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Induction and Function of the Phage Shock Protein Extracytoplasmic Stress Response in *Escherichia coli**

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The phage shock protein (Psp) F regulon response in *Escherichia coli* is thought to be induced by impaired inner membrane integrity and an associated decrease in proton motive force (pmf). Mechanisms by which the Psp system detects the stress signal and responds have so far remained undetermined. Here we demonstrate that PspA and PspG directly confront a variety of inducing stimuli by switching the cell to anaerobic respiration and fermentation and by down-regulating motility, thereby subtly adjusting and maintaining energy usage and pmf. Additionally, PspG controls iron usage. We show that the Psp-inducing protein IV secretin stress, in the absence of Psp proteins, decreases the pmf in an ArcB-dependent manner and that ArcB is required for amplifying and transducing the stress signal to the PspF regulon. The requirement of the ArcB signal transduction protein for induction of *psp* provides clear evidence for a direct link between the physiological redox state of the cell, the electron transport chain, and induction of the Psp response. Under normal growth conditions PspA and PspD control the level of activity of ArcB/ArcA system that senses the redox/metabolic state of the cell, whereas under stress conditions PspA, PspD, and PspG deliver their effector functions at least in part by activating ArcB/ArcA through positive feedback.

Transcription of the PspF regulon in *Escherichia coli*, which consists of the *pspABCDE* operon and *pspG* gene, is driven by DNA-dependent RNA polymerase containing the alternative σ factor, σ^{54} (for review, see Refs. 1 and 2). The enhancer-binding protein PspF activates Psp expression (for review, see Ref. 1). PspF is constitutively expressed but is autogenously negatively regulated to maintain a low intracellular concentration (3). Integration host factor facilitates control of the *psp* operon and *pspG* (4–6).

The *psp* operon and *pspG* are strongly induced in response to protein IV (pIV)[3] from filamentous phage and a number of pIV homologues, which are often secretins from type II and type III bacterial secretion systems (for review, see Refs. 1 and 2). PspA is induced under conditions that block or reduce the efficiency of the protein export apparatus. Mutants in *secA*, *secD*, and *secF*, depletion of YidC, and mutations in components of the twin-arginine translocation (Tat) pathway lead to PspA induction. Additionally, *psp* operon expression can be transiently induced by a variety of membrane-altering stresses including extreme heat shock (50 °C), hyperosmotic shock, ethanol treatment (10%), inhibition of fatty acid biosynthesis, and exposure to hydrophobic organic solvents (7) or proton ionophores such as CCCP. In *E. coli*, PspB and PspC are absolutely required for the expression of the PspF regulon when induced with a secretin (for review, see Refs. 1 and 2) but are only partially required when *psp* is induced by ethanol treatment or hyperosmotic shock and are not required for induction by extreme heat shock. The common factor that may link *psp*-inducing conditions is an uncoupling or depletion of the proton motive force (pmf).

In *E. coli* PspF is a cytoplasmic protein, PspA is a peripheral inner membrane protein and resembles a coiled coil protein, PspB, PspC, and PspD are inner membrane proteins containing putative leucine zipper motifs, PspE is periplasmic, and PspG is predicted to be an integral inner membrane protein (Ref. 8 and for reviews, see Ref. 1 and 2). PspA specifically and directly binds to the AAA+ transcription activation domain of PspF, inhibiting *pspA-E* and *pspG* transcription (Ref. 9 and for review, see Ref. 1 and 2). Under Psp inducing conditions, the interaction between PspA and PspF is disrupted, allowing activation of the *pspA-E* and *pspG* promoters (for review, see Ref. 2). With pIV, the inducing signal is transduced via PspB and PspC, positive regulators of *psp* transcription, and signal transduction is most likely achieved via protein-protein interactions (for review, see Ref. 1 and 2). PspD, PspE, and PspG are not known to have any major involvement in *psp* transcription regulation.

PspA acts as an effector of the *psp* system and is thought to serve to prevent proton loss during conditions where the *psp* operon is induced, but the precise mechanism used is unknown (for review, see Ref. 2). PspG is also thought to act as an effector of the Psp system (6). The *psp* genes may be important for infection. The *psp* operon genes are up-regulated during swarming in *Salmonella typhimurium* (10) and during biofilm formation in *E. coli* and are among the most highly up-regulated genes in *S. typhimurium* during macrophage infection. *pspC* mutants of *Yersinia enterocolitica* are severely attenuated

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1 and Tables 1–5.

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[3] The abbreviations used are: pIV, protein IV; CCCP, carbonyl cyanide *p*-chlorophenylhydrazone; pmf, proton motive force; IPTG, isopropyl-β-D-galactopyranoside; $\Delta\psi$, electron potential; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodine.

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TABLE 1
***E. coli* K-12 strains and plasmids used in this study**

Strain or plasmid	Relevant characteristics	Reference
Strain		
XL1-Blue	tet^r	Laboratory collection
MC4100λpsp3	MC4100 λϕ(*pspA-lac*) (amp^r)	11
JWK5716	BW25113 *pspG*::Kan (kan^r)	12
JWK5536	BW25113 *arcB*::Kan (kan^r)	12
JWK3882	BW25113 *cpxA*::Kan (kan^r)	12
JWK2226	BW25113 *ubiG*::Kan (kan^r)	12
JW5536	BW25113(pJW5536)	13
MG1655	Wild-type	CGSC# 7740
MG1655Δ*pspA*	Δ*pspA*	6
MG1655Δ*pspBC*	Δ*pspBC*	6
MG1655Δ*pspD*	Δ*pspD*	14
MG1655Δ*pspF*	Δ*pspF*	6
MVA40	MG1655 *pspG*::Kan (kan^r)	6
MVA42	MG1655Δ*pspA pspG*::Kan (kan^r)	6
MVA47	MG1655Δ*pspD pspG*::Kan (kan^r)	This work MG1655Δ*pspD*xJWK5716
MVA59	MG1655 *arcB*::Kan (kan^r)	This work MG1655xJWK5536
MVA61	MG1655Δ*pspF arcB*::Kan (kan^r)	This work MG1655Δ*pspF*xJWK5536
MVA69	MG1655Δ*pspF ubiG*::Kan (kan^r)	This work MG1655Δ*pspF*xJWK2226
MVA27	MG1655Δ*pspA* λϕ(*pspA-lac*) (amp^r)	This work MG1655Δ*pspA*xMC4100λpsp3
MVA44	MG1655 λϕ(*pspA-lac*) (amp^r)	This work MG1655xMC4100λpsp3
MVA45	MG1655Δ*pspBC* λϕ(*pspA-lac*) (amp^r)	This work MG1655Δ*pspBC*xMC4100λpsp3
MVA62	MVA27 *arcB*::Kan (amp^r, kan^r)	This work MVA27xJWK5536
MVA63	MVA44 *arcB*::Kan (amp^r, kan^r)	This work MVA44xJWK5536
MVA60	MVA44 *cpxA*::Kan (amp^r, kan^r)	This work MVA44xJWK3882
MVA67	MVA44 *ubiG*::Kan (amp^r, kan^r)	This work MVA44xJWK2226
Plasmid		
pGZ119EH	IPTG-inducible *tac* promoter expression vector, (cam^r)	A gift from M. Russel
pPMR129	pGZ119EH harbouring *gIV* (pIV), (cam^r)	A gift from M. Russel
pAPT110	IPTG-inducible *lacUV5* promoter expression vector (spc^r, kan^r)	16
pPB9	pAPT110 harbouring *pspA* (spc^r, kan^r)	9
pBR325D	Recombinant cloning vector, (cam^r, tet^r, amp^r)	Laboratory collection
pJLB4	P_{lacUV5}-*gIV*	A gift from M. Russel
pGJ4	P_{lacUV5}-*gIV* (pIV) (1.5 kb) from pJLB4 cloned into the pBR325D (EcoRI-EcoRI) (tet^r, amp^r)	This work
pBAD18-cm	Expression vector, pBAD *ara* promoter	A gift from J. Beckwith
pSLE18A	pspA in pET28b+	17
pPB10	P_{ara}-*pspA*; XbaI-HindIII fragment carrying *pspA* cloned into pBAD18-cm (cam^r)	This work
pLL10	P_{ara}-*pspD*; XbaI-HindIII fragment carrying *pspD* cloned into pBAD18-cm (cam^r)	This work
pLL11	P_{ara}-*pspG*; XbaI-HindIII fragment carrying *pspG* cloned into pBAD18-cm (cam^r)	This work
pJW5536	$P_{T5/lac}$-6x*his-arcB-gfp*	13

for virulence during infection and exhibit growth defects when the type III secretion system is expressed (for review, see Ref. 2).

Although regulation of the PspF regulon has been extensively studied, little is known about the biological function of the Psp proteins, and the nature of the signal transduction process involved in the induction of the Psp response remains undetermined. Here we have analyzed the roles of Psp proteins in *E. coli* under either normal or stress growth conditions (i) by using transcription profiling, (ii) by measuring cell motility, and (iii) by determining the electron potential component of pmf in individual *E. coli* cells. We have analyzed the signal transduction pathway required for the induction of the PspF regulon and identified ArcB as required for induction of Psp.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Media, and Growth Conditions—Bacterial strains used in this study are listed in Table 1. Transduction by $P1_{vir}$ (from P. Genevaux) was as by Miller (15). Strains were grown in Luria-Bertani (LB) broth or on LB agar plates at 30 or 37 °C (15). Arabinose promoters were induced with 0.02, 0.1, or 0.4% arabinose *lac* promoters with 0.1 or 1 mM isopropyl-β-D-galactopyranoside (IPTG). Antibiotics used were ampicillin (100 mg ml^{-1}), kanamycin (30 mg ml^{-1}), chloramphenicol (25 mg ml^{-1}), tetracycline (10 mg ml^{-1}), and spectinomycin (50 mg ml^{-1}).

DNA Manipulations—Plasmids used in this study are listed in Table 1. Transformation was as by Miller (15). For pGJ4, *gIV* (encoding pIV) was subcloned from pJLB4 (from M. Russel) using EcoRI into pBR325D. For pPB10, *pspA* was subcloned from pSLE18A (17) using XbaI-HindIII into pBAD18-cm. *pspD* was amplified from *E. coli* MG1655 using primers PspD-F (5′-GAAAATCTAGAGGAGTGAAACGATGAATACTCGCT-G-3′ (the XbaI site is underlined) and PspD-R 5′-GAAAAAAGCTTTCACCTTTTATAACGCTGTGCCAG-3′ (the HindIII site is underlined) and ligated into pBAD18-cm to create pLL10. *pspG* was amplified from *E. coli* MG1655 using primers 5′-GAAAATCTAGAGGAGTGAAACGATGCTGGAACTA-C-3′ (the XbaI site the underlined) and PspG-R1 5′-GAAAA-AAGCTTTTAGTAACGCCAGCGGTCATAAC-3′ (the HindIII site is underlined) and ligated into pBAD18-cm to create pLL11. Constructs were verified by sequencing.

