strain the *pspA::* Ω -Sp insertion reduced Φ (*pspABC'-lacZ*) expression by 50 %. Taken together, all of these data suggest the following. First, RpoN-dependent expression originates

only from upstream of the Ω -Sp insertion site within *pspA*. Second, significant RpoN-independent expression originates both upstream and downstream of the Ω -Sp insertion site within *pspA*. Therefore, we next analysed the non-coding regions upstream and downstream of *pspA* (Fig. 2) for the presence of RpoN-dependent and RpoN-independent transcription initiation sites.

Identification of RpoN-dependent and RpoN-independent 5' mRNA ends expressed from the *pspA* control region

Interposon analysis had suggested that both RpoN-dependent and RpoN-independent transcription initiation might originate upstream from *pspA* (Fig. 1). Therefore, we used primer extension analysis to identify 5' mRNA ends originating from the *pspF-pspA* intergenic region of the intact *psp* locus. RNA was isolated from both *rpoN*⁺ and Δ *rpoN* strains and analysed with a primer complementary to the 5' end of *pspA* (see Methods). The most abundant mRNA molecule isolated from the *rpoN*⁺ strain had a 5' end at the expected position downstream of the predicted RpoN-binding site (P1 in Fig. 3). This confirms the location of the RpoN-dependent promoter. As expected, this mRNA was undetectable in the *rpoN* null mutant (Fig. 3). We also identified two additional 5' mRNA ends separated by 2 nucleotides, which mapped a short distance upstream of P1 (P2 in Fig. 3), indicating that they did not arise from processing of the RpoN-dependent transcript. Furthermore, these mRNAs were detected in both *rpoN*⁺ and *rpoN* null strains. Sequences similar to the -10 element of a σ^{70} -dependent promoter were identified upstream (Figs 2 and 3). All of these results were also confirmed by 5' RACE

Fig. 1. Interposon analysis of *psp* gene expression. The diagrams on the left represent the location of the Ω -Sp insertions and their predicted effects on transcription (thin arrows indicate predicted transcripts). The *lacZY* operon fusions were integrated into the *ara* locus as described in Methods (i.e. the native *pspA* operon of each strain is unaffected). The data on the right are from β -galactosidase assays of each fusion construct in *rpoN*⁺ or Δ *rpoN* mutant strains [determined as described in Methods and expressed in arbitrary (Miller) units as the means $\pm$ SD of three assays]. Strains with the *tacp* vector pVLT33 ($-$ YscC) or the *tacp-yscC*⁺ expression plasmid pAJD509 ($+$ YscC) were grown in the presence of 0.2 mM IPTG, as described in Methods. Note that a strain with a promoterless *lacZY* operon fusion integrated into the chromosome had approximately 5–10 Miller units of endogenous β -galactosidase activity.

Fig. 2. Large intergenic regions of the *Y. enterocolitica* *psp* locus. (a) Genetic structure of the *psp* locus. Genes are shown as thick arrows to indicate the direction of transcription. The *pspF*–*pspA* and *pspA*–*pspB* intergenic regions are labelled by their sizes in bp. (b) Nucleotide sequence surrounding the *pspF*–*pspA* intergenic region. Numbering is with respect to the RpoN-dependent transcription initiation site (P1), which is also shown in bold type overlined with >. RpoN-independent 5' mRNA ends (P2) are labelled similarly. The *pspF* and *pspA* translation initiation codons are underlined and labelled. A putative –10 element for P2 and –24/–12 elements for P1 are also underlined and labelled. Deletion end-points of constructs used in this study are indicated. Downward-facing arrows and bold type indicate nucleotide substitutions used in this study. The putative PspF-binding region is overlined and labelled (assigned by alignment with the PspF-binding site in the *E. coli* *pspA* control region; Jovanovic *et al.*, 1999). (c) Nucleotide sequence surrounding the *pspA*–*pspB* intergenic region. Numbering is with respect to the transcription initiation site (+1), which is also shown in bold type overlined with >. The *pspA* termination codon, *pspB* translation initiation codon and putative –10 and –35 elements are underlined and labelled. Deletion end-points of constructs used in this study are indicated. A trinucleotide substitution of the putative –10 element used in this study is indicated by downward-facing arrows and bold type.

