THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 53, Issue of December 31, pp. 55707–55714, 2004
Printed in U.S.A.

Identification of a New Member of the Phage Shock Protein Response in *Escherichia coli*, the Phage Shock Protein G (PspG)*[S]

Received for publication, August 6, 2004, and in revised form, October 5, 2004
Published, JBC Papers in Press, October 13, 2004, DOI 10.1074/jbc.M408994200

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The phage shock protein operon (*pspABCDE*) of *Escherichia coli* is strongly up-regulated in response to overexpression of the filamentous phage secretin protein IV (pIV) and by many other stress conditions including defects in protein export. PspA has an established role in maintenance of the proton-motive force of the cell under stress conditions. Here we present evidence for a new member of the phage shock response in *E. coli*. Using transcriptional profiling, we show that the synthesis of pIV in *E. coli* leads to a highly restricted response limited to the up-regulation of the *psp* operon genes and *yjbO*. The *psp* operon and *yjbO* are also up-regulated in response to pIV in *Salmonella enterica* serovar *Typhimurium*. *yjbO* is a highly conserved gene found exclusively in bacteria that contain a *psp* operon but is physically unlinked to the *psp* operon. *yjbO* encodes a putative inner membrane protein that is co-controlled with the *psp* operon genes and is predicted to be an effector of the *psp* response in *E. coli*. We present evidence that *yjbO* expression is driven by σ^{54}-RNA polymerase, activated by PspF and integration host factor, and negatively regulated by PspA. PspF specifically regulates only members of the PspF regulon: *pspABCDE* and *yjbO*. We found that increased expression of YjbO results in decreased motility of bacteria. Because *yjbO* is co-conserved and co-regulated with the *psp* operon and is a member of the phage shock protein F regulon, we propose that *yjbO* be renamed *pspG*.

The phage shock protein operon (*pspABCDE*) was first characterized in *Escherichia coli* (1) and is highly conserved in many Gram-negative bacteria including several pathogens. There is good evidence that the *psp* genes are involved in protecting the bacterial cell during infectious processes. For example, *pspC* mutants of *Yersinia enterocolitica* are severely attenuated for virulence during infection (2) and exhibit growth defects when the type III secretion system is expressed (3). Significantly, the *psp* genes are among the most highly up-regulated genes in *Salmonella typhimurium* during macrophage infection (4). The *psp* operon is also up-regulated during swarming in *S. typhimurium* (5) and during biofilm formation in *E. coli* (6).

Expression of the *psp* operon in *E. coli* is induced by protein IV (pIV),[1] a secretin from filamentous phage f1 (1). pIV forms a pore in the bacterial outer membrane that is required for the assembly and export of filamentous phage (7, 8). The pIV protein is the founding member of a large family of bacterial secretins, all of which form large multimeric export channels in the outer membrane. Overexpression of several secretins, often components of the type II and type III bacterial secretion systems, has also been shown to induce expression of the *psp* operon (*e.g.* Refs. 7 and 9) establishing that the response is not restricted to a phage protein. Expression of the *psp* operon can also be induced following overexpression of mutant forms of the outer membrane protein PhoE that are not efficiently secreted (10). PspA synthesis is switched on under conditions that block or reduce the efficiency of the export apparatus, for example, mutants in *secA*, *secD*, and *secF* (10) and depletion of YidC (11, 12). Mutations in components of the twin-arginine translocation pathway also leads to PspA induction under anaerobic conditions (Ref. 13 and see also Ref. 12). Other more general stresses including extreme heat shock (50 °C), hyperosmotic shock, ethanol treatment (10%), and uncouplers of proton-motive force induce *psp* (reviewed in Ref. 14). The common factor that may link *psp*-inducing stresses is their effect in dissipating proton-motive force. Indeed, it is significant that PspA, an effector protein of the phage shock response, is known to be involved in maintaining proton-motive force under stress conditions (15). In addition to Psp protein homologues in other Gram-negative bacteria, a PspA homologue (VIPP1) has been found in *Synechocystis*, which is thought to be important in thylakoid formation, consistent with a role of PspA in sustaining membrane function (16).

Psp proteins mediate regulation of the *psp* operon (17, 18). Transcription of the *psp* operon is driven by the σ^{54}-RNA polymerase (σ^{54}-RNAP) (17), which is activated by the enhancer binding protein PspF (19) and facilitated by integration host factor (IHF) (20). The expression of PspF is negatively autogenously controlled (21). PspA negatively regulates *psp* transcription by binding to the activator protein PspF (22, 23). Conversely, PspB and PspC act as positive regulators of *psp* operon transcription by overcoming the negative regulation

* This work was supported by The Wellcome Trust, a Biotechnology and Biological Sciences Research Council Core Strategic grant, and National Institutes of Health Grant GM38361 (to S. Kustu). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains Supplemental Tables I–III and Fig. S1.

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[1] The abbreviations used are: pIV, protein IV; IHF, integration host factor; IPTG, isopropyl 1-thio-β-D-galactopyranoside; RT, reverse transcriptase.

