Involvement of σ^S accumulation in repression of the *flhDC* operon in acidic phospholipid-deficient mutants of *Escherichia coli*

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Escherichia coli *pgsA* mutations, which cause acidic phospholipid deficiency, repress transcription of the flagellar master operon *flhDC*, and thus impair flagellar formation and motility. The molecular mechanism of the strong repression of *flhDC* transcription in the mutant cells, however, has not yet been clarified. In order to shed light on this mechanism we isolated genes which, when supplied in multicopy, suppress the repression of *flhD*, and found that three genes, *gadW*, *metE* and *yeaB*, were capable of suppression. Taking into account a previous report that *gadW* represses σ^S production, the level of σ^S in the *pgsA3* mutant was examined. We found that *pgsA3* cells had a high level of σ^S and that introduction of a *gadW* plasmid into *pgsA3* cells did reduce the σ^S level. The *pgsA3* cells exhibited a sharp increase in σ^S levels that can only be partially attributed to the slight increase in *rpoS* transcription; the largest part of the effect is due to a post-transcriptional accumulation of σ^S . *GadW* in multicopy exerts its effect by post-transcriptionally downregulating σ^S . *YeaB* and *MetE* in multicopy also exert their effect via σ^S . Disruption of *rpoS* caused an increase of the *flhD* mRNA level, and induction from P_{trc} -*rpoS* repressed the *flhD* mRNA level. The strong repression of *flhD* transcription in *pgsA3* mutant cells is thus suggested to be caused by the accumulated σ^S .

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INTRODUCTION

Escherichia coli membranes contain only three major phospholipids. Molecular genetic approaches to correlate mutationally modified lipid compositions with altered membrane functions have been successfully used to understand the biological roles of the individual phospholipids (for reviews, see Shibuya, 1992; Cronan, 2003; Dowhan *et al.*, 2004). Lack of phosphatidylethanolamine, a zwitterionic phospholipid, which accounts for about 70 % of total phospholipids, is lethal, but the lethality is suppressed by supplementation with high concentrations of divalent cations (DeChavigny *et al.*, 1991; Saha *et al.*, 1996). The lack of phosphatidylethanolamine causes a defect in motility (Shi *et al.*, 1993), an activation of the Cpx two-component signal transduction pathway (Mileykovskaya & Dowhan, 1997), a defect in division site selection, probably due to the concomitant increase in cardiolipin content that affects the localization of MinD (Mileykovskaya & Dowhan, 2005), and a defect in the native folding of lactose permease LacY, which causes the loss of active transport of the substrate (Dowhan *et al.*, 2004).

Lack of, or deficiency in, the major acidic phospholipid phosphatidylglycerol, brought about by the mutations

pgsA30::kan or *pgsA3*, respectively, is lethal, due to the absence of, or the missense defect in, phosphatidylglycerophosphate synthase, which catalyses the committed step in the biosynthetic pathway for this acidic phospholipid (Shibuya, 1992; Usui *et al.*, 1994). However, simultaneous lack of the major outer membrane lipoprotein (Braun's lipoprotein), the most abundant protein, encoded by *lpp*, suppresses the lethality (Shibuya, 1992; Kikuchi *et al.*, 2000; Matsumoto, 2001). Since phosphatidylglycerol is required for modification of the prolipoprotein, the modification cannot be carried out in the mutant cells, hampering processing for maturation, and the thus unmodified prolipoproteins accumulating in the inner membrane cause cell lysis after fusing the inner membrane to murein (Suzuki *et al.*, 2002). Hence, if the major outer membrane lipoprotein is lacking, both *pgsA* mutants are viable by substituting phosphatidylglycerol in its vital roles with other acidic biosynthetic intermediates, such as phosphatidic acid, CDP-diacylglycerol and *N*-acylphosphatidylethanolamine, which have been shown to accumulate in the mutant cells (Matsumoto, 2001; Mileykovskaya *et al.*, 2009).

In *pgsA3* mutant cells, the deficiency in phosphatidylglycerol causes retarded translocation of newly synthesized

outer membrane proteins across the inner membrane (de Vrije *et al.*, 1988), since activation of the amphitropic protein SecA in the translocation machinery is hampered (Dowhan *et al.*, 2004). The *pgsA3* mutation also causes a significant decrease in the level of OmpF protein in the outer membrane and a serious impairment of flagellar formation that makes the cells non-motile (Nishino *et al.*, 1993). The first phenomenon is caused by the elevation of the *micF* RNA level, but the level of transcriptional