Reverse Transcription-PCR—Total RNA was isolated and purified after digestion with RNase-free DNase I (Promega) using an RNeasy kit (Qiagen). Qiagen Onestep reverse transcription-PCR kit was used to amplify *pspD* (35 cycles; 206-nucletide DNA fragment). For *pspD* the primers D-RTPCR-F

(5′-GGCAACAGGCCGGGCAAAAGG-3′) and D-RTPCR-R (5′-CCTTTTATAACGCTGTGCC-3′) were used.

Western Blot Analysis—Western blotting was as described (17) using antibodies to PspA (14), pIV (6), or PspG. PspG peptide antibodies were generated by Eurogentec (Ougree, Belgium) against the sequence NH_2-CAPKVPKYQRYDRWRY-COOH.

β-Galactosidase Assay—Cells were grown overnight at 37 °C in LB broth containing the appropriate antibiotic and diluted 100-fold into the same medium. After growth to mid-exponential phase ($A_{600} = 0.4$) cultures were assayed for β-galactosidase activity as described by Miller (15).

Motility Assay—Motility assays were carried out using motility agar SA (soft agar) (1% Tryptone, 0.5% NaCl, and 0.3% agar) with the appropriate antibiotic and 1 mM IPTG or 0.02% arabinose where required. 2 μl of a 10× concentrated LB culture at $A_{600} \sim 0.6$ was pipetted into the motility agar, plates were incubated at 37 °C for 6 h, and zones of motility were measured in millimeters (6). Because growth rate and density of cells can influence the motility of bacteria, growth was measured after 1, 2, 4, 6, 8, 16, and 24 h by A_{600} and counting the colony forming units. Numbers of cells used were adjusted to be the same, according to estimates of colony forming units.

Microarray Analysis—Microarray experiments were as described (6). These experiments were performed in quadruplicate, consisting of two biological replicates and two technical replicates using PCR product microarrays printed at IFR (Norwich, UK).

Bioinformatics—Microarray data were preprocessed to identify genes changing >2-fold in their differential expression level using GeneSpring 6.1 (Silicon Genetics), genes were ordered by expression level, and then separate genes or biologically linked sets of genes were tested for co-regulation phenotypes. We used a conservative approach to estimate an upper boundary for the p values; in each case we determined the minimum rank (maximum rank when testing for down-regulation) of the set of genes under consideration (*e.g.* the 43 flagellar genes) in the ordered list of n-fold increase in expression level (*e.g.* for the Δ*pspA* mutant the smallest relative expression level of a flagellar gene was ≈0.7, and this was the 3571th highest ratio of $n = 4340$ observed values). We calculated the probability of observing all m genes under consideration among the top n highest expression levels, which yields an upper boundary for the p value $p \leq (n/m)/(N/m)$ (for the flagellar genes in the Δ*psp*A mutant, this results in an upper bound for the p value of $p < 0.0005$). The alternative would be to use a histogram-based test or to estimate the density, which would also only be approximate, or would require computationally expensive simulations. Because the p value is only estimated, it is not straightforward to adjust for multiple comparisons. If we only consider the tests performed, then a simple Bonferroni corrections allowed us to consider sets of genes with $p < 0.001$ for further biological analysis and interpretation.

Confocal Fluorescence Microscopy to Assess Membrane Potential (Δψ)—The Δψ was measured as described by Becker *et al.* (18) with the following modifications. Cells from an overnight LB culture were subcultured into fresh LB and grown to an A_{600} of 0.8. 1 ml of culture was spun down and resuspended in 1 ml of permeabilization buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 10 mM glucose). 2 μl of 5 mg ml^{-1} JC-1 (Molecular Probes) was added for 30 min at room temperature. Cells were spun down and resuspended in 500 μl of permeabilization buffer. Microscope slides were prepared as described by Glaser *et al.* (19). Fluorescent bacteria were examined using a Leica TCS-NT confocal microscope (Leica Microsystems) equipped with a krypton/argon laser with an excitation wavelength of 485 nm. Leica confocal software (Leica Microsystems) was used to calculate the green/red fluorescence emission ratio from 100 individual cells taken from three independently grown cultures of each strain.

RESULTS

Strategy for Experimental Design and Transcriptome Data Analysis—Previous results demonstrated that the transcriptome response of the PspF regulon after induction by pIV secretin overproduction is largely limited to the members of the PspF regulon (6), suggesting a fine-tuning adaptation of the cell to stress growth conditions. The main aim of this work was to determine whether there is a genetic program specific to the Psp response and so elucidate a possible biological function for this system. To assess whether the Psp proteins predicted to have an effector function, PspA and PspG, or with no ascribed function, PspD, exert their roles under normal growth conditions, we compared the transcriptome profiles of *pspA*, *pspG*, and *pspD* mutants to that of wild type cells. To analyze the transcriptome profiles of cells expressing Psp effectors at levels higher than those found for the Psp response under pIV inducing conditions, we overexpressed the Psp effectors PspA and PspG. We reasoned that a more pronounced expression of Psp effectors than seen under physiological conditions and in the absence of stimuli will force the cell to respond at a measurable level. We assumed that the stress-inducing stimulus acts to release the PspA-imposed negative regulation of the PspF regulon in a PspB/PspC-dependent manner and that subsequently intracellular levels of Psp effector proteins then increase. We identified genes that showed at least a 2-fold differential expression between wild type *versus* either Δ*pspA* or Δ*pspD* or Δ*pspG* cells and wild type *versus* wild type overproducing either PspA or PspG and with a p cut-off value of 0.05 (supplemental Tables 1–5). From this initial list we analyzed either single gene expression or used hierarchical clustering and self-organized maps to arrange genes in groups or clusters (based solely on the similarity of their gene expression and function) with a fold change ≥2 compared with expression in wild type cells and a p cut of value <0.001. The biological function of up-regulated (Up) or down-regulated (Down) genes or sets of genes is given in Table 2. By focusing on gene sets (groups of genes that share common biological function or regulation), we successfully revealed the changes occurring in orchestrated gene expression underlying the basic cellular processes controlled by PspA, PspD, and PspG. All genes referred to here are annotated on the EcoCyc™ web site.

Transcriptional Profiles of ΔpspA, ΔpspD, and ΔpspG Mutants—To compare the transcription profile of wild type (MG1655) cells to that of cells lacking the *pspA* gene (MG1655Δ*pspA*; a non-polar deletion of *pspA*), to cells lacking the *pspD* gene (MG1655Δ*pspD*), or to cells lacking the *pspG*

TABLE 2
Microarray analyses

A, gene groups up-regulated (Up), down-regulated (Down), or not affected (NA) in MG1655Δ*pspA* (Δ*pspA*), MG1655Δ*pspD* (Δ*pspD*), MVA40 (Δ*pspG*) strains compared to MG1655 (wild type). -Fold change with respect to wild type cells is presented in parentheses. B, gene groups Up, Down, or NA in strains overexpressing PspA (from pPB10 in MG1655) or PspG (from pLL11 in MG1655) compared to MG1655 wild type carrying control vector plasmid pBAD18-cm. -Fold change with respect to wild type cells carrying control vector is presented in parentheses.

A	Δ*pspA*	Δ*pspD*	Δ*pspG*
Motility and chemotaxis*	Up p<0.0005 *fli, flh, flg, mot, ycgR, yhjH, che, tar, tap, tsr* (2-11.5)	Up p<0.0001 *fli, flh, flg, mot, ycgR, yhjH, che, tar, tap, tsr* (2-16)	NA
Energy generation aerobic	Up p<1.9e-7 *glpF* (3.3) *glpK* (5.3) *glpD* (6.5)	Up p<1.52e-7 *glpF* (3.6) *glpK* (6.3) *glpD* (10) *arcB* (2.7) *ydhY* (11)	Up p<2.8e-9 *yghK* (4.8)
anaerobic	NA	NA	Down p<0.0001 *hlyE* (0.1) *nikR/yhhG* (0.3) *sseA* (0.1) Up p<0.001 *flu* (5.9)
Low pH response	Down p<1.8e-14 *gadA* (0.3) *gadB*(0.2) *gadC/xasA* (0.4) *slp* (0.3) *pyrI* (0.3) *pyrB* (0.2) *hdeA* (0.2) *hdeB* (0.2) *hdeD* (0.2)	Down p<1.1e-6 *gadB* (0.2) *gadC/xasA* (0.4) *slp* (0.4) *hdeA* (0.2) *hdeB* (0.1)	

B	PspA overexpression	PspG overexpression
Motility and chemotaxis	NA	Down p<9e-9 *fliY* (0.5) *fliM* (0.4) *fliI* (0.4) *fliE* (0.4) *flgK* (0.5) *flhD* (0.5) *motA* (0.2)
Energy generation aerobic	Down p<3.1e-9 *icdA* (0.4) *glcD* (0.3) *fdoI* (0.3)	Down p<2.2e-7 *cyoC* (0.5) *cadA* (0.4) *qor* (0.3) *yhaI* (0.2) *fdoI* (0.5)
anaerobic	Up p<2.8e-7 *nirD* (2.4) *pspE* (2.3) Down p<9.4e-6 *flu* (0.2)	Up p<7e-15 *yfiD* (8.6) *focA* (3.3) *gpsA* (2.8) *dcuB* (2.6) *nirB* (9.6) *nirD* (2.2) *narG* (9.2) *dmsA* (2.5) *dmsB* (2.2) *napD* (2.6) *narP* (4.5) *glpE* (3)
Transport systems iron	NA	Down p<0.0005 *gpmA* (0.1) *entF* (0.3) *fepE* (0.4) *nrdH* (0.3) *nrdI* (0.3) *nrdE* (0.2) *nrdF* (0.2) *entC* (0.1) *entE* (0.2) *entB* (0.3) *entA* (0.2) *ybiL/b0805* (0.3)
cations and H^+ (H^+ generation)	Up p<2.8e-7 *nikE* (2.8) *modF* (2.2)	Up p<1.3e-14 *nikE* (8.2) *nikB* (2.1) *modF* (3) *amtB* (3.2) *yebL* (5.1) *yebM* (4.9) *kdgT* (3.6) *nupG* (4.4) *kefC* (5.1) *gabP* (2.5) *ynfK* (9.1)
spermidine/putrescine (catabolism)	Up p<1.7e-7 *potD* (69.8) *potC* (13.5) Down p<0.00025 *gabD* (0.4)	Up p<3.7e-7 *potD* (4.3) *speE* (2.4) Down p<0.00015 *gabD* (0.3)
oligopeptides/amino acids (and amino acid synthesis)	Up p<3.5e-7 *oppC* (3.1) *oppF* (3) *dppA* (3.1)	Up p<1.34e-5 *livH* (2.7) *livK* (2.4) *proW* (7.2) *dppA* (5) *argB* (2.7) *argG* (2)
drug/H^+ antiporters	NA	Up p<0.0002 *mdtN/yjcR* (18.7) Down p<0.00063 *emrD* (0.3) *emrK* (0.5) *lytB* (0.4)
putative transporters	Down p<2e-6 *yheS* (0.2)	Up p<1e-14 *yjgN* (10.7) Down p<2e-6 *yheS* (0.2)
High pH response formate neutralisation	NA	Up p<0.0002 *yodA* (12.8) Down p<0.0005 *hycF* (0.3) *hycG* (0.4) *hycI* (0.5)
Assembly and secretion of proteins	Up p<1.6e-24 *dnaK* (3.1) *dnaJ* (2.4) *grpE* (3.5) *clpB* (5) *lon* (3.1) *ilvL* (3.2) *leuL* (3.8) *msyB* (5.9) *fdrA* (2.4) *secE* (2.4)	Up p<1e-9 *clpB* (2.3) *eco* (2.2) *msyB* (6.5) *secE* (2.5)
Lipids, lipoprotein and murein synthesis; cell envelope integrity	Up p<0.001 *lpp* (2) *murG* (3.3) *ddg* (3) Down p<1.8e-7 *mltC* (0.35) *cutF* (0.37)	Up p<0.0002 *ddg* (3.4)
DNA replication	Down p<7.6e-6 *dnaC* (0.4) *priB* (0.4) *polA* (0.4)	NA

*For complete set of genes affected see Supplemental Tables 1 and 2.

gene (MVA40), MG1655, MG1655Δ*pspA* MG1655Δ*pspD*, and MVA40 were grown to A_{600} 0.6–0.8, and cells were harvested for RNA extraction. The deletion mutants did not show any growth rate defects or reduced yields of cells compared with the wild type strain, establishing that these deletion mutations are not toxic to the cell (data not shown). The results of this transcriptome analysis are presented in Table 2A and supplemental Tables 1–3.