TABLE I
Bacterial strains and plasmids

Strain or plasmid	Genotype or relevant characteristics	Ref. or source
Bacterial strains		
E. coli		
BW25113	Wild-type	CGSC# 7739 Gift from Hirotada Mori 26
JWK3169_1 (Km+)	BW25113 *rpoN*::*kn*	Gift from Hirotada Mori
JWK5716_1 (Km+)	BW25113 *pspG*::*kn*	Gift from Hirotada Mori
MC1061	*lac*$^-$	Gift from Majorie Russel (27)
MC1068	MC1061Δ*himA*::*Tn10* (tc^r)	A gift from Michael Chandler
MG1655	Wild-type	CGSC No. 7740 (28)
MG1655Δ*pspA*	MG1655 Δ*pspA*	This work
MG1655Δ*pspBC*	MG1655 Δ*pspBC*	This work
MVA29	MG1655Δ*pspABC*::*kn*	This work
MVA19	MG1655Δ*pspABC*::*kn pspF*::mTn*10-tet* (*pspF*Δ*HTH*)	This work
MG1655Δ*pspF*	MG1655 Δ*pspF*	This work
MVA40	MG1655 *pspG*::*kn*	This work
MVA42	MG1655Δ*pspA pspG*::*kn*	This work
S. typhimurium		
LT2	Wild-type	29
Plasmids		
pGZ119EH	IPTG-inducible *tac* promoter expression vector. cm^r.	Gift from Marjorie Russel (30)
pPMR129	pGZ119EH habouring *pIV*. cm^r	Gift from Marjorie Russel (31)
pMR25	*lacZ* transcriptional fusion vector. tet^r	12
pSJ1	pMR25 with *pspA* promoter region plus the first 21 amino acids of PspA cloned into the mcs (EcoRI-EcoRI). tet^r	12
pMC1403	*lacZ* translational fusion vector. ap^r	32
pLL1	The *pspG* promoter region plus the first 6 amino acids of PspG cloned in-frame into the mcs of pMC1403 (EcoRI-BamHI). ap^r	This work
pLL2	The *pspG* promoter region plus the first 6 amino acids of PspG cloned out of frame into the mcs of pMC1403 (EcoRI-BamHI). ap^r	This work
pSLE1	*pspA* promoter region (EcoRI-BamHI)cloned into the vector pTE103 (56) ap^r	23
pJH2	*pspG* promoter region (EcoRI-BamHI) subcloned from pLL1 into pTE103 ap^r	This work
pBAD18-cm	Vector cm^r	A gift from Jonathan Beckwith
pLL8	*pspG* (XbaI-HindIII) cloned into the mcs of pBAD18-cm. cm^r	This work

imposed by PspA under specific inducing conditions (*e.g.* pIV) (17, 24, 25). Phenotypes of cells lacking the *psp* operon are very subtle and include reduced survival in stationary phase at alkaline pH and changed motility (14).

Here we have used whole genome transcriptional profiling to determine the global effect of pIV synthesis in *E. coli*. In the highly restricted response we have identified one new gene associated with the *psp* system, *pspG* (previously *yjbO*). *pspG* is physically unlinked with the *psp* operon but is co-conserved and co-regulated with the *psp* operon genes by σ^{54}, PspF, IHF, and PspA. Several lines of evidence suggest that PspG is an effector of the phage shock system and not a regulator of *psp* expression.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—The bacterial strains and plasmids used in this study are described in Table I. MG1655Δ*pspA*, MG1655Δ*pspBC*, and MG1655Δ*pspF* were constructed as described in Ref. 12. MVA29 was constructed by transducing Δ*pspABC*::*kn* from J134 (17) into MG1655. MVA19 was constructed by transducing Δ*pspABC*::*kn* from J134 and *pspF*::mTn*10-tet* from K1527 (19) into MG1655. MVA40 was constructed by transducing *pspG*::*kn* from JWK5716_1 (Km+) into MG1655. MVA42 was constructed by transducing *pspG*::*kn* from JWK5716_1 (Km+) into MG1655Δ*pspA*. Strains were grown aerobically with shaking at 37 °C. For microarray analyses, strains were grown to mid-exponential phase in N-C- minimal media (33) supplemented with 0.4% glucose as carbon source and 10 mM NH_4Cl as nitrogen source. For all other experiments, strains were grown in Luria-Bertani (LB) media (34). Antibiotics were used at the following concentrations: ampicillin (ap), 100 μg ml^{-1}; chloramphenocol (cm), 25 μg ml^{-1}; tetracycline (tet), 10 μg ml^{-1}; and kanamycin (kn), 30 μg ml^{-1}. IPTG was added to a final concentration of 1 mM and arabinose was added to a final concentration of 0.4% when required unless otherwise stated. Transformations and $P1_{vir}$ transductions were performed as described in Ref. 35.

Microarray Analysis—Growth of cultures was halted with 1/10 volume of 5% phenol in ethanol and RNA was extracted with hot phenol/SDS (36). RNA was treated with DNase I for 1 h at 37 °C. For the initial microarray experiments, RNA was fluorescently labeled during reverse transcription and cDNA was hybridized to *E. coli* PCR product microarrays according to S. Kustu and co-workers (37). Hybridization, scanning, and normalization were carried out as described (38) and genome images were prepared (37). Experiments were performed in duplicate with a dye swap. The microarray data for the pIV experiments were generated with fluorescently labeled genomic DNA as a reference channel in each experiment using *E. coli* and *S. typhimurium* PCR product microarrays printed at IFR (39–41). Experiments were performed in quadruplicate, consisting of two biological replicates and two technical replicates. Microarray slides were scanned with a Genepix 4000B scanner (Axon Instruments). Fluorescent spot and local background intensities were quantified using Genepix Pro software. For labeling, hybridization, and data analysis protocols and details of statistical filtering procedures, see the online site (ifr.bbsrc.ac.uk/Safety/Microarrays/#Protocols). Further statistical analysis was carried out using Cyber-T (visitor.ics.uci.edu/genex/cybert/).

RT-PCR—Qiagen® One-step RT-PCR kit was used according to the manufacturer' instructions to amplify *pspA* (20 cycles) and *pspG* (35 cycles) from RNA samples. For amplifying *pspA* the primers RT-PspA(a) (5′-CTCGCTTTGCCGACATCGTGAATG-3′) and RT-PspA(b) (5′-TGCCAGTTGTTCGCTGATTGCATC-3′) were used. For amplifying *pspG* the primers RT-PspG(a) (5′-GCTGGAACTACTTTTTGTGATTGG-3′) and RT-PspG(b) (5′-CGCCAGCGGTCATAACGCTGATAT-3′) were used.

Western Blotting—Western blotting was carried out as described (23) using primary antibodies to PspA (12) and pIV (a gift from Marjorie Russel). pIV antibodies were used at a 1:10,000 dilution with donkey anti-rabbit secondary antibodies (Amersham Biosciences).

β-Galactosidase Assays—β-Galactosidase assays were carried out as described (35).

Bioinformatics Methods—Fuzzpro (EMBOSS programs) was used to search for consensus sequences in regions of DNA by allowing small numbers of mismatches to be introduced to the search.