Fig. 3. Primer extension analysis of the *pspA* control region. RNA was extracted from *rpoN*⁺ or *rpoN* null strains containing the multicopy *psp* locus plasmid pAJD113 and the *tacp-yscC* expression plasmid pAJD126. Arrows indicate the major primer extension products. G, A, T, C indicate the DNA sequencing reactions. The nucleotide sequence surrounding the putative transcription initiation sites is shown at the bottom, with a predicted RpoN-binding site for P1 and -10 element for P2 underlined. Consensus sequences for the -10 element of an *E. coli* σ^{70} -dependent promoter and for an RpoN-binding site are shown for comparison. The gel lane images have been rearranged from the original order, but the data are from the same gel and a single experiment.

analysis of mRNA initiated from a different template [a multicopy Φ (*pspA-lacZ*) fusion plasmid; data not shown].

5' deletion analysis of the *pspA* control region

The primer extension analysis had identified RpoN-dependent and RpoN-independent 5' mRNA ends within the *pspA* control region (Fig. 3). Next we undertook a 5' deletion analysis in order to identify how much upstream DNA was required for RpoN-dependent and RpoN-independent expression of a Φ (*pspA-lacZ*) operon fusion made with the isolated *pspA* control region. A set of single-copy Φ (*pspA-lacZ*) operon fusion strains was constructed with different 5' deletions of the *pspA* control region fragment (Fig. 2). Strains were grown under both non-Psp-inducing (-YscC) and Psp-inducing (+YscC) conditions and β -galactosidase activities were determined (Table 2). YscC overproduction does not induce Φ (*pspA-lacZ*) expression as much as some other proteins (Maxson & Darwin, 2004, 2005, 2006). However, it does cause a severe growth defect in some *psp* null mutants (Darwin & Miller, 2001; Green & Darwin, 2004; Maxson & Darwin, 2006) and so its

Table 2. Effects of control region alterations on Φ (*pspA-lacZ*) expression

Promoter*	Mutation†	β -Galactosidase specific activity‡			
		<i>rpoN</i> ⁺		Δ <i>rpoN</i>	
		-YscC	+YscC	-YscC	+YscC
Δ 278	None	57 ± 3	180 ± 11	29 ± 1	31 ± 1
Δ 252	None	55 ± 1	180 ± 10	29 ± 1	29 ± 1
Δ 132	None	52 ± 4	130 ± 2	34 ± 1	32 ± 2
Δ 71	None	52 ± 2	54 ± 1	78 ± 3	78 ± 2
Δ 39	None	8 ± 1	8 ± 1	8 ± 1	8 ± 1
Δ 12	None	8 ± 1	9 ± 1	9 ± 1	9 ± 1
Δ 278	P1 (-12/-24)	34 ± 1	35 ± 1	30 ± 2	32 ± 1
Δ 278	P2 (-10)	34 ± 1	130 ± 9	10 ± 1	10 ± 1

*Each strain has a Φ (*pspA-lacZ*) fusion integrated on the chromosome with differing amounts of DNA upstream of the RpoN-dependent transcription initiation site (P1) as shown in Fig. 2.

Each control region fragment has either the wild-type sequence (none), or mutations within the putative RpoN-binding site of P1 (-12/-24) or the putative -10 motif of P2 (-10) as indicated in Fig. 2.

†Strains with the *tacp* vector pVLT35 (-YscC) or the *tacp-yscC*⁺ expression plasmid pAJD126 (+YscC) were grown as described in Methods. β -Galactosidase was determined as described in Methods and expressed in arbitrary (Miller) units as the means ± SD of three assays.

effect on *pspA* operon expression is probably physiologically significant.