The microarray data of Δ*pspA*, Δ*pspD*, and Δ*pspG* clearly show for the first time that the protein products of *pspA*, *pspD*, and *pspG* function under normal growth conditions and not only after encountering stress, since relative changes in gene expression are Psp-dependent and are apparent without any added inducing stimulus or stress present. The transcriptional profiles of Δ*pspA* and Δ*pspD* are strikingly similar (Table 2A and supplemental Table 1 and 2), and so it is likely that PspA and PspD have overlapping functions or might work synergistically. A possible synergistic relationship between PspA and PspD is further reasoned by results from motility assays and $\Delta\psi$ measurements (see below). In general, Δ*pspD* cells showed more pronounced up-regulation of all genes affected compared with Δ*pspA* cells (Table 2A). The transcription profile of Δ*pspG* cells does not show any clear similarity to the profiles of Δ*pspA* and Δ*pspD* cells with only a small set of genes significantly changing (Table 2A and supplemental Table 3).

Microarray analyses showed increased levels of *pspB*, *pspC*, *pspD*, *pspE*, and *pspG* transcripts in cells lacking *pspA* compared with wild type (supplemental Table 1). Comparing the transcription profile of wild type cells to cells lacking *pspD* or to cells lacking *pspG*, the level of transcription of the *psp* genes remained constant (supplementary Tables 2 and 3), establishing that PspD or PspG do not control transcription from the *psp* operon or *pspG* promoters under normal growth conditions (6, 14). The level of *pspF* transcription remained constant in all three experiments, consistent with control of Psp being exerted at the level of PspF activity (3).

It is clear from the transcriptional profiles of Δ*pspA* and Δ*pspD* cells that there is up-regulation of genes involved in the motility of bacteria (Table 2A). These genes include the *fliA*, *flhC*, and *flhD* flagellar master regulators, genes for flagellar function and biosynthesis, and chemotaxis genes including *tar*, *tap* and *tsr* receptors. In Δ*pspG* cells, transcription of flagellar genes remains unchanged compared with the wild type, consistent with the observation that Δ*pspG* cells do not show changes in motility compared with wild type cells (6).

Genes *glpF*, *glpK*, and *glpD* involved in the uptake and metabolism of glycerol during the most efficient aerobic respiration reaction, "glycerol shift," in Δ*pspA* and Δ*pspD* cells are up-regulated compared with wild type (Table 2A). In Δ*pspD* the gene encoding putative oxidoreductase, *ydhY*, which is predicted to contain a Fe-S cluster and be involved in aerobic respiration, is highly up-regulated, and the putative pmf-dependent transporter (*yghK*) is up-regulated in Δ*pspG* cells (Table 2A). Notably, in Δ*pspA* and Δ*pspD* cells expression of the *arcB* gene is up-regulated (Table 2A). The up-regulation of ArcB can deactivate ArcA-P (20), and the inactivation of ArcA can then cause up-regulation of genes involved in aerobic respiration (such as *glpD*).

The expression of genes either involved in anaerobic respiration (such as one coding for rhodanese-like enzyme, *sseA*) or activated by Fnr, the regulator of genes involved in fermentation and anaerobic respiration (*hlyE* and *nikR*), is down-regulated in Δ*pspG* cells (Table 2A). The *flu* gene, which is negatively regulated by OxyR and Fnr, is one of the most highly up-regulated genes in Δ*pspG* cells (Table 2A). Because no other genes regulated by OxyR are affected, we infer that this change as well could be due to decreased activity of Fnr.

Genes involved in responding to low pH (*gad*, *slp*, and *hde*), some involved in pathogenesis (*hde*), are down-regulated in Δ*pspA* and Δ*pspD* cells (Table 2A). Notably, under aerobic growth, *gad* genes are implicated in survival of extreme acid conditions, but under anaerobic growth they are involved in biosynthesis of γ-aminobutyric acid and, consequently, anaerobic respiration and resistance to high pH. Genes involved in the catabolism of fatty acids (which in addition decreases the high pH response), *atoA* and *atoE*, are up-regulated in Δ*pspG* cells.

Taken together these results suggest that under normal growth conditions, at basal level expression, even though PspA and PspD affect different sets of genes than PspG, these three Psp proteins function to subtly adjust the redox state of the cell and energy usage by down-regulating cell motility and aerobic respiration. This is consistent with a proposed role for Psp response in maintaining the energy generation and pmf under induced stress conditions (for review, see Ref. 2) when Psp proteins are at high intracellular concentrations. Our results underline that under normal growth conditions intracellular concentration of PspA is sufficient for this protein to exert effective negative regulation and low level effector function.

Transcriptional Profiles of Wild Type Cells Overexpressing PspA or PspG—To examine the transcriptional response to the overexpression of the predicted Psp effector proteins (PspA and PspG) in wild type *E. coli* cells, MG1655 containing the plasmids pPB10 (PspA), pLL11 (PspG), or pBAD18-cm (vector control) were grown to A_{600} 0.4–0.6 and induced with 0.4% arabinose for 1 h, and cells were harvested for RNA extraction. The synthesis of PspA or PspG was detectable after 1 h by Western blotting (supplemental Fig. 1) and did not lead to reduced growth rates or reduced yields of cells, indicating a lack of toxicity of these overproduced proteins. The results of the transcriptome analysis are presented in Table 2B and supplementary Tables 4 and 5. Taken together, the transcription profile of cells overexpressing PspA and PspG shows the inverse profile of Δ*pspA*, Δ*pspD*, and Δ*pspG* cells, with additional genes affected. For example, certain genes involved in aerobic energy generation are up-regulated in Δ*pspA* and down-regulated in PspA-overexpressing cells, whereas genes involved in anaerobic respiration are down-regulated in Δ*pspG* and up-regulated in PspG-overexpressing cells (compare Table 2, A and). Although in many instances PspA and PspG affect the same functional gene clusters, there are distinct differences in both numbers of genes affected among the same cluster and clusters of genes affected (Table 2B).

Microarray analyses of PspA and PspG overexpression showed increased levels of *pspA* transcription (287-fold) or *pspG* transcription (239-fold), respectively, compared with the vector control (supplemental Tables 4 and 5). Notably, after pIV induction, the level of PspA transcription is increased

100-fold, and the level of PspG transcription is increased 20-fold (6). *pspBCDE* and *pspG* transcription remains essentially unchanged in cells overexpressing PspA, demonstrating that this increased level of PspA does not increase the negative regulation imposed on the PspF regulon. Cells overexpressing PspG show a low (<10%) induction of the PspF regulon (supplemental Table 5) compared with wild type cells overproducing pIV secretin (6). This increase in *pspA* transcription after overexpression of PspG is dependent on the presence of PspB and PspC in the cell (data not shown).

When PspG is overexpressed, some genes involved in motility are down-regulated (Table 2B). This is consistent with results demonstrating that PspG overexpression results in decreased motility (6). Notably, these motility genes are up-regulated in Δ*pspA* and Δ*pspD* mutants compared with wild type but not in Δ*pspG* (Table 2A).

Upon overexpression of PspG, some ArcA-positively regulated genes for anaerobic respiration (formate metabolism) are up-regulated (*yfiD*, *focA*) (Table 2B). Also, a small portion of ArcA-negatively regulated genes for aerobic respiration are down-regulated (*icdA*, *glcD*, *cyoC*, and *cadA*) in PspA- and PspG-overexpressing cells (Table 2B). These results suggest an activation of the ArcB/ArcA system by overexpressing PspA and PspG. In *E. coli*, changes in gene expression in response to changing respiratory conditions of growth is partially mediated by the ArcB/ArcA two-component signal transduction system, which comprises the transmembrane ArcB sensor kinase and its cytosolic cognate response regulator ArcA (for review, see Ref. 21). Under anaerobic or microaerobic conditions, ArcB transphosphorylates ArcA, which represses genes involved in aerobic respiration (*e.g.* enzymes of electron transport and the tricarboxylic acid cycle) and activates genes that sustain anaerobic growth and respiration (*e.g.* pyruvate formate lyase (*pfl*) and hydrogenase I (*hdn*)). Under aerobic conditions, oxidized forms of quinone electron carriers in the membrane inhibit the autophosphorylation of ArcB and, therefore, mediation of the Arc metabolic response by ArcB (22, 23). In PspG-overexpressing cells, *gpsA* is up-regulated (Table 2B). GpsA, involved in the glycerol shift reaction, facilitates quinone reduction and biosynthesis of the phospholipid precursor, glycerol 3-phosphate, activities that oppose action of the protein product of ArcA-regulated *glpD* (the aerobic respiration) up-regulated in Δ*pspA* and Δ*pspD* cells (Table 2A). Interestingly the gene adjacent to *pspG*, which is a quinone oxidoreductase (*qor*) and involved in aerobic respiration, is down-regulated when PspG is overexpressed (Table 2B). Additionally, genes for the preferential uptake of the polycation spermidine (*potD* and *potC*) and biosynthesis of sperimidine from putrescine (*speE*) are up-regulated, whereas the gene for putrescine catabolism (*gabD*) is down-regulated (Table 2B). Also, genes that direct the import of H^+ by means of cations/H^+ or substrate/H^+ symport or antiport (*kdgT*, *nupG*, *kefC*, *gabP*, and *dcuC*) or produce H^+ (*ynfK*/*b*1593) are up-regulated specifically in cells overexpressing PspG (Table 2B). These changes in gene expression can increase the reducing capacity of the cytoplasm and, therefore, favor an anaerobic respiration. Notably, mostly PspG-overexpressing cells also show up-regulation of some genes involved in anaerobic respiration and fermentation, which are positively controlled by Fnr (*nikE*, *nikB*, *dcuB*, *nirB*, *nirD*, *narG*, *dmsAB*, and *napD*) (Table 2B). Among these genes are the NarL- or NarP-controlled anaerobic respiration and fermentation genes, *nirB*, *nirD*, *narG*, and *napD*. The molybdenum transport *modF* gene required for the function of NapA (assembly of which is assisted by NapD) is up-regulated in PspA- and PspG-overexpressing cells. Also up-regulated is the *narP* regulator. Collectively these genes are involved in the metabolism of nitrogen and anaerobic respiration. In PspG-overexpressing cells, ammonium (*amtB*) and γ-aminobutyric acid (*gabP*) transport as well as arginine biosynthesis (*argB* and *argG*) are up-regulated, probably facilitating the provision of a nitrogen source.