DNase I Footprinting Assays—DNase I footprinting reactions (10 μl) were carried out at 37 °C in STA buffer (25 mM Tris acetate, pH 8.0, 8 mM magnesium acetate, 10 mM KCl, 1 mM dithiothreitol, 3.5% (w/v) polyethylene glycol 8000) essentially as described (42). Briefly, 0–400

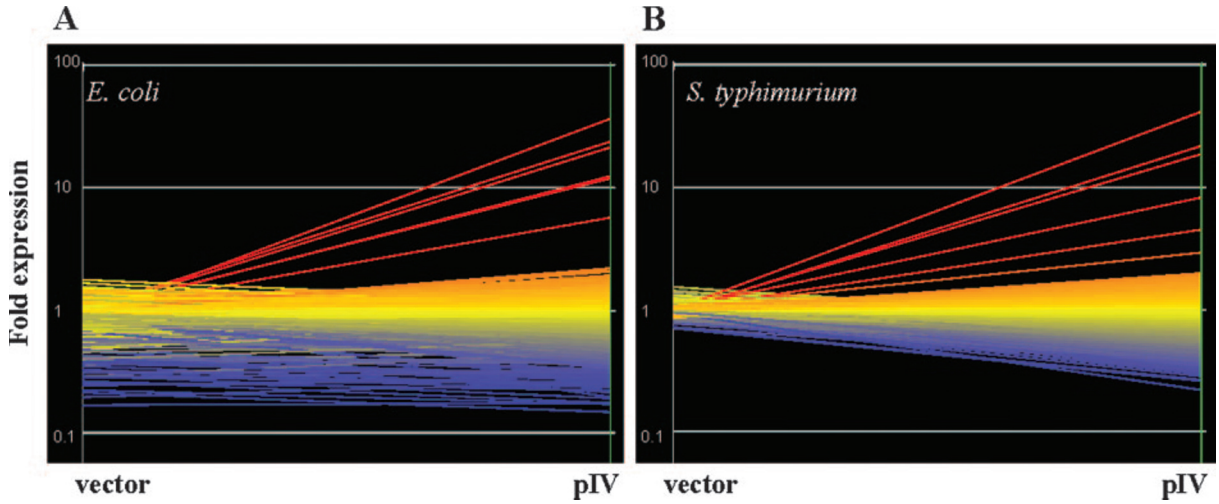

FIG. 1. **Whole genome expression profiles for *E. coli* MG1655 cells (*A*) and *S. typhimurium* LT2 (*B*).** Cells were grown in N-C- media supplemented with 0.4% glucose and 10 mM NH_4Cl to mid-log phase and pIV expression was induced with IPTG for 1 h. Extracted RNA was converted to cDNA, labeled, and hybridized to the *E. coli* and *S. typhimurium* microarrays (from J. C. H.). Gene expression in cells expressing pPMR129 (pIV) is normalized to expression in cells expressing pGZ119EH (vector control). Genes up-regulated in response to pIV stress are indicated in *red*, genes down-regulated in response to pIV stress are indicated in *blue*, and genes not changing in expression in response to pIV stress are indicated in *yellow*. The six genes indicated in *red* are *pspA*, *pspB*, *pspC*, *pspD*, *pspE*, and *pspG*.

nM Eσ^{54} (reconstituted *in situ* with 1:2 molar ratio of E and σ^{54}) or 0–1 μM *E. coli* PspF was incubated with 15 nM pLL1 for 10 min and treated with 1.75×10^{-3} units of DNase I (Amersham Biosciences) for 2 min. The DNase I reaction was quenched by the addition of DNase I stop buffer (400 mM NaCl, 30 mM EDTA, 1% SDS) and the DNA was purified using QIAquick spin columns (Qiagen) according to the manufacturer's instructions. DNase I protected regions were identified by primer extension PCR as described (42) using 0.5 μl of 1 μM γ-^{32}P-end labeled primers pPspG1 (5′-GAACACGCGCTCAAACTGGTGGCGG-3′) (for σ^{54} binding) and pPspG2 (5′-CTGGCGCGCGGCAGTGGCGGC-3′) (for PspF binding).

In Vitro Transcription Assay—*In vitro* transcription reactions (10 μl) were carried out as described (43) with a 1:5 ratio RNAP to σ^{54} and with plasmids pSLE1 (*pspA*) or pJH2 (*pspG*).

Motility Assay—Motility assays were carried out using motility agar (1% tryptone, 0.5% NaCl and 0.3% agar) plus the appropriate antibiotic. 2 μl of a fully grown LB overnight culture was pipetted into the motility agar, plates were incubated at 37 °C for 6 h, and zones of motility were measured in millimeters.

RESULTS

pIV Secretin Stress Results in the Up-regulation of pspABCDE and pspG—To examine the transcriptional response to pIV-induced stress in *E. coli* wild type MG1655 cells containing the plasmids, pPMR129 (pIV) or pGZ119EH (vector control) were grown to mid-log phase, expression from the plasmids was induced with IPTG for 1 h, and cells were harvested for RNA extraction. The synthesis of pIV reached high levels after 1 h (see Supplementary Materials and Fig. 1) indicating that it should elicit a full cellular response but did not lead to reduced growth rates, or reduced yields of cells, indicating a lack of toxicity. Microarray analyses showed increased levels of *psp* operon transcripts in pIV-expressing cells compared with the vector control (Fig. 1*A*). We correlated activation of the *pspA* promoter with increased levels of the PspA protein using Western blotting (data not shown). Other than the *psp* operon genes, only a single gene, *yjbO*, showed a significant and sizeable up-regulation in response to pIV secretin stress (Fig. 1*A*; see Supplementary Materials Tables I and III). This data indicates that large transcriptional responses of *E. coli* to pIV are very rare and identify a new gene involved in the phage shock response, *yjbO*. We propose to rename this gene *pspG*.

To confirm that transcript levels of *pspG* are increased in wild type MG1655 cells expressing pIV, RT-PCR was carried out on the RNA samples used for the microarray experiments. RT-PCR clearly demonstrates that *pspG* transcription is up-regulated, along with *pspA* transcription, in pIV-expressing MG1655 cells compared with the vector control (Fig. 2*A*). β-Galactosidase assays using a translational reporter for PspG (pLL1) confirm that PspG is produced in response to pIV in MC1061 cells (Fig. 2*B*).