In an *rpoN*⁺ strain the β -galactosidase activities of deletion constructs Δ 252, Δ 132 and Δ 71 were indistinguishable from that of the original construct (Δ 278) under uninduced conditions (-YscC). However, the YscC-induced activities of constructs Δ 132 and Δ 71 were slightly reduced and completely abolished, respectively (Table 2). This shows that DNA upstream of position -71 is needed for regulated expression of Φ (*pspA-lacZ*). PspF is required for YscC-dependent induction. In *E. coli* the PspF-binding site overlaps the *pspF* translation initiation codon (Jovanovic *et al.*, 1999). Sequence alignment suggests that the same is probably true in *Y. enterocolitica* (Fig. 2 and data not shown). Therefore, the PspF-binding site is likely to have been compromised in construct Δ 132 and completely removed in construct Δ 71 (Fig. 2). All Φ (*pspA-lacZ*) expression was completely abolished in deletion constructs Δ 39 and Δ 12 (Table 2). The remaining 8–9 Miller units can be attributed to endogenous *Y. enterocolitica* β -galactosidase activity and basal expression from an empty Φ (-*lacZ*) operon fusion (Maxson & Darwin, 2005 and data not shown). From this it can be concluded that the region upstream of position -39 is required for all basal Φ (*pspA-lacZ*) expression.

The major goal of these experiments was to characterize RpoN-independent Φ (*pspA-lacZ*) expression. To more

clearly identify the region required specifically for RpoN-independent expression the deletion constructs were analysed in an *rpoN* null strain (Table 2). The results confirmed that the region upstream of position -39 is required for RpoN-independent expression of $\Phi(\textit{pspA-lacZ})$. This region includes almost the entire DNA region upstream from where the RpoN-independent 5' mRNA ends were mapped (Figs 2 and 3). Furthermore, the $\Delta 39$ construct removes part of a putative -10 element (Fig. 2). Together, all of these data suggest that one or both of the 5' mRNA ends represents an RpoN-independent transcription initiation site. Finally, in the *rpoN* null strain only, we noticed that $\Phi(\textit{pspA-lacZ})$ expression was elevated in the $\Delta 71$ construct (Table 2). We do not yet understand the explanation for this, and do not draw any conclusions from it. It may be an artifact resulting from the relative positioning of vector sequences upstream of the control region end-point in this particular deletion construct.

Site-directed mutagenesis of the *pspA* control region

To complete the analysis of the *pspA* control region, putative promoter elements were targeted by site-directed mutagenesis. Mutations were introduced into the original $\Delta 278$ $\Phi(\textit{pspA-lacZ})$ construct and β -galactosidase activities were determined (Table 2). First, the highly conserved -24 and -12 dinucleotides of the predicted RpoN-binding site were disrupted (Fig. 2). In an *rpoN*⁺ strain this mutation abolished YscC-dependent induction and reduced basal expression by almost 50 % (Table 2). This effect was indistinguishable from that of an *rpoN* null mutation on expression of the original $\Phi(\textit{pspA-lacZ})$ construct with the wild-type control region sequence. In an *rpoN* null strain the -24/-12 mutation had no effect on $\Phi(\textit{pspA-lacZ})$ expression (Table 2). Therefore, as expected the mutation only affected RpoN-dependent expression.

The region immediately upstream of the putative RpoN-independent transcription initiation sites has several overlapping sequences that might represent -10 elements (CATTATATTTT; Fig. 2), although there is no recognizable -35 element. To test the functionality of the putative -10 elements, and to distinguish between them, we made three different trinucleotide substitutions. Two of these (CATTAT to GCGTAT) and (TATATT to CCAATT) did not reduce RpoN-independent expression of $\Phi(\textit{pspA-lacZ})$ (data not shown). However, a TATTTT to TTGGTT mutation (Fig. 2) essentially abolished $\Phi(\textit{pspA-lacZ})$ expression in an *rpoN* null strain and also reduced expression in an *rpoN*⁺ strain (Table 2). Therefore, we tentatively assigned the TATTTT motif as a -10 element. The TATATT to CCAATT mutation, which also disrupts the first position of this -10 element, did not affect expression, possibly because it leaves the most highly conserved positions intact (Harley & Reynolds, 1987; Hawley & McClure, 1983). It also remains possible that multiple sequences serve as -10 elements. Nevertheless, when taken together the primer extension, 5' deletion and site-directed mutagenesis data strongly

suggest the presence of at least one RpoN-independent transcription initiation site upstream of *pspA*.