Clearly the data from overexpression studies and the results with deletion mutants are in concert and show that one action of the Psp effectors is to increase gene expression for anaerobic respiration and a reducing environment and to decrease gene expression for aerobic respiration and one pmf-consuming process (motility), potentially to maintain the redox state of the cell and pmf.

Additional observations suggest that a switch toward an anaerobic respiration mode occurs in PspA- and PspG-overexpressing cells. As noted above, cells overexpressing PspG show up-regulation of genes involved in formate biosynthesis (*yfiD*, which replaces *pflD* under low pH stress conditions) and control of formate transport to the periplasm (*focA*). These genes are positively regulated by ArcA and induced by low pH (*yfiD*) (for review, see Ref. 21). In cells overexpressing PspA or PspG, the gene encoding aerobic formate dehydrogenase, CytB 556 (*fdoI*), is down-regulated, and genes involved in the neutralization of formate (FhlA regulon, *hycFGI*) are down-regulated in cells overexpressing PspG. The synthesis of formate and import of γ-aminobutyric acid (see above) can create a low pH environment, and up-regulation of low pH-induced genes is evident (*e.g. yfiD* and *yodA*), an outcome that mirrors the transcriptome results from Δ*pspA*, Δ*pspD*, and Δ*pspG*. In PspA-overexpressing cells, *flu*, a gene implicated in phase variation and cell autoaggregation that is negatively regulated by Fnr, is highly down-regulated (Table 2B), whereas in Δ*pspG* cells *flu* is up-regulated (Table 1, upper).

An increased reducing environment can be responsible for the result showing that genes involved in iron transport and metabolism (the Fur regulon: *gpmA*, *entFfepE*, *nrdHIEF*, and *entCEBA*) are highly down-regulated when PspG is overexpressed (Table 2B). Expression of the Fur regulon is negatively controlled by the Fur regulator with iron (Fe^{2+}) as the corepressor. Expression of *fur* itself is not affected; hence, the down-regulation of iron transport is likely to be a consequence of an increased concentration of available intracellular ferrous iron that is more stable and less toxic in a reducing environment. In addition, a putative outer membrane iron transport receptor *ybiL* (*b*0805) is down-regulated (Table 2B). Genes involved in the reconstitution and formation of Fe-S clusters (rhodaneses) are up-regulated in PspA (*pspE*)- and PspG (*glpE*)-overexpressing cells (Table 2B).

Finally, some changes in gene expression may serve to specifically confront the Psp-inducing stimuli. In cells overexpressing PspG, genes involved in the transport of branched chain amino acids (*livH* and *livK*) and Pro/Gly/betaine osmo-

TABLE 3
Motility phenotype of wild type and *psp* mutants overproducing the pIV secretin

Strain	Relevant genotype	Motility (Change in %)[a]
MG1655(pGJ4)[b]	wild type(*gIV*)	−40 ± 1
MVA40(pGJ4)	*pspG*::Kan(*gIV*)	−30 ± 1
MVA42(pGJ4)	Δ*pspA pspG*::Kan(*gIV*)	−20 ± 1
MG1655Δ*pspF*(pGJ4)	Δ*pspF*(*gIV*)	−16 ± 1
MG1655Δ*pspBC*(pGJ4)	Δ*pspBC*(*gIV*)	−15 ± 2

[a] Motility assays were carried out on SA (soft agar) plates (0.3%) at 37 °C for 6 h. Percentage change with S.D. in motility of strains was calculated from at least six independent motility assays and compared to the isogenic strain carrying the control vector plasmid pBR325D; in this table controls with change in motility quoted as zero are not presented.

[b] Plasmid pGJ4 carries *gIV* (pIV secretin gene) under control of IPTG-inducible *lac* promoter. pIV expression was induced by 1 mM IPTG for 1 h before assay (for details see "Experimental Procedures)."

protectants (*proW*) are up-regulated, and oligopeptide transport (*oppC*, *oppF*, and *dppA*) is up-regulated in cells overexpressing PspA or PspG (Table 2B). This can contribute to confronting hyperosmotic shock, a condition that induces the Psp response. PspA- or PspG-overexpressing cells show up-regulation of protein translocation suppressor genes (*msyB* and *fdrA*), *secE,* the serine protease inhibitor gene *eco*, leucine and isoleucine leader peptide genes (*ilvL* and *leuL*), and heat shock-induced molecular chaperones (*e.g. dnaK, dnaJ, grpE,* and *clpB*) or protease (*lon*) genes (Table 2B). Expression of these genes can confront extreme heat shock, one Psp-inducing stimulus. The gene that encodes the major murein lipoprotein (*lpp*) is up-regulated in cells overexpressing PspA. In addition, a murein biosynthesis-related gene (*murG*) is up-regulated, whereas a gene involved in murein degradation (*mltC*) is down-regulated (Table 2B). These changes might increase the integrity of the cell envelope and, hence, confront many Psp-inducing stimuli. Finally, the gene encoding the lipoprotein NlpE (*cutF*) involved in copper homeostasis and required for activation of Cpx signaling for adhesion is down-regulated in PspA overexpressing cells (Table 2B).

Response of the PspF Regulon Decreases the Motility of Bacteria upon Induction by Secretin pIV—To determine whether induction of the Psp response under physiological conditions down-regulates motility, we used either wild type or *psp* mutant cells and compared their motility phenotypes with cells grown under one Psp-inducing stress condition. As shown in Table 3, pIV overproduction reduced the motility of wild type bacteria (40%). We used a single Δ*pspG* or a double Δ*pspA* Δ*pspG* mutant to help determine the basis of the motility phenotype caused upon induction by pIV. In a single Δ*pspG* mutant overexpressing pIV secretin, motility is decreased 30% (compared with 40% in wild type), whereas in a double mutant Δ*pspA* Δ*pspG* overexpressing the pIV secretin, motility is decreased 20% (compared with 40% in wild type and 30% in Δ*pspG* alone) (Table 3). This result is in agreement with a joint action of PspA and PspG on motility and confirms that upon induction by pIV secretin, PspA and PspG both contribute to decreased motility. A Δ*pspF* mutant, unable to activate the PspF regulon, upon pIV overproduction showed a decrease in motility similar to Δ*pspA* Δ*pspG* (16% compared with 20%) (Table 3). As anticipated, Δ*pspBC,* which is defective in transducing the pIV-inducing signal, under the same stress conditions showed a similar decrease in motility as the Δ*pspF* mutant (15%) (Table 3). This suggested that a 15–20% decrease in motility is solely due to stress caused by pIV secretin overproduction, independent of PspF regulon expression. This is consistent with results determining that pIV-inducing stress decreases pmf in the absence of Psp response (see below). The results presented in this section and the previous section together with results presented earlier (6) justify a functional link between the Psp response, the functionality of the proposed Psp effectors (PspA and PspG), and the motility phenotype, which is dependent on pmf usage.

Correlation between Psp Protein Expression and Motility Phenotype—Clusters of genes involved in motility are up-regulated in Δ*pspA* and Δ*pspD* and down-regulated in cells overexpressing PspG. Highly up-regulated in Δ*pspA* or Δ*pspD* cells is a major structural flagellar biosynthesis gene, *fliC* (see supplemental Tables 1 and 2). Because induction of the Psp response by pIV secretin (see above) and because PspG up-regulation (in Δ*pspA*, Δ*pspABC*) or overexpression down-regulates motility (6), it is likely that there is a functional link between Psp protein expression, expression of flagellar genes, and motility phenotype. We briefly inspected Δ*pspA* and Δ*pspD* cells using electron microscopy. All cells show a flagellar morphology comparable with wild type cells (data not shown).

The microarray profile of Δ*pspA* showed up-regulation of genes involved in the motility and chemotaxis of *E. coli*. This should correlate with increased motility of Δ*pspA* cells, but this phenotype could be suppressed by increased expression of other Psp proteins (*e.g.* PspG) since PspA is a negative regulator of the PspF regulon. In agreement with this is the decrease in motility seen in Δ*pspA* but not in the double mutant Δ*pspA* Δ*pspG* (6). Indeed, overexpression of PspG results in a decrease in motility independent of other Psp proteins (in Δ*pspF* mutant) (6), and in cells overexpressing PspG motility genes are down-regulated (Table 2B). However, the motility phenotype of Δ*pspA* cells suggests that PspG affects motility at a post-transcriptional level; even though in the absence of PspA expression of motility genes is up-regulated, motility *per se* is decreased by the effect of PspG. Therefore, a direct correlation between the motility phenotype and the function of PspG is evident. Here, we showed the same for PspA. Although the transcriptome profile of cells overexpressing PspA did not show changes in the expression of flagellar genes, PspA expression in either wild type or Δ*pspF* cells from pPB10 or pPB9 constructs greatly decreases motility (Table 4). Notably, overexpression of PspA abolished motility of wild type and Δ*pspF* cells. As expected, co-expression of PspA and PspG (in the absence of IPTG or arabinose, respectively) decreased motility to a greater extent compared with expression of PspA or PspG alone (Table 4). This is consistent with results showing that both PspA and PspG contribute to decreased motility under stress growth conditions (Table 3).