To determine whether the response to pIV detected in *E. coli* is conserved in other bacteria that contain the *psp* operon, a pIV expression experiment was carried out in *Salmonella enterica* serovar *Typhimurium* LT2. There is significant up-regulation of *psp* operon and *pspG* transcripts in the pIV-expressing *S. typhimurium* cells (Fig. 1*B*; see Supplementary Materials Tables I and III). The transcriptional response to pIV in *S. typhimurium* resembles that of *E. coli* in that the response to pIV secretin stress is highly restricted. Our comparative transcriptomic analysis of responses of *E. coli* and *S. typhimurium* to pIV shows that the common core of up-regulated genes are *pspABCDE* and *pspG*. Transcription of *pspF* does not show any change in response to pIV expression, consistent with control of PspF being exclusively at the level of activity (21). Such a limited and specific response to pIV stress resembles the response of *E. coli* cells to IPTG, a gratuitous inducer of the *E. coli lac* operon. We performed a microarray experiment to show that IPTG only causes significant increased expression of *lac* operon genes in MG1655, no other transcriptome changes occur (see Supplementary Materials Table II). As with *lac* promoter activity induced by IPTG, the effect of pIV inducing stimulus under our growth conditions in *E. coli* appears to be close to gratuitous (44).

pspG Transcription Is Regulated by psp-encoded Proteins—To examine the effect of overexpression of the *psp* genes on the transcriptome, transcripts from cells lacking the negative regulator PspA (MG1655Δ*pspA*) were compared with transcripts from cells lacking the positive regulator PspF (MG1655Δ*pspF*). In MG1655Δ*pspA*, the *psp* operon is expressed at high levels because the negative regulator of its transcription has been removed. Conversely, in MG1655Δ*pspF*, expression of the *psp* operon is completely absent because the activator protein required for σ^{54}-RNAP driven transcription has been removed. Levels of *psp* expression in the MG1655Δ*pspA* strain are therefore close to levels in wild type cells expressing pIV, but without the production of PspA. It is clear from Fig. 3 that *pspBCDE* is transcribed at high levels in MG1655Δ*pspA* compared with MG1655Δ*pspF*. As with the re-

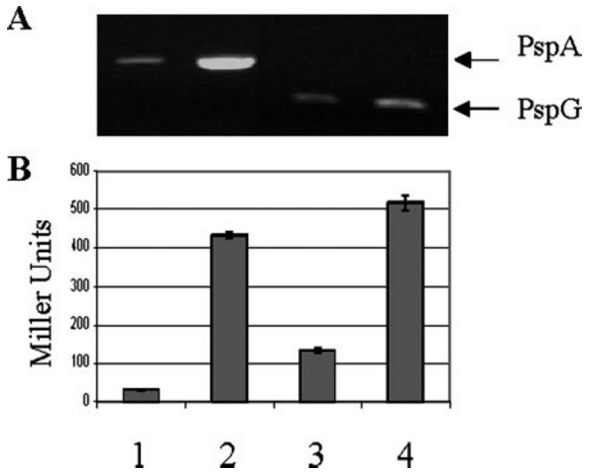

FIG. 2. ***pspA* and *pspG* transcription is up-regulated in response to pIV-secretin stress.** *A*, *pspA* and *pspG* transcripts were amplified from total RNA samples from MG1655 cells by RT-PCR as described under "Experimental Procedures." *B*, β-galactosidase assays were carried out on MC1061 cells to detect transcription of *pspA* (pSJ1) and translation of *pspG* (pLL1). *Lane 1*, *pspA* detected in MG1655 expressing pGZ119EH (vector) for 1 h; *lane 2*, *pspA* detected in MG1655 expressing pPMR129 (pIV) for 1 h; *lane 3*, PspG detected in MG1655 expressing pGZ119EH for 1 h; *lane 4*, PspG detected in MG1655 expressing pPMR129 for 1 h.

sponse of wild type MG1655 cells to pIV stress, there is very little transcriptional change across the whole genome in response to overexpression of *psp* genes in MG1655Δ*pspA*, with the clear exception of the gene *pspG*, which is strongly up-regulated. The level of expression from the *pspA* promoter and the *pspG* promoter was similar when we compared wild type cells expressing pIV to cells lacking PspA, the only known negative regulator of *psp* expression. This result establishes that pIV is a strong and effective inducing signal.

We considered that the synthesis of pIV in mutants unable to mount a wild type Psp response might result in additional changes in the transcriptome to compensate for the inability of the cell to adapt to stress arising through the failure to express the *psp* genes. MG1655Δ*pspA*, MG1655Δ*pspBC*, and MG1655Δ*pspF* cells containing pPMR129 (pIV) and pGZ119-EH (vector control) were grown to mid-log phase and induced with IPTG. The synthesis of pIV reached high levels after 1 h (see Supplementary Materials Fig. 1), which is consistent with the observation that filamentous phage grow normally in *psp* mutants (1). The *psp* mutant strains did not show any growth defects on expression of pIV. Microarray analysis showed that synthesis of pIV in MG1655Δ*pspA*, MG1655Δ*pspBC*, and MG1655Δ*pspF* does not cause any pIV-dependent changes in the transcriptome attributable to the loss of PspA, PspBC, or PspF, respectively (see Supplementary Materials Table I). *pspG* was not further up-regulated in MG1655Δ*pspA*, MG1655Δ*pspBC*, or MG1655Δ*pspF* cells when expressing pIV, probably because the *psp* operon, and therefore *pspG*, were constitutively on in MG1655Δ*pspA* and always off in MG1655Δ*pspBC* and MG1655Δ*pspF*. This data shows that expression of *pspG* is negatively regulated by PspA and its transcription may be activated by PspF via a σ^{54} promoter and positively regulated by PspBC.