Identification of 5' mRNA ends expressed from the *pspA-pspB* intergenic region

Interposon analysis had also suggested that some RpoN-independent transcription initiation might originate downstream from *pspA* (Fig. 1). Therefore, we also used primer extension analysis to identify 5' mRNA ends originating from the *pspA-pspB* intergenic region of the intact *psp* locus. RNA was analysed by primer extension analysis with an oligonucleotide complementary to the 5' end of *pspB* (see Methods). Surprisingly, the 5' ends of mRNA molecules mapped to a single C residue that was just within the *pspB* gene (Fig. 4). We are confident that the *pspB* translation initiation codon has been correctly identified. It has a Shine-Dalgarno (SD) motif, there are no alternative downstream ATG or GTG codons with SD motifs, and the predicted PspB amino terminus is conserved between different species (data not shown). We also confirmed the primer extension result by 5' RACE analysis of mRNA initiated from a different template [a multicopy $\Phi(\textit{pspB-lacZ})$ fusion plasmid; data not shown]. This 5' mRNA end may be generated from a σ^{70} -dependent promoter because putative -35 and -10 elements were identified upstream (Fig. 4).

Fig. 4. Primer extension analysis of the *pspA-pspB* intergenic region. RNA was extracted from an *rpoN*⁺ strain containing the multicopy *psp* locus plasmid pAJD113 and the *tacp-yscC* expression plasmid pAJD126. An arrow indicates the primer extension (PE) product. G, A, T, C indicate the DNA sequencing reactions. The nucleotide sequence surrounding the putative transcription initiation site is shown at the bottom. The *pspB* translation initiation codon is double underlined and possible -10 and -35 elements are single underlined. The arrangement of a consensus *E. coli* σ^{70} -dependent promoter (-35 and -10) is also shown for comparison. The gel lane images have been rearranged from the original order, but the data are from the same gel and a single experiment.

5' deletion analysis and site-directed mutagenesis of the *pspA-pspB* intergenic region

To investigate the function of the putative promoter identified by mRNA analysis we first constructed a single-copy Φ (*pspB-lacZ*) operon fusion strain. The fragment used for this fusion encompassed the 3' end of *pspA* (position -270 in Fig. 2) to 63 bp within the 5' end of *pspB*. The single-copy Φ (*pspB-lacZ*) fusion strain produced 180 Miller units of β -galactosidase activity when grown to mid-exponential phase at 26 °C in LB broth (Table 3). This compares to only 5–10 Miller units due to endogenous *Y. enterocolitica* β -galactosidase activity and basal expression of an empty Φ (-*lacZ*) operon fusion (Maxson & Darwin, 2005 and data not shown). Therefore, these results support the hypothesis that the '*pspA-pspB*' fragment contains an active promoter. Φ (*pspB-lacZ*) expression was not affected by *rpoN* and *pspF* null mutations, or by *yscC* overexpression (data not shown).

Next we did a 5' deletion analysis of the '*pspA-pspB*' fragment to determine how much upstream DNA was important for Φ (*pspB-lacZ*) expression. For this we constructed a set of single-copy Φ (*pspB-lacZ*) operon fusion strains with different 5' deletions of the '*pspA-pspB*' fragment (Fig. 2). The strains were grown to mid-exponential phase at 26 °C in LB broth and β -galactosidase activities were determined (Table 3). Deletion constructs Δ 177, Δ 115 and Δ 55 expressed β -galactosidase activities similar to that of the original construct (Δ 270). This suggests that DNA upstream of position -55 is not required for Φ (*pspB-lacZ*) expression, making it unlikely as a target for a regulatory protein under the growth conditions we used. However, the β -galactosidase activity of deletion construct Δ 17 was reduced by 60 % in comparison to the full-length fragment (Δ 270).