To correlate the transcriptional profile of the genes involved in motility of bacteria with the phenotype of the Δ*pspD* strain, the motility of Δ*pspD* was measured (Table 4). The motility of Δ*pspD* is significantly increased (31%) compared with wild type, in agreement with the microarray analysis, suggesting that the expression profile of motility genes in Δ*pspD* mutant is a direct consequence of PspD effector action. Because PspD is not a regulator, this effect again is likely to be at post-transcrip-

TABLE 4
Effects of PspA, PspG, and PspD on motility phenotype

Strain	Relevant genotype	Motility (% change)[a]	
		−Arabinose[b]	+Arabinose[b]
MG1655(pLL11)[c]	wt(*pspG*)	−58 ± 3	NM[d]
MG1655Δ*pspF*(pLL11)	Δ*pspF*(*pspG*)	−30 ± 5	−84 ± 8
MG1655(pPB10)[e]	wt(*pspA*)	−50 ± 5	NM
MG1655Δ*pspF*(pPB10)	Δ*pspF*(*pspA*)	−50 ± 6	NM
MG1655Δ*pspF*(pPB9[f]+pLL11)	Δ*pspF*(*pspA* + *pspG*)	−82 ± 5	
MG1655	Wild type	0 ± 2	
MG1655Δ*pspD*	Δ*pspD*	+31 ± 5	
MVA47	Δ*pspD pspG*::Kan	+30 ± 3	
MG1655(pLL10)[g]	wt(*pspD*)	+5 ± 1	+8 ± 1
MG1655Δ*pspF*(pLL10)	Δ*pspF*(*pspD*)	−5 ± 1	−3 ± 4
MG1655Δ*pspD*(pLL10)	Δ*pspD*(*pspD*)	+3 ± 2	+4 ± 2
MG1655Δ*pspD*(pPB10)	Δ*pspD*(*pspA*)	−52 ± 1	−77 ± 2
MG1655Δ*pspD*(pLL11)	Δ*pspD*(*pspG*)	−5 ± 2	−15 ± 2
MG1655Δ*pspF*(pPB9 + pLL10)	Δ*pspF*(*pspA* + *pspD*)	−80 ± 4	
		−IPTG[h]	+IPTG[h]
MG1655(pPB9)	wt(*pspA*)	−55 ± 3	NM
MG1655Δ*pspF*(pPB9)	Δ*pspF*(*pspA*)	−50 ± 1	NM
MG1655Δ*pspD*(pPB9)	Δ*pspD*(*pspA*)	−37 ± 1	−88 ± 3
MG1655(pGJ4)[i]	wt(*gIV*)	−29 ± 1	−42 ± 4
MG1655Δ*pspD*(pGJ4)	Δ*pspD*(*gIV*)	−11 ± 6	−32 ± 3
MG1655Δ*pspD*(pPB9, pGJ4)	Δ*pspD*(*pspA*, *gIV*)	−47 ± 6	NM
MVA47(pGJ4)	Δ*pspD pspG*::Kan(*gIV*)	−10 ± 2	−28 ± 0.5

[a] Motility assays were carried out on SA (soft agar) plates (0.3%) at 37 °C for 6 h (6). Percentage change with S.D. in motility of strains was calculated from at least six independent motility assays and compared to isogenic strains carrying the control vector plasmid(s); in this table strains carrying the control vector plasmid with change in motility quoted as zero are not presented.
[b] The cells were induced (+) by 0.1% arabinose for 1 h.
[c] Plasmid pLL11 carries *pspG* on pBAD18-cm under control of pBAD arabinose-inducible promoter.
[d] NM, no motility.
[e] Plasmid pPB10 carries *pspA* on pBAD18-cm under control of pBAD arabinose-inducible promoter.
[f] Plasmid pPB9 carries *pspA* on pAPT110 under control of IPTG-inducible *lac* promoter.
[g] Plasmid pLL10 carries *pspD* on pBAD18-cm under control of pBAD arabinose-inducible promoter. wt, wild type.
[h] The cells were induced (+) by 1 mM IPTG for 1 h.
[i] Plasmid pGJ4 carries *gIV* that encodes pIV secretin on pBR325D under control of IPTG-inducible *lac* promoter.

tional level. A double mutant Δ*pspD* Δ*pspG* exhibited no additional increase in motility in comparison to the single *pspD* mutant (Table 4), implying that the lack of PspG (not up-regulated in the Δ*pspD*) in addition to Δ*pspD* does not contribute to the motility phenotype seen in Δ*pspD*. This agrees with the microarray profile for Δ*pspG* where motility genes are not affected.

The expression of *pspD* from pLL10 was confirmed using reverse transcription-PCR (data not shown), and overexpression of PspD does not detectably induce the Psp response in wild type cells carrying the chromosomal *pspA-lac* transcriptional fusion (data not shown). The overproduction of PspD did not significantly change motility of either wild type, Δ*pspF*, or Δ*pspD* cells (Table 4). However, from the relatedness in the transcription profiles, it appears that PspA and PspD might work synergistically. Indeed, co-expression of PspA and PspD (in the absence of IPTG or arabinose, respectively) showed the cumulative effect with a greater decrease in motility compared with the expression of PspA alone (Table 4). Moreover, PspA was able to complement the Δ*pspD* motility phenotype both in the absence or presence of inducer and decrease the wild type motility up to 42% (pPB10) or 57% (pPB9) (Table 4). Induction of the Psp response by overproduction of the pIV secretin from plasmid pGJ4 fully complemented the Δ*pspD* motility phenotype in the presence of inducer (Table 4). Complementation of Δ*pspD* motility phenotype in the presence of pIV-inducing stimulus appears due to PspA and not PspG action. Overexpression of pIV in a double Δ*pspD* Δ*pspG* mutant gave a similar result as that for the Δ*pspD* strain with intact *pspG* (Table 4). Overproduction of PspA and pIV together showed complementation of the Δ*pspD* to a higher extent than with PspA or pIV overexpression alone (Table 4). PspG overexpression did not complement the motility phenotype of Δ*pspD* efficiently, only decreasing the motility up to 15% of Δ*pspD* in the presence of inducer (Table 4). This again highlights some of the differences seen between PspA and PspG functions at the transcriptome level and enhances the view that PspA and PspG have a common target for their biological function but have somewhat distinct mechanisms of action.

ArcB Is Required for Induction of the Psp Response—Transcriptome analyses revealed that there is a link between the ArcB/ArcA-regulated genes and Psp, both under normal growth conditions (Δ*pspA*, Δ*pspD*, Δ*pspG*) and after overexpression of PspA or PspG (likely to mimic in part the Psp response). The ArcB/ArcA system controls genes involved in aerobic respiration and some genes required for anaerobic respiration and fermentation (for review, see Ref. 21). We reasoned that the ArcB sensor might be responsible for recognizing the Psp-inducing stimulus. Hence, we introduced a Δ*arcB* mutation into a strain wild type for *pspF*, *pspABCDE*, and *pspG* carrying a chromosomal *pspA-lac* transcriptional fusion (MVA63). and β-galactosidase assays were carried out either under normal and pIV-, extreme temperature (50 °C)-, ethanol treatment (10%)- or CCCP (80 mM)-inducing conditions (Table 5). The results show that Psp cannot be induced in Δ*arcB* cells by pIV, extreme temperature, or ethanol shock compared with wild type cells. However, the addition of the ionophore CCCP that directly dissipates the pmf induces the Psp response in an

TABLE 5
ArcB is required for induction of the *psp* operon

Strain and growth conditions	Relevant genotype	β-Gal activity[a] (Miller units)
MVA44	MG1655 ϕ(*pspA-lac*)	68 ± 3
+50 °C[b]		299 ± 4
+10% EtOH[c]		244 ± 19
+80 μM CCCP[d]		400 ± 60
MVA44(pGZ119EH)	MG1655 ϕ(*pspA-lac*)(control)	65 ± 5
MVA44(pPMR129)[e]	MG1655 ϕ(*pspA-lac*)(*gIV*[e])	473 ± 19
MVA63	MG1655 Δ*arcB* ϕ(*pspA-lac*)	40 ± 2
+50 °C		47 ± 3
+10% EtOH		68 ± 3
+80 μM CCCP		148 ± 7
MVA63(pGZ119EH)	MG1655 Δ*arcB* ϕ(*pspA-lac*)(control)	44 ± 4
MVA63(pPMR129)	MG1655 Δ*arcB* ϕ(*pspA-lac*)(*gIV*)	47 ± 1
MVA44(pJW5536)[f]	MG1655 ϕ(*pspA-lac*)(*arcB*)	36 ± 2 (64 ± 2)[g]
+10% EtOH		220 ± 11
MVA44(pJW5536, pBR325)	MG1655 ϕ(*pspA-lac*)(*arcB*, control)	38 ± 3
MVA44(pJW5536, pGJ4)[e]	MG1655 ϕ(*pspA-lac*)(*arcB*, *gIV*)	398 ± 22
MVA63(pJW5536)	MG1655 Δ*arcB* ϕ(*pspA-lac*)(*arcB*)	42 ± 11 (62 ± 5)[g]
+10% EtOH		212 ± 18 (329 ± 6)[g]
MVA63(pJW5536, pBR325)	MG1655 Δ*arcB* ϕ(*pspA-lac*)(*arcB*, control)	53 ± 8 (67 ± 4)[g]
MVA63(pJW5536, pGJ4)	MG1655 Δ*arcB* ϕ(*pspA-lac*)(*arcB*, *gIV*)	380 ± 10 (615 ± 9)[g]

[a] The β-galactosidase activity in LB medium was assayed after growing cells at 37 °C except for cells carrying pJW5536 where β-galactosidase activity was assayed after growing cells at 30 °C. Mean values of three independent assays with S.D. are shown.
[b] Psp induction by 50 °C for 5 min.
[c] Psp induction by 10% ethanol for 30 min.
[d] Psp induction by 80 μM CCCP for 15 min.
[e] Plasmids pPMR129 and pGJ4 carry *gIV* (*gIV* encodes pIV secretin) under control of IPTG-inducible *tac* promoter.
[f] Plasmid pJW5536 carries *6his-arcB-gfp* under control of IPTG-inducible pT5/*lac* promoter.
[g] *6his-arcB-gfp* expression from pJW5536 was induced by 0.1 mM IPTG for 1 h (these results are presented in parentheses).

ArcB partially dependent manner (Table 5). Δ*pspA* Δ*arcB* cells carrying a *pspA-lac* transcriptional fusion was used as a control to demonstrate the ArcB independence of Psp expression when it is not controlled by the negative regulator PspA. Furthermore, a plasmid encoding ArcB complemented the Δ*arcB* mutation and restored Psp induction (results shown for the ethanol treatment and pIV secretin; Table 5). Clearly ArcB is required for relief of the repression imposed by PspA and is part of or impacts upon the signal transduction pathway involved in the Psp response. Results with CCCP suggest that a signal threshold might exist, depending on the stimulus (see also below).

The sensor protein CpxA is part of the two-component CpxA/CpxR signal transduction system. The system senses and responds to aggregated and misfolded proteins in the bacterial envelope (for review, see Ref. 24). In addition, CpxA may activate ArcA, the ArcB/ArcA system response regulator. It has been shown, with one exception, that Psp-inducing stimuli (*e.g.* secretins) do not induce the CpxA/CpxR system in *Y. enterocolitica* (25). Because our results implicated ArcB in the Psp signal transduction pathway, we wanted to learn whether CpxA might be also involved. Hence, we introduced a Δ*cpxA* mutation into a wild type strain carrying a chromosomal *pspA-lac* transcriptional fusion (MVA60), and β-galactosidase assays were carried out either under normal, pIV, extreme temperature, or ethanol treatment-inducing conditions. In Δ*cpxA* the Psp response can be induced normally (data not shown).