The psp Operon and pspG Are Co-conserved and Co-regulated—pspG and *pspFpspABCDE* are not physically linked on the chromosome, but *pspG* is highly conserved among bacteria in which the *psp* operon is conserved. Furthermore, all bacteria containing a recognizable *psp* operon carry a *pspG* homologue, and *pspG* homologues are not present in bacteria lacking a *psp* operon. This shows that the *pspG* and *psp* loci are co-conserved. PspG is a small (~9 kDa) highly hydrophobic protein that is predicted to be an inner membrane protein (enzim.hu/hmmtop/http://www.cbs.dtu.dk/services/TMHMM/).

Because our experiments indicated that *pspG* transcription is regulated by the same elements that regulate *psp* operon transcription, we used a bioinformatic approach to search the *pspG* promoter region for the control elements that are present in the *psp* operon promoter, which are binding sites for σ^{54}, PspF, and IHF. Using the program fuzzpro (EMBOSS programs) and the consensus sequence WWWTCAA[N4]TTR for IHF binding (45) and sequences GGCACGCAAATTGT for σ^{54} binding and TAGTGTAATTCGCTAACT for PspF binding (based on the σ^{54} and PspF binding sites in the *pspA* promoter) (20, 46) we found potential binding sites for σ^{54}, IHF, and PspF (Fig. 4).

Using the translational fusion for *pspG* (pLL1) we found that σ^{54}-RNAP and activation by PspF are required *in vivo* for pIV-induced PspG expression. In the wild type strains the basal level of PspG expression is extremely low, but is up-regulated upon induction with pIV. In mutant strains for σ^{54} and PspF, *pspG* expression is abolished both before and after induction (data not shown). DNase I protection assays using purified components confirmed that the *pspG* upstream DNA region was bound by PspF (Fig. 5*A*, *lane 3*) and by the σ^{54}-RNAP (Fig. 5*B*, *lane 3*) at the promoter sequence predicted by bioinformatics. *In vitro* transcription assays established that a transcript originated from the predicted *pspG* promoter region, dependent upon σ^{54}, PspF, and ATP (Fig. 6*A*). Transcripts from the *pspG* promoter increased with increasing concentrations of PspF (Fig. 6*B*) and it appears that in the absence of IHF the *pspG* promoter is much more sensitive to PspF concentration than the *pspA* promoter. Addition of IHF to the *in vitro* transcription assay increases the level of transcripts from the *pspG* promoter indicating that the predicted IHF binding site in the *pspG* promoter region is functional (Fig. 6*C*) and that the binding of IHF to the *pspG* promoter facilitates *pspG* transcription. IHF enhances *psp* operon transcription (20, 46) and facilitates binding of PspF to its upstream activation sequences in the *psp* operon regulatory region, autogenously down-regulating *pspF* transcription (20, 21). Consistent with this data and the increased sensitivity of the *pspG* promoter to PspF in comparison to the *pspA* promoter (Fig. 6*B*), PspG expression in an IHF mutant (MC1068) is increased both before and after induction by pIV *in vivo* (data not shown). Combined, these results provide strong evidence that *pspG* is tightly co-regulated with the *psp* operon and is a member of the PspF-dependent regulon.

PspG Is Not a Regulator of the PspF Regulon—To test whether PspA can be induced by pIV, extreme heat shock, or ethanol shock in cells lacking PspG function, we carried out β-galactosidase assays using the transcriptional reporter for *pspA* (pSJ1) in JWK5716_1 (Km+) (Δ*pspG*) and its parent strain, BW25113. Before induction, basal levels of PspA were equally low (~50 Miller units) in both wild type and Δ*pspG* strains. PspA can be induced by pIV stress (~10-fold), extreme heat shock (~8-fold), and ethanol shock (~5-fold) in cells that cannot produce PspG to the same level as in wild type cells. This demonstrates that PspG is not essential for *psp* operon transcription when the operon is induced using either a specific secretin stimulus or general membrane stress stimuli. Because *psp* operon transcription is not affected by the absence of PspG in the cell, then PspG is clearly not acting as a regulator of *psp* operon transcription. Using the translational reporter for PspG expression (pLL1), we show that PspG expression is unchanged in the Δ*pspG* strain compared with wild type both before induction (~150 Miller units) and after induction (~700 Miller