Table 3. Effects of control region alterations on Φ (*pspB-lacZ*) expression

Promoter*	Mutation†	β -Galactosidase specific activity‡
Δ 270	None	180 ± 2
Δ 177	None	180 ± 13
Δ 115	None	190 ± 7
Δ 55	None	200 ± 12
Δ 17	None	71 ± 1
Δ 270	-10	32 ± 2

*Each strain has a Φ (*pspB-lacZY*) fusion integrated on the chromosome with differing amounts of DNA upstream of the transcription initiation site as shown in Fig. 2.

†Each control region fragment has either the wild-type sequence (none), or a TAT to GCG mutation within the putative -10 motif (-10) as indicated in Fig. 2.

‡Determined as described in Methods and expressed in arbitrary (Miller) units as the means ± SD of three assays.

This may be because this deletion removed a putative -35 element (Fig. 2).

Finally, if the putative promoter within *pspB* is authentic then disruption of the predicted -10 element should reduce expression. Therefore, we tested whether Φ (*pspB-lacZ*) expression was affected by a trinucleotide substitution in the -10 region of the full-length Δ 270 construct (Fig. 2). This mutation reduced Φ (*pspB-lacZ*) expression by over 80 % (Table 3), which further corroborates the identification of a putative σ^{70} -dependent promoter.

DISCUSSION

This work was motivated by data from a previous study that suggested some PspF-independent expression of *pspC* (Darwin & Miller, 2001). The phenotypes of an *rpoN* null mutant further supported this hypothesis (Fig. 1 and data not shown). The *pspF-pspABCDycjXF* locus contains two large non-coding regions that we decided to focus on for this study (Fig. 2): the region containing promoters for the divergently transcribed *pspF* and *pspA* genes (187 bp) and the *pspA-pspB* intergenic region (161 bp). RpoN-independent promoters were identified both upstream and downstream of *pspA*. The expected location of the PspF/RpoN-dependent *pspA* promoter was also confirmed.

Three 5' mRNA ends were identified upstream of *pspA* (Fig. 3). The most abundant was expressed from the RpoN/PspF-dependent promoter and served to confirm its anticipated location. This RpoN-dependent promoter is apparently highly conserved upstream of *pspA* in different bacteria (Green & Darwin, 2004). Two RpoN-independent 5' mRNA ends were also identified, separated by only 2 nucleotides, and mapping approximately 30 bp upstream of the RpoN-dependent transcription initiation site. These two mRNA ends might represent two different promoters or alternative transcription initiation sites for the same promoter. Another possibility is that there is a single initiation site and the shorter transcript arose from a processing event. Multiple putative -10 elements were found upstream, but only one mutation reduced RpoN-independent Φ (*pspA-lacZ*) expression (Table 2 and data not shown). Although a -35 element could not be identified we are confident that at least one of the 5' mRNA ends is indicative of a promoter. First, RpoN-independent Φ (*pspA-lacZ*) expression was abolished by deletion of upstream DNA (Table 2). Second, RpoN-independent Φ (*pspA-lacZ*) expression was also abolished by the -10 mutation (Table 2). RpoN-independent *pspA* transcription initiation sites were not detected in *E. coli* by ribonuclease protection assays (Weiner *et al.*, 1991). It is possible that the sensitivity was insufficient. *E. coli pspA* primer extension experiments were also only reported to have identified the RpoN-dependent 5' mRNA end, but the data were not shown (Weiner *et al.*, 1991). Either RpoN-independent expression upstream of *pspA* is unique to *Y. enterocolitica*, or it also occurs in *E. coli* but was below the limit of detection in the published experiments.