In Δ*pspF* (with no Psp expression) or in Δ*pspBC* (with no signal transduction and Psp induction) motility is decreased around 15% on induction by pIV (see above). Because *arcB* mutants (no signal transduction and Psp induction) might show a similar motility decrease upon pIV induction, we performed motility assays using Δ*arcB* cells. Motility decreased 50% in Δ*arcB* under pIV-imposed stress conditions compared with Δ*arcB* under normal conditions, which displayed slightly increased motility (15%) compared with wild type. Clearly ArcB is not solely involved in signaling to Psp but is also involved in responding to the pIV stimulus, either Psp-dependently or Psp-independently, in agreement with (i) microarray data showing the ArcB/ArcA system is partially activated upon PspG and PspA overproduction (see above) and (ii) the proposed function of Psp effectors in conserving the pmf and energy usage.

Changes in the Electron Potential Component of pmf in Cells Responding to pIV Secretin and CCCP Stimuli or Overproduction of Psp Effectors—The membrane potential (Δψ) component of the pmf can be measured in cells using the cationic dye JC-1 (Molecular Probes) (18). JC-1 indicates membrane depolarization by shifting its fluorescence emission from red (~590 nm) to green (~530 nm) after excitation at 485 nm (Fig. 1*A*). Because our transcriptome profiles, motility assays, and ArcB experiments along with previous reports (Ref. 18 and for review, see Ref. 2) suggest that Psp proteins are important in maintaining the pmf across the inner membrane of *E. coli* cells, we have employed fluorescence ratio imaging with JC-1 to measure the Δψ.

We determined that cells lacking *pspF*, *pspA*, *pspD*, *pspG*, *pspBC* (a double mutant), or *pspA pspG* (a double mutant) do not have a Δψ significantly changed relative to wild type cells (data not shown). This establishes that under normal physiological conditions, the lack of Psp proteins does not substantially contribute to maintenance of Δψ. Overexpression of PspA in either wild type also appears to have little effect on the Δψ of

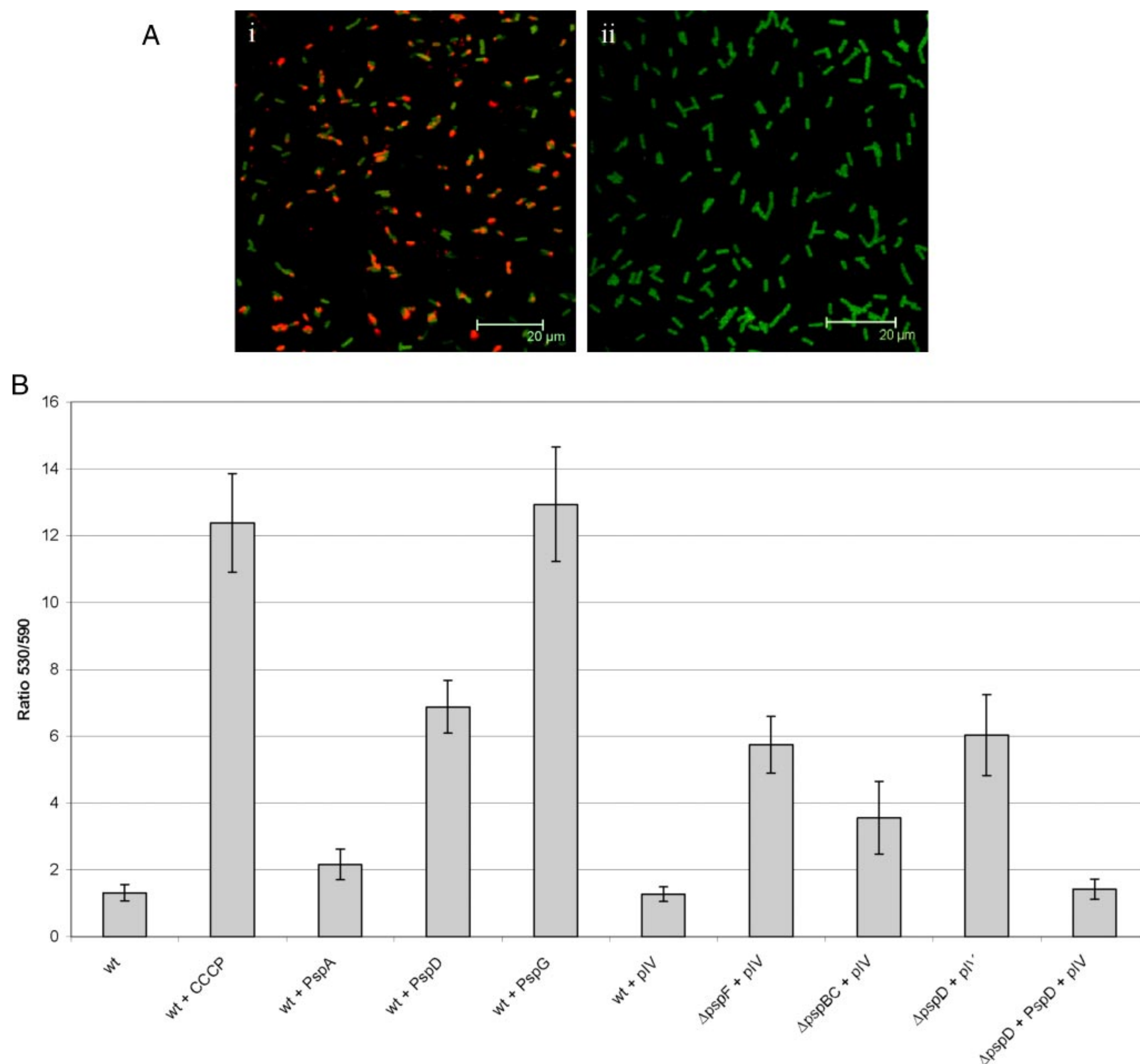

FIGURE 1. **Determination of the electron potential (Δψ).** *A*, *E. coli* cells treated with JC-1 MG1655 wild type (*i*) and MG1655 overexpressing PspG from pLL11 (*ii*) in the presence of 0.4% arabinose for 1 h. *B* and *C*, changes in Δψ are presented as *green*/*red* (530/590 nm) ratio. CCCP was added at a concentration of 80 mM for 15 min, PspA, PspD, and PspG were expressed from pPB10, pLL10, and pLL11, respectively, for 1 h with 0.4% arabinose, and pIV was expressed from pGJ4 or pPMR129 for 1 h with 1 mM IPTG at 37 °C. An increase in the ratio 530/590 indicates a decrease in Δψ. wt, wild type.

the cell (Fig. 1*B*). However, after overexpression of PspD or PspG in wild type cells, the Δψ decreases significantly and, in the case of PspG overexpression, to low levels evident in cells treated with the ionophore CCCP (Fig. 1, *A* and *B*). Overproduction of an inner membrane protein PspD and a putative inner membrane protein PspG *per se* may impair the membrane integrity and cause a decrease in Dy. However, overproduction of other Psp inner membrane proteins PspB and PspC did not show any effect on Δψ.[4]

After pIV overexpression in wild type cells there is no discernable change in Δψ (Fig. 1*B*); however, overexpression of pIV in Δ*pspF* cells resulted in decreased Δψ (Fig. 1*B*). Indeed, Δ*pspF* cells exhibit impaired growth (mutant/wild type colony forming units, 10^{-2}) under stress conditions caused by prolonged overproduction of the pIV secretin compared with wild type (data not shown). Overexpression of pIV in Δ*pspBC* (used so as to not transduce the pIV stress signal) shows a decrease in Δψ compared with wild type (Fig. 1*B*), but this decrease is not as marked as that in Δ*pspF* cells (Fig. 1*B*), and growth of these cells is not impaired by pIV. Δ*pspD* cells overexpressing pIV secretin showed decreased Δψ, suggesting that cells lacking PspD cannot cope with pIV synthesis as successfully as wild type cells (Fig. 1*B*). PspD is not required for Psp induction (14); hence, this result can be attributed solely to the Psp response. This decrease in Δψ can be rescued by introducing PspD expressed from the plasmid pLL10 (Fig. 1*B*). These results show that pIV secretin overexpression decreases pmf in the absence of

[4] G. Jovanovic, L. J. Lloyd, A. J. Mayhew, and M. Buck, unpublished data.

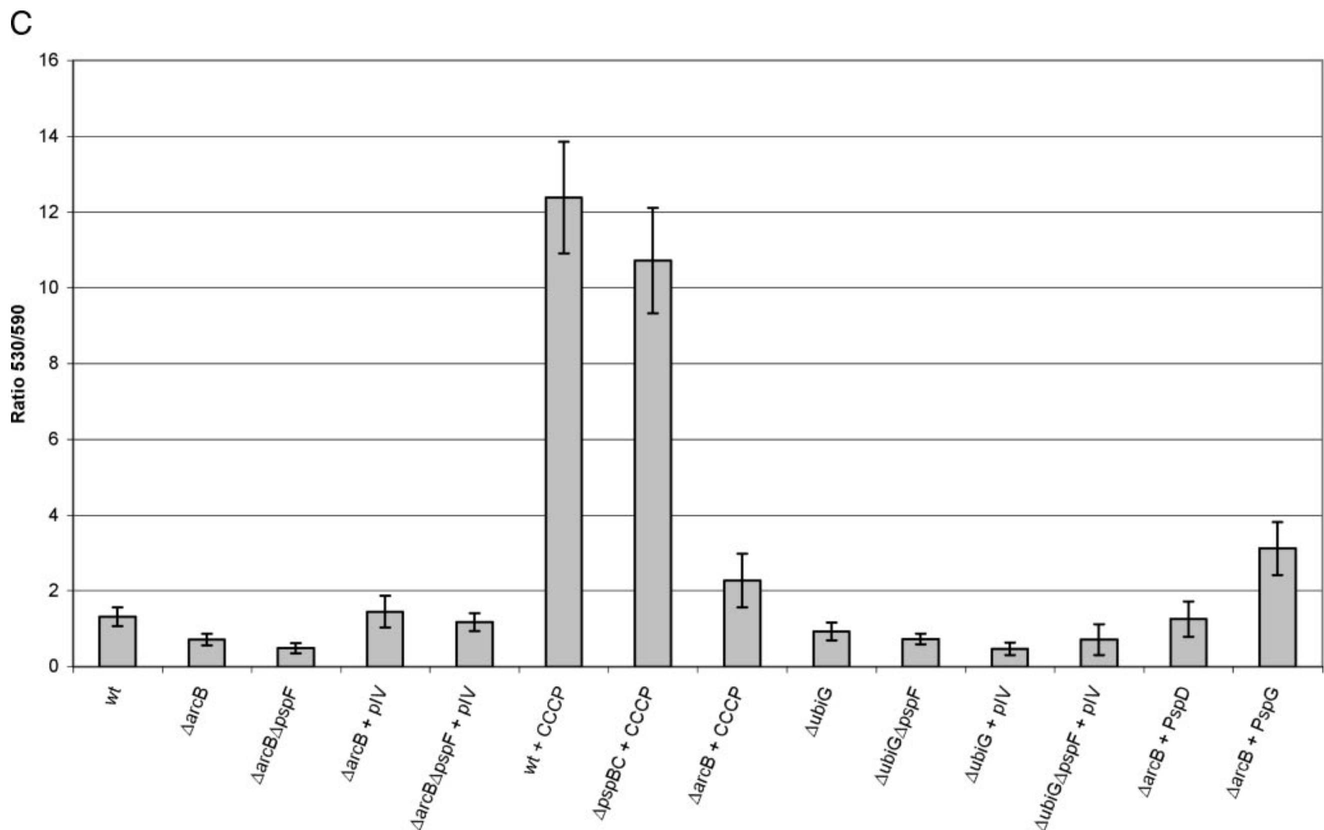

FIGURE 1—*continued*

Psp and that the Psp response is required for the maintenance of $\Delta\psi$ under stress growth conditions, as previously suggested for PspA (for review, see Ref. 2).