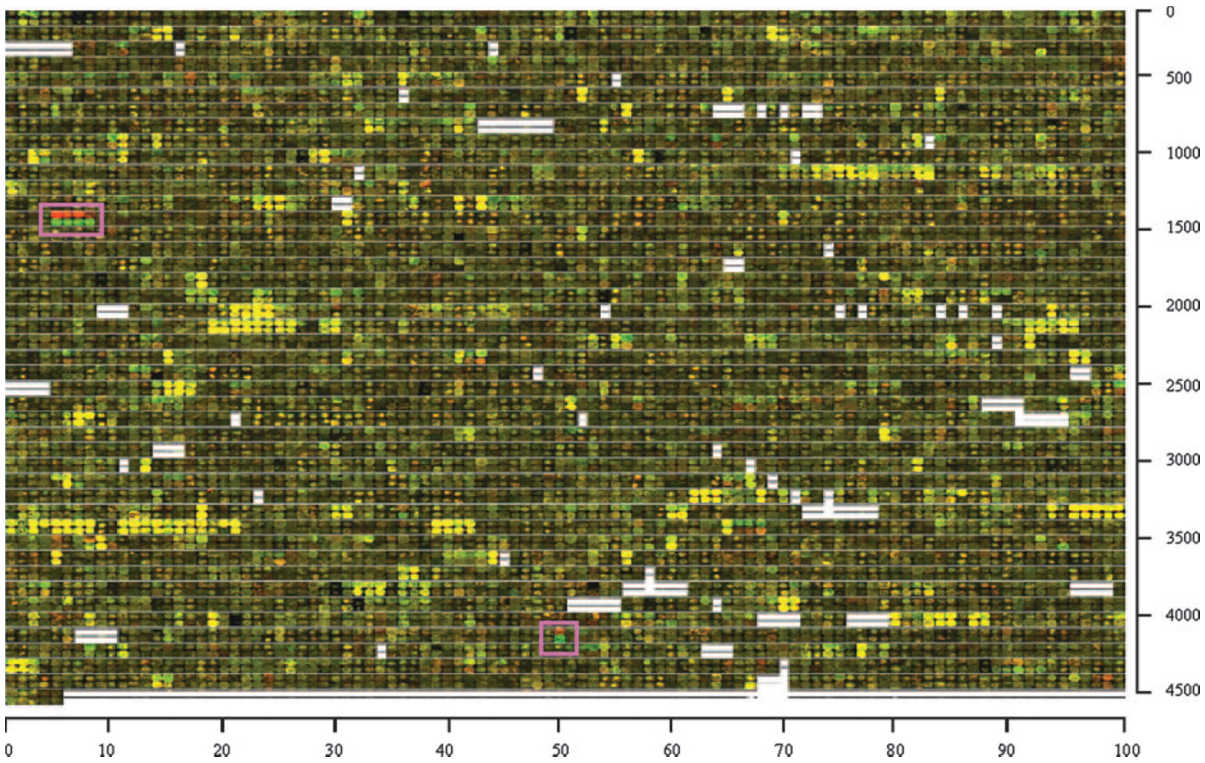

FIG. 3. **pIV secretin stress results in a highly restricted transcriptional response.** *E. coli* microarrays (from the laboratory of S. Kustu) were probed with mixtures of cDNAs from MG1655Δ*pspA* and MG1655Δ*pspF* grown on N-C- media supplemented with 0.4% glucose and 10 mM NH_4Cl to mid-log phase. Spots from fluorescence scanning of the microarrays were rearranged in genome order. The *b* numbers are indicated. Spots are arranged in doublets as a dye-swap experiment was carried out. Those *b* numbers with a *red spot* in the *top row* of the doublet and a *green spot* in the *bottom row* of the doublet are up-regulated in MG1655Δ*pspA* compared with MG1655Δ*pspF* and vice versa. For highly expressed genes, spots appear intense *yellow* because of image saturation. *pspB*, *C*, *D*, *E* (b1305–1308), and *pspG* (b4050) (highlighted) are clearly up-regulated in MG1655Δ*pspA* compared with MG1655Δ*pspF*. There is little change in gene expression across the rest of the genome.

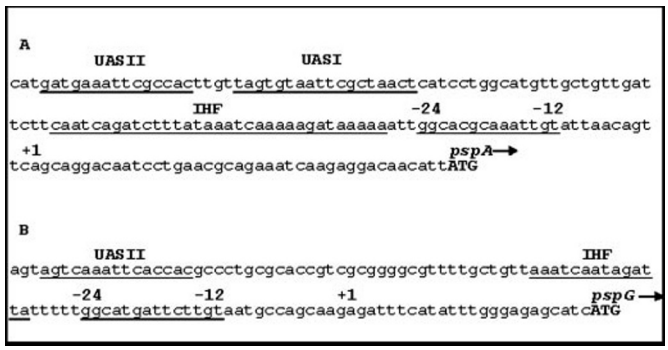

FIG. 4. **The *pspG* promoter region contains regions predicted to bind PspF, IHF, and σ^{54}.** *A*, the *pspA* promoter region of *E. coli* (20, 57). *B*, the *pspG* promoter region of *E. coli*. Sites for PspF, IHF, and σ^{54} in the *pspG* promoter region were predicted by bioinformatics (fuzzpro, EMBOSS programs). Consensus sequence for IHF binding is WWWTCAA[N4]TTR (45) and the sequences for σ^{54} (GGCACGCAAATTGT) and PspF (TAGTGTAATTCGCTAACT) binding are present in the *pspA* promoter.

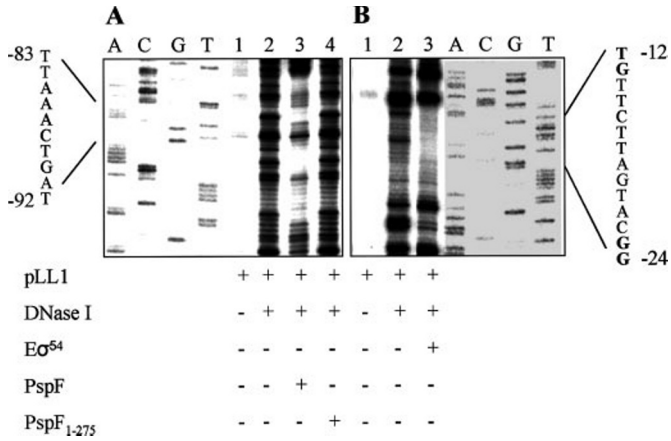

FIG. 5. **PspF and σ^{54}-RNA polymerase bind the *pspG* promoter region.** *A*, PspF (used at 400 nM) footprint on the *pspG* promoter between positions −76 and −109 is shown in *lane 3*. Control reactions that do not contain PspF are shown in *lanes 1* and *2*. The reaction in *lane 4* was conducted with 1000 nM $PspF_{1-275}$ (PspF lacking the DNA binding domain). *B*, Eσ^{54} (used at 200 nM) footprint on the *pspG* promoter between positions −8 and −30 is shown in *lane 3*. Control reactions that do not contain Eσ^{54} are shown in *lanes 1* and *2*. In *A* and *B* the lanes marked *A*, *C*, *G*, and *T* contain chain termination DNA sequencing reactions conducted with pLL1 and the chain terminating ddATP, ddCTP, ddGTP, and ddTTP, respectively. DNase I-treated (+) and -untreated (−) reactions are marked at the *bottom*. The DNA sequence shown on the side was predicted using bioinformatics (fuzzpro, EMBOSS programs) to bind to PspF (*A*) and Eσ^{54} (*B*). In *B*, the consensus promoter −12 and −24 regions of σ^{54}-dependent promoters are shown.

units). Therefore we conclude that PspG is not involved in controlling the PspF regulon.