The close proximity of RpoN-dependent and RpoN-independent promoters upstream of *pspA* (Figs 2 and 3) raises the question of whether they can be occupied (active) simultaneously. Mutation of the putative -10 element of the RpoN-independent promoter reduced $\Phi(\textit{pspA-lacZ})$ expression in both *rpoN*⁺ and $\Delta\textit{rpoN}$ strains (Table 2). This suggests that this promoter does contribute to $\Phi(\textit{pspA-lacZ})$ expression when the RpoN-dependent promoter is active. However, it is also possible that this mutation independently compromises both promoters. Alternatively, only one promoter may be occupied at any one time and the total β -galactosidase activity measured reflects the average of the population of cells. It might be interesting to investigate this question in future experiments, particularly with an *in vitro* transcription system.

The literature contains numerous examples of bacterial genes with multiple upstream promoters, including the *E. coli rpoE* operon, which, like the *pspA* operon, is involved in extracytoplasmic stress response (Raina *et al.*, 1995). In many cases the precise physiological function of the multiple promoters is unknown and this is certainly true for RpoN-independent expression of the *Y. enterocolitica pspA* operon. RpoN-independent expression from upstream of *pspA* might ensure low-level constitutive expression of some *psp* genes or regulation under unknown conditions. Constitutive expression might indicate that low levels of some Psp proteins are critical under non-inducing conditions, perhaps to facilitate a fast response to inducing stress conditions.

Another RpoN-independent promoter was identified downstream from *pspA*. Unexpectedly, the promoter overlaps the 5' end of *pspB* (Fig. 2). There are many examples of promoters within bacterial genes, but the location of this one at the extreme 5' end of *pspB* was surprising. Nevertheless, we are confident of the veracity of this promoter. The 5' mRNA end was mapped from different templates by both primer extension analysis (Fig. 2) and 5' RACE (data not shown). Putative -35 and -10 elements were identified at the appropriate locations and their disruption by deletion (-35) or mutation (-10) significantly reduced $\Phi(\textit{pspB-lacZ})$ expression (Table 3). Expression of $\Phi(\textit{pspB-lacZ})$ was not completely abolished by these disruptions. However, it is not unprecedented for control region mutations in -35 and -10 elements to leave some promoter function intact. The location of this promoter indicates that it can only control expression of *pspC* and the downstream genes. Its activity was unaffected by *pspF* and *rpoN* null mutations, by YscC overproduction or by heat or osmotic shock (Fig. 1 and data not shown). Therefore, the promoter may drive low-level constitutive expression of *pspC* and downstream genes. Alternatively, it could be responsible for regulated expression under as-yet-unidentified conditions.

Examination of the genome sequences from closely related *Enterobacteriaceae* suggests that the relatively large size of the *pspA-pspB* intergenic region is a signature of *Yersinia* species. It occurs in *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis* (data not shown). Furthermore, the -10

element of the promoter is perfectly conserved in all three of these species. In contrast, the *pspA-pspB* intergenic region is approximately 100 bp shorter in both *E. coli* and *Salmonella enterica* serovar Typhimurium. Therefore, the RpoN-independent promoter and any upstream control elements identified here may not be present in all species. However, it is possible that different internal promoters are present in the *psp* loci of other species. For example, Northern hybridization analysis of the *E. coli pspABCDE* operon detected a constitutively expressed transcript encoding only *pspBCDE*, although its origin was not determined (Brissette *et al.*, 1991).

Appropriate expression of the Psp response system is probably critical. Indeed, *pspA* operon expression is highly regulated in both *E. coli* and *Y. enterocolitica*. This regulation is mediated entirely by a conserved RpoN/PspF-dependent promoter upstream of *pspA*. In addition, the PspA, PspB and PspC proteins all modulate PspF activity via a putative signal transduction pathway, which further increases the complexity of *pspA* operon regulation. This study has revealed yet another level of transcriptional complexity within the *Y. enterocolitica pspA* operon. RpoN-independent promoters are present both upstream and downstream of *pspA*. These promoters may only be responsible for a relatively small amount of the total level of *psp* gene expression in comparison to the highly inducible PspF/RpoN-independent promoter (e.g. see Maxson & Darwin, 2004). Nevertheless, a goal for future experiments will be to investigate the role of these promoters and whether there are any conditions under which their activity is altered.

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