ArcB is absolutely required for induction of the Psp response by a variety of inducing stimuli (Table 5). There is a small increase of $\Delta\psi$ in Δ*arcB* cells compared with the wild type (Fig. 1*C*) consistent with lower basal level expression from *pspA* promoter (Table 5). A more pronounced increase of $\Delta\psi$ in Δ*arcB* Δ*pspF* cells (Fig. 1*C*) is consistent with the proposed role for Psp effector proteins under normal growth conditions (Table 1, upper). pIV overproduction in Δ*pspF* cells greatly decreased $\Delta\psi$ (Fig. 1*B*), but Δ*arcB* or Δ*arcB* Δ*pspF* cells overexpressing pIV did not show any change in $\Delta\psi$ compared with wild type cells (Fig. 1*C*). However, as seen for Δ*pspF*-overproducing pIV (Fig. 1*B*), Δ*arcB* (the absence of Psp induction and response) overproducing pIV also shows impaired growth (mutant/wild type colony forming units, 10^{-3}) compared with wild type (data not shown). Production of ArcB from a plasmid in Δ*arcB* cells overproducing pIV restored growth (data not shown). This shows that the decrease in $\Delta\psi$ upon pIV induction is ArcB-dependent and that ArcB has to be present in cells to cope with at least one stress condition that induces the Psp response. Because induction of Psp response by pIV absolutely required ArcB (Table 5), these results suggest that Psp-inducing signal is related to an ArcB-dependent decrease in $\Delta\psi$ (pmf).

Unlike the pIV stress condition, CCCP induction of the Psp response only partially depends on ArcB (Table 5), raising the possibility of the existence of a signal threshold. Therefore, we treated the wild type, Δ*pspBC*, and Δ*arcB* cells with CCCP and looked for changes in $\Delta\psi$. In Δ*arcB* cells $\Delta\psi$ is not decreased to the level evident in wild type or Δ*pspBC* cells under the same conditions of CCCP addition (Fig. 1*C*). Notably, wild type or Δ*pspBC* cells could not maintain the $\Delta\psi$ upon the addition of strong Psp inducer CCCP (Fig. 1*C*), suggesting that CCCP is a more severe stress than is pIV overproduction. These results confirm that Psp induction with a signal that will directly dissipate the pmf (*e.g.* CCCP) only partially depends on ArcB and that such a signal may partially bypass the PspBC-dependent signal transduction pathway.

Psp Response Activates ArcB—Cumulative results indicate that signaling, Psp induction, and the Psp response itself relies on ArcB activity. ArcB sensor activity is inactivated by quinones (22, 23), and *ubi* mutants in turn fully activate the ArcB kinase activity and ArcB/ArcA system (22). To see whether it is possible to induce the Psp response simply by strongly activating ArcB, we assayed Δ*ubiG* mutant cells carrying a *pspA-lac* chromosomal fusion before and after induction. The *ubi* mutation and the presumed consequent strong activation of ArcB/ArcA system does not in itself induce the Psp response (wild type = 80 ± 5 Miller units *versus* Δ*ubiG* = 77 ± 4 Miller units) and greatly diminishes activation of the PspA promoter by pIV secretin (90 ± 2 Miller units compared with 473 ± 19 in WT cells). This is in agreement with observations that anaerobic growth conditions are not sufficient to induce the Psp response.[4] It seems that a low level activation of ArcB might function to amplify, generate, and transduce the signal to Psp. We showed that in the absence of Psp protein expression (Δ*pspF* mutant), cells cannot maintain the $\Delta\psi$ under stress

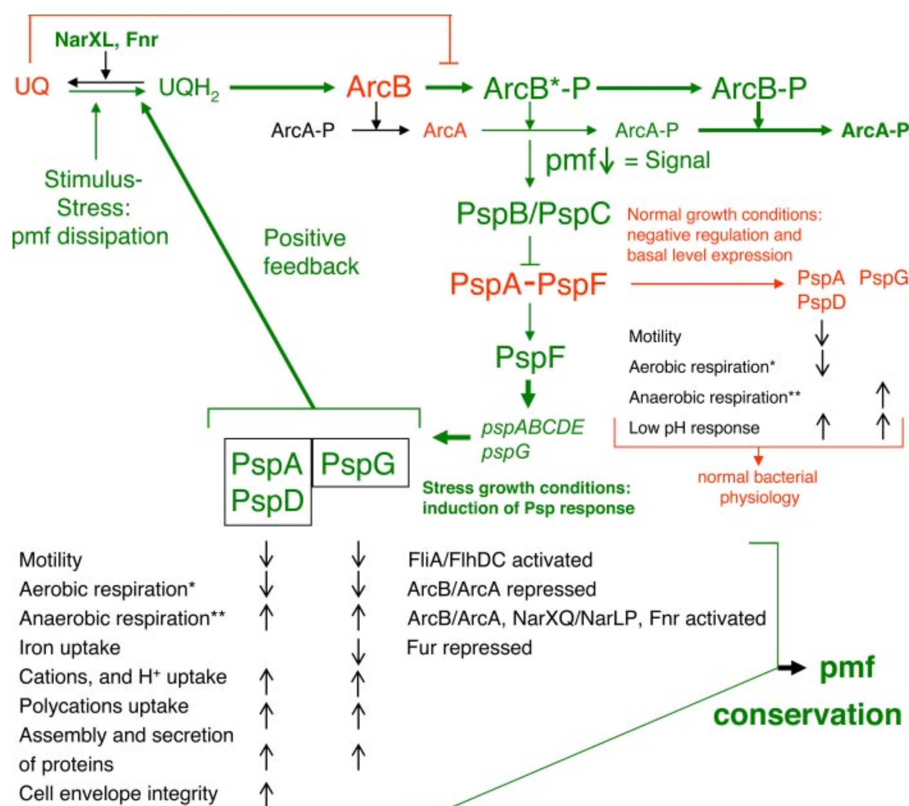

FIGURE 2. **Model for induction and function of the PspF regulon response.** Under normal growth conditions (in *red*) PspA imposes negative regulation on PspF regulon, and basal level expression of Psp effectors affect some cellular processes at a low level, *e.g.* motility, aerobic and anaerobic respiration, and low pH response. Under stress growth conditions (in *green*) (impaired inner membrane (*IM*) integrity and consequent pmf dissipation (decrease in electron potential ($\Delta\psi$) and increase in reducing capacity)), ArcB senses the redox state of the quinones where the ratio of UQ-oxidized/UQH_2-reduced (ubiquinol:ubiquinone pool) will trigger low level activation of ArcB/ArcA (*ArcB**-*P*), generation of the signal (further decrease in pmf due to activation of ArcA, ArcA-P), PspB/PspC-dependent release of PspF activator from the PspA-PspF complex, and induction of the PspF regulon (*pspABCDE* operon and *pspG*). Consequently Psp effectors PspA, PspD, and PspG in increased concentrations will act through positive feedback to further activate ArcB (*ArcB-P*), ArcA (*ArcA-P*), NarXQ/NarLP, Fnr, etc. regulated genes and to control *e.g.* tricarboxylic acid (*) or glycerol shift and formate-nitrate respiratory chain (**) reactions. This will result in pmf conservation under stress growth conditions. See "Discussion" for details. Up (↑) and down (↓) regulation of genes is indicated.

growth conditions such as pIV secretin overproduction (Fig. 1*B*). If ArcB/ArcA activation and re-routing the cell metabolism to formate/nitrate anaerobic respiration and fermentation is one of the major outcomes of the Psp response to enable management of stress, then a *ubi* mutation in a Δ*pspF* strain should be able to cope with stress conditions such as pIV overproduction. Therefore, we measured the $\Delta\psi$ of D*ubiG* Δ*pspF* strain upon induction with pIV. This strain can indeed maintain normal $\Delta\psi$ under pIV-inducing stress conditions (Fig. 1*C*). The $\Delta\psi$ in Δ*ubiG*, Δ*ubiG* Δ*pspF*, and Δ*ubiG* + pIV cells did show a moderate increase compared with wild type cells (Fig. 1*C*). Furthermore, to determine whether PspD or PspG overproduction under non-stress conditions decreases the $\Delta\psi$ in an ArcB-dependent manner, we overexpressed either PspD or PspG in Δ*arcB* cells. The $\Delta\psi$ in Δ*arcB* cells overexpressing PspD or PspG is decreased compared with Δ*arcB* containing the control vector (Fig. 1*C*) but not decreased to the same extent as in wild type cells overexpressing PspD or PspG (Fig. 1*B*). At least partially, ArcB is required for the PspD or PspG overproduction-dependent decrease of $\Delta\psi$, in agreement with transcriptional profile analyses where, besides the ArcB/ArcA system, other gene clusters regulated by *e.g.* Fnr and Fur are implicated in the Psp response.

DISCUSSION

ArcB Is Required for Generating and Transducing the Signal for Psp Induction—Important issues regarding the mechanism by which the Psp-inducing stimulus is converted to signal, the mechanism of signal transduction, the biological function of the Psp response, and adaptation to stress conditions have not been elucidated to date. Several lines of evidence now show for the first time the nature of the pIV secretin Psp-inducing stimulus and reveal that ArcB is required (with the exception of CCCP), most likely as a redox sensor, to transduce the inducing signal to Psp (Fig. 2). The role of PspB and PspC might be to sense the outcome of the signal produced by ArcB activity and release the negative regulation of the PspF regulon (Fig. 2). The results showed that pIV overproduction in the absence of an active Psp response decreases the electron potential and cell motility to some extent, consistent with the fact that the Psp response will confront this stimulus (i) by maintaining the $\Delta\psi$ and (ii) by further down-regulating pmf-consuming processes such as motility. We showed that in an *arcB* mutant, induction of the Psp response by PspBC-dependent (pIV), partially-dependent (ethanol treatment), or independent (extreme heat shock) stimuli failed. However, induction of Psp by a strong stimulus such as the ionophore CCCP (for review, see Ref. 1) that directly uncouples pmf from ATP synthesis only partially depended on ArcB, providing evidence for a stimulus threshold. Wild type cells cannot cope with the CCCP stimulus as successfully as with pIV and can probably only partially mitigate the effects of CCCP. We showed that Δ*arcB* cells overproducing pIV do not have decreased $\Delta\psi$ but have severely impaired growth. Growth in these cells is even more impaired than in a Δ*pspF* mutant alone under the same conditions. Reconstitution of the growth and decrease in $\Delta\psi$ is ArcB-dependent. However, Δ*arcB* cells treated with CCCP had their $\Delta\psi$ decreased to some extent, suggesting that indeed the CCCP stimulus can bypass an ArcB requirement and decrease $\Delta\psi$ only in a partially ArcB-dependent manner. Importantly, bypassing ArcB strongly suggests that depending on the stimulus threshold, ArcB activation generates the signal, evident as a more pronounced decrease of $\Delta\psi$ (Fig. 2) and in addition can partially substitute for the loss of Psp

response under stress conditions. Oxidized forms of quinones are ArcB-specific signals that silence ArcB kinase activity (Fig. 2), and we showed that in a Δ*ubiG* mutant the Psp response cannot be induced by pIV and that loss of $\Delta\psi$ in Δ*pspF* cells overproducing pIV can be recovered in a double Δ*ubiG* Δ*pspF* mutant. Subsequently, in a Δ*arcB* mutant, motility is increased, and pIV secretin stress does not reduce the $\Delta\psi$ but causes impaired growth.