PspG Is an Effector of the PspF Regulon—Considering the putative membrane location of PspG, and that PspG is not involved in *psp* regulation *per se*, it is likely that this protein is an effector of the *psp* system. It has been shown that the *psp* operon is up-regulated during swarming in *Salmonella* (5) and *psp* mutants have altered motility (14) therefore we employed a motility assay to compare wild type cells to strains mutant for various *psp* genes to explore a possible effector function of PspG (Fig. 7). Cells lacking the negative regulator (Δ*pspA* and Δ*pspABC*) (therefore with increased PspG expression) show decreased motility. Note that in the presence of PspBC, motility is less decreased. As a control, strains deleted for *pspBC* show no change in motility. Double mutants for the activator PspF and the negative regulator PspA (therefore no PspG expression)

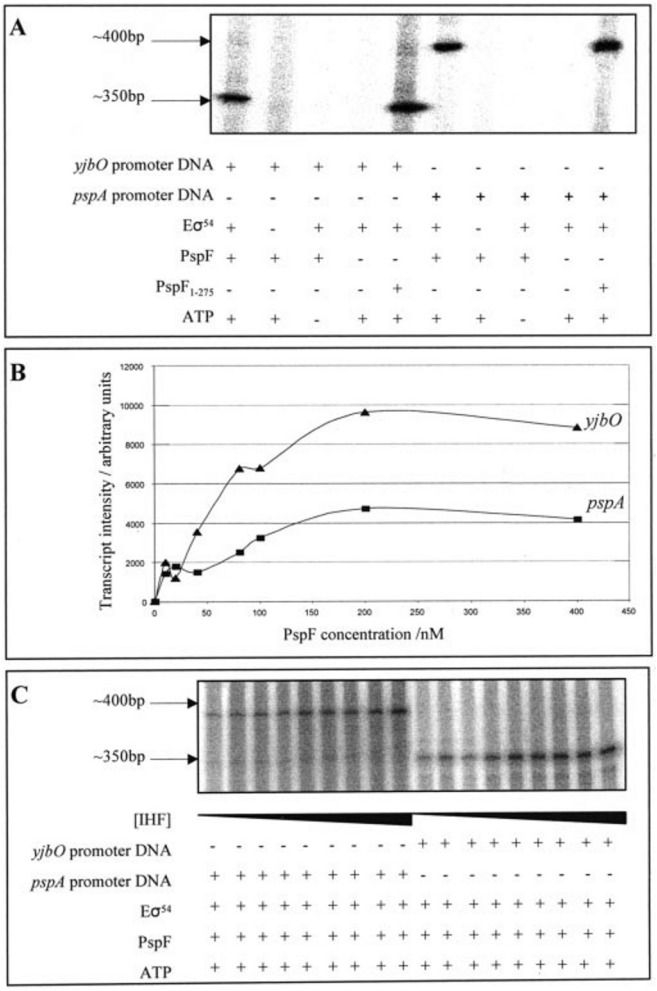

FIG. 6. ***pspG* transcription requires σ^{54}-RNA polymerase, PspF, and ATP and is facilitated by IHF.** *A*, transcripts from the *pspA* promoter (pSLE1) (~350 bp) and the *pspG* promoter (pJH2) (~400 bp) are dependent on Eσ^{54} PspF and ATP. *B*, the addition of increasing concentrations (0–400 nM) of wild type PspF to the transcription assay in *A*. *C*, the addition of increasing concentrations (0–200 nM) of IHF to the transcription assay in *A*.

show slightly increased motility. Cells deleted for *pspG* or for both *pspA* and *pspG* also show slightly increased motility. Strains mutant for the activator PspF (no PspG and no Psp operon expression) show unchanged motility. When PspG is expressed from pLL8, motility in both wild type and Δ*pspF* cells is greatly decreased (Fig. 7). Induction of PspG expression by 0.4% arabinose decreased motility to a higher extent compared with non-inducing conditions (data not shown). PspG up-regulation does not induce the PspF regulon response,[2] therefore we assume that PspG can function independently of the inducing signal and the Psp response. In summary, these results establish that increased expression of PspG causes decreased motility, whereas the lack of PspG or abolished expression of PspG results in slightly increased motility implying that PspG is an effector of the *psp* system.

DISCUSSION

Our data show that expression of pIV in both *E. coli* and *S. typhimurium* results in the significant up-regulation of *pspABCDE* and *pspG* (*yjbO*) transcripts. Previously it has been shown that the *pspABCDE* operon and *pspG* are among the 25 most highly up-regulated genes in *S. typhimurium* infecting macrophages (4). Similarly high levels of expression of the *psp* operon and *pspG* are also seen in *S. typhimurium* infecting epithelial cells and in *Shigella flexneri* infecting macrophages and epithelial cells.[3] Therefore, the *psp* response linked with *pspG* is observed in a range of enteric bacteria, with potentially important roles for these genes in bacterial virulence. In contrast to the response of *E. coli* to stresses such as nitrogen limitation (37) and specialized growth conditions (47–49), which cause substantial changes on a transcriptional level, the synthesis of pIV causes a very restricted change in gene transcription. Restricted responses in microarray experiments have been reported previously, for example, the limited transcriptional response to cell division inhibitors (50). It is possible that a range of pIV-dependent changes in the cell do occur, but only at a translational or post-translational level. Identifying gene regulators through expression profiling may well prove to be a generally challenging problem (51).

As it has been shown for the *pspA* promoter, both the transcriptional activator, PspF, and the σ^{54}-RNAP physically interact *in vitro* with the *pspG* promoter and activation is dependent on PspF and σ^{54}-RNAP *in vivo*. *pspG* expression is also subject to negative regulation imposed by PspA. This is in agreement with the results of Green and Darwin (52) in *Y. enterocolitica*. Here we have shown that the *pspG* promoter is more sensitive to PspF activation than the *pspA* promoter implying that under stress conditions and release of PspA negative regulation, *pspG* responds rapidly. The transcription of *pspG in vitro* is enhanced by IHF in a concentration-dependent manner. However, *in vivo* the basal level of *pspG* expression in IHF mutants is increased. IHF works to enhance transcriptional activation of the *psp* operon (20, 46) and facilitates the binding of PspF to upstream activation sequences I and II in the *psp* operon regulatory region. Thus IHF enhances both the activation of *psp* transcription and the negative autogenous control of *pspF* keeping the PspF concentration at a low level (20, 21). Strains lacking functional IHF will therefore under normal growth conditions have increased concentrations of the activator PspF and decreased concentrations of the negative regulator PspA. Hence, the increased level of *pspG* expression in IHF mutants should be because of the high sensitivity of the *pspG* promoter to PspF activation and diminished negative regulation by PspA. To summarize, our data show that *pspG* is a member of the PspF regulon in *E. coli* and *Salmonella* and is tightly regulated in concert with the *psp* operon. Because the expression of the activator PspF is constant under all growth conditions, the key regulatory point under normal growth conditions is strong negative regulation imposed by PspA, whereas under inducing conditions this regulation is lifted leading to the coordinated expression of the *psp* operon and *pspG*. In fact, PspF specifically regulates only *pspABCDE* and *pspG*. *pspG* is physically separated from the *psp* operon on the chromosome, but is conserved in all bacteria harboring the *psp* operon and there are no obvious PspG homologues in bacteria that lack a recognizable *psp* operon. Therefore, the co-regulated expression of the *psp* operon and *pspG* by PspF and PspA is likely to be a widely conserved and important feature of cellular adaptation to secretin-induced stress. It is striking that the *psp* operon and the *pspG* σ^{54} promoters, which are physically unlinked, are both regulated by PspF, PspA, and IHF in exactly the same fashion. Darwin and Miller (3) suggest that in *Y. enterocolitica*, another genetic locus is involved in the *psp* response to secretin stress because a double *pspF*/*psp* operon mutant showed a more severe growth defect than the *psp* operon mutant alone. Further evidence to support this has been reported (52). Our

[2] L. J. Lloyd, G. Jovanovic, and M. Buck, unpublished data.

[3] J. Hinton, unpublished results.

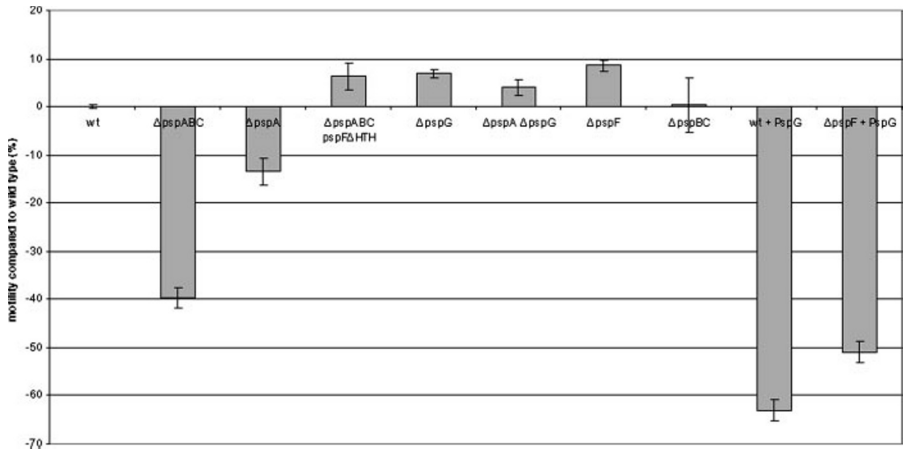

FIG. 7. **PspG overexpression results in decreased *E. coli* cell motility.** Percentage change in motility of strains mutant for various *psp* genes, or wild type cells overexpressing PspG compared with their respective wild type controls (motility of control cells is quoted as 0). The percentage change in motility for each strain is calculated from at least three independent motility assays. Wild type (Wt), MG1655; Δ*psp*ABC, MVA29; Δ*psp*A, MG1655Δ*pspA*; Δ*psp*ABC *psp*FΔHTH, MVA19; Δ*psp*G, MVA40; Δ*psp*GΔ*psp*A, MVA42; Δ*psp*F, MG1655Δ*pspF*; ΔBC, MG1655Δ*pspBC*; wt + PspG, MG1655/pLL8; Δ*psp*F + PspG, MG1655Δ*pspF*/pLL8.

data suggests that the separate genetic locus could be the *Y. enterocolitica* homologue of *pspG*.

pspG is dispensable for basal level expression and induction of the PspF regulon by pIV, ethanol shock, or extreme heat shock under normal growth conditions and so PspG is not acting to regulate the expression of *psp* genes. PspG was not toxic, judged by growth rates and yields. In this study we demonstrate that increased expression of PspG results in decreased motility, whereas the lack of PspG expression causes slightly increased motility. It has been shown that swarming in *Salmonella* induces the *psp* operon (5), and therefore up-regulation of PspG (this paper). PspA has been implicated in maintaining proton-motive force under stress conditions (15) and proton-motive force is proportional to cell motility (53–55). The precise function of PspG in motility is not clear from our data, but because PspA upon induction is an effector involved in proton-motive force maintenance we assume that PspG could play an additional role as an effector of the Psp response. The greatest reduction in motility is seen in strains that overproduce PspG but it is likely that under different growth conditions the reduction in motility might be because of synergistic actions of PspA and PspG. The major difference between PspA and PspG could be that PspA requires induction to switch between being a negative regulator to being an effector, whereas PspG, according to our results, is constantly in an effector state. Because PspA and the previously unknown PspF regulon member, PspG, are so tightly co-regulated, our results raise issues about previously described phenotypes attributed to PspA, in particular the contribution of PspG to these phenotypes. We propose that stimuli that induce the *psp* operon disrupt the integrity of the inner membrane and affect the proton-motive force of the cell. To test the role of PspG as an effector of the phage shock response it will be important in future work to study, using microarray analysis, the response of wild type, *pspABCDE*, and *pspG* mutant strains to stresses that change or uncouple the proton-motive force.

Acknowledgments—We thank Adriane Jones (University of California, Berkeley) for advice on DNA microarrays, Simon Cutting (Royal Holloway, University of London) for raising antibodies against PspA, Hajime Niwa (Imperial College London) for the gift of full-length PspF, Michael Stumpf (Imperial College London) for statistical work on microarray data, Derek Huntley (Imperial College London) for assistance with bioinformatics work, Brett Pennell (Imperial College London) for work on motility assays, and Sydney Kustu for comments on the manuscript. We also acknowledge the gift of clones, strains, and antibodies from Hirotada Mori (Nara Institute of Science and Technology, Japan), Marjorie Russel (The Rockefeller University, New York), Jonathan Beckwith (Harvard Medical School), and Michael Chandler (IPBS, Toulouse, France).

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