Psp Response Maintains pmf by Switching Cell Metabolism to Anaerobic Respiration Mode—One major aspect of the PspF regulon response is to cause a fine adjustment of respiratory enzyme gene expression and reduce the expression of high energy/pmf-consuming processes such as motility by favoring anaerobic over aerobic respiration. This allows the cell to manage the Psp-inducing conditions that presumably impair the inner membrane integrity and dissipate the pmf (Fig. 2). Under pIV stress conditions the Psp response indeed maintains $\Delta\psi$ and most likely the pH gradient, which together constitute the pmf. In the absence of signal transduction (Δ*pspBC*, Δ*arcB*) and/or the activator, PspF, cells are unable to cope with pIV stress, failing to maintain the $\Delta\psi$ and normal growth. The transcriptome results revealed that low level expression of the ArcB/ArcA system controlled by PspA and PspD under normal growth conditions is elevated by overexpressing PspA or PspG effectors (Fig. 2). Hence, PspA, PspD, and PspG are the effectors necessary for a proper Psp response arguing that PspA and PspD act synergistically and somewhat distinctly from PspG (Fig. 2). However, PspA and PspG functions overlap in many instances, including activation of the Arc modulon (Fig. 2). Notably, in the absence of stress, overexpression of PspD or PspG (but not PspA) decreases the $\Delta\psi$ in an ArcB-dependent fashion, which is consistent with a more pronounced PspG/Arc modulon relationship in cells overexpressing PspG than PspA (Fig. 2). An important outcome of these experiments is that an effector function can be attributed to PspD, a protein that has no previously ascribed function. Also, it is clear that ArcB/ArcA-regulated genes are significantly active under the aerobic growth conditions used in our work. This is consistent with results recently presented by Perrenoud and Sauer (26), suggesting that metabolic processes including the tricarboxylic acid cycle are under considerable negative regulation by the ArcB/ArcA system during aerobic growth. Importantly the function of PspA and PspG effectors in decreasing motility is consistent with transcriptome profiles and the motility phenotype obtained after pIV induction of the Psp system. Clearly the overexpression of PspA and PspG effectors resembles the actual induction of the Psp response under stress conditions. It appears that induction simply relieves the PspA-imposed negative regulation on PspF and consequently increases the level of Psp effector proteins, otherwise active under normal growth conditions but only expressed at a low basal level. Therefore, we anticipate that the redox state of ArcB and the actual level of ArcB/ArcA system activity (22, 23) will be crucial for both generating and transducing the signal necessary for PspF regulon induction and the actual Psp response (Fig. 2). Under normal growth conditions it is likely that Psp proteins impose a fine control on the ArcB/ArcA modulon activity. This is significant to understanding that the stress response pathways such as Psp are important to normal bacterial physiology, not only to radical stresses (Fig. 2). Induction of the PspF regulon would result in positive feedback control on ArcB/ArcA activity (Fig. 2). Psp signaling and response to some extent may resemble the order of events seen in stationary growth phase that causes low level activation of ArcA to prevent degradation of the stationary phase genes master regulator, σ^S factor, which in turn through positive feedback stimulates ArcA activity (27).

In addition to the ArcB/ArcA modulon, the PspG effector protein causes an up-regulation of Fnr- and NarL/NarP-regulated genes involved in anaerobic respiration (Fig. 2). Respiratory enzyme synthesis in enterobacteria is controlled in response to electron acceptor availability. The iron-sulfur protein Fnr and the sensor-regulator proteins ArcB-ArcA control respiratory gene transcription in response to oxygen and quinone pool redox status, respectively. Notably, the capacity of *E. coli* to adapt its catabolism to prevailing redox conditions resides mainly in pyruvate-formate lyase, an enzyme more active during microaerobiosis than anaerobiosis (28). Apparently, pyruvate formate lyase expression is mainly positively controlled by the Arc system (*pflBfocA* and *yfiD*), and from our results it appears that *focA* and *yfiD* are up-regulated in PspG-overexpressing cells. Also, under aerobic growth conditions, Fnr activity can be triggered by reduced glutathione (29). Therefore, our results suggest that under Psp-inducing stress conditions, increased reducing environment in the cytosol (increased Arc system activity) may trigger the signal generation for the PspF regulon response, and one consequence of the response may be a further increase in the reducing capacity of the cell. Such changes can account for the activity of Arc and Fnr (30) modulons, both depending on reducing conditions, and introduction of the anaerobic respiration mode under Psp-inducing stress conditions. The sensor-regulator proteins NarX-NarL and NarQ-NarP, in conjunction with Fnr control anaerobic respiratory gene expression in response to nitrate and nitrite and together with ArcA, activate the energetically most efficient anaerobic respiratory chain, formate-nitrate oxidoreductase (for review, see Ref. 31). Interestingly, NarQ sensor, like ArcB, also responds to aeration (32). According to our microarray results, the formate-nitrate anaerobic respiratory chain is up-regulated in PspA- and PspG-overexpressing cells. Notably, as is evident for many σ^{54}-dependent genes in *E. coli*, Psp may have a function that is related to nitrogen metabolism. The pmf-dissipating stress conditions may favor the general anaerobic respiration mode over aerobic and activation of Arc, Fnr, and Nar systems through the Psp response can be used to co-ordinate a conservation of the cells energy and pmf (Fig. 2).

Psp Response Controls Iron Metabolism—In cells overexpressing PspG, genes involved in iron uptake are strongly down-regulated (Table 1, lower), suggesting that the intracellular concentration of iron is modulated in cells during the Psp response (Fig. 2). Iron can be used for constitution of new and reconstitution of old Fe-S clusters that can be used in respiration. Fe-S cluster synthesis may be increased in a Psp-dependent manner since both known rhodaneses (PspE and GlpE) are up-regulated in PspA- and PspG-overexpressing cells, respectively, supporting a proposed role for PspE (for review, see Ref. 2).

Psp Response Down-regulates Motility—The high pmf-consuming process, motility, is down-regulated under pIV stress-induced Psp response in a PspA- and PspG-dependent manner. The results of microarray analyses and motility assays established a clear link between expression and function of PspA and PspG effectors and decrease in motility (Fig. 2), most likely achieved at a post-transcriptional level. Motility phenotype, function, and the level of expression of the PspA and PspG correlate. Decreases in motility are indeed a consequence of the Psp response, and under physiological conditions, when the Psp responds to pIV secretin overproduction, the level of changes in motility is different compared with PspA and/or PspG overexpression. This is consistent with measurements of $\Delta\psi$ of *psp* mutants and previous microarray analyses (6) where significant transcriptome changes in wild type cells overexpressing pIV are not observed. Presumably, the expression of motility genes, motility phenotype, and function of the PspA and PspG proteins are correlated *in vivo* and fine-tuned to maintain and conserve pmf. Motility appears to be a hypersensitive sensor of intracellular energy/pmf status and can be used for analysis of Psp effector function.

Psp Response Directly Confronts the Inducing Stimuli—The cellular response after PspA and PspG overexpression mainly overlaps with the cellular response to high extracellular pH (33), *e.g.* the ArcB/ArcA-dependent genes for anaerobic respiration are up-regulated, spermidine/putreceine and γ-aminobutyric acid, and cation import are up-regulated, whereas the genes for motility and formate dehydrogenase are highly down-regulated. Also, at high pH the uptake of protons is highly up-regulated to compensate for the inverted ΔpH and loss of $\Delta\psi$ (33). Our microarray results and $\Delta\psi$ measurements show that in cells overexpressing PspA or PspG and PspD or PspG, the uptake of protons is up-regulated, and $\Delta\psi$ is decreased, respectively. Hence, in addition to $\Delta\psi$, the Psp response may sustain the pH gradient to directly maintain the pmf and confront the pmf-dissipating conditions. Microarray data showed that overexpression of PspA or PspG might specifically confront the majority of Psp-inducing stimuli as well. These include stimuli (for review, see Ref. 1 and 2) such as impaired envelope integrity, block of phospholipid or lipoprotein biosynthesis, protein translocation defects, hyperosmotic shock, prolonged stationary growth phase, and the addition of metals to *rpoE* mutants (see Table 2, A and B).

pspD, *pspE*, and *pspG* are not conserved in all bacteria that contain *pspF pspABC* (for review, see Ref. 2). This suggests that PspF, PspA, PspB, and PspC are indispensable for Psp regulation and function, whereas different species may have evolved a slightly different Psp response compared with *E. coli*. However, *pspG* is conserved in all enterobacteria containing *pspF pspABC*, whereas *pspD* and *pspE* are not. Therefore, it is likely that PspG plays an important role in the Psp response in these species. This also might explain why PspA and PspD appear to have overlapping functions in *E. coli*.

Is Psp Part of a General Stress Response Network?—Lack of the heat shock protein response master regulator, σ^{32}, increases and prolongs the Psp response (for review, see Ref. 1), suggesting either a negative effect of heat shock protein response upon Psp or help in maintaining a rapid Psp response. We showed that either PspA or PspG overexpression up-regulates the σ^{32}-controlled genes encoding molecular chaperons (*dnaKJ*, *grpE*, *clpB*) and protease (*lon*) involved in folding of proteins under heat shock, suggesting that the heat shock protein response supports the Psp response under Psp inducing stress conditions. Most Psp-inducing stimuli do not activate Cpx and RpoE responses (25), and our results confirmed that CpxA is not required for Psp induction. Moreover, upon overexpression of PspA or PspG, NlpE implicated in envelope stress and induction of the Cpx response is down-regulated, whereas the Lpp is up-regulated. Therefore, although Cpx may sense the envelope damage through NlpE (for review, see Ref. 24), Psp might do this through Lpp. However, Psp induction in a *rpoE* mutant strain by either addition of metals (18) or in stationary phase growth (34) as well as concerted induction of Cpx, RpoE, and Psp systems after severely impaired envelope biosynthesis (25) suggests a connection of the Psp, Cpx, and RpoE responses and the management of severe extracytoplasmic stress. The PspF regulon may play a role in one large concerted stress response network, including the Cpx, RpoE, and heat shock protein responses, in which the unifying element is coordination of protein turnover and energy/pmf conservation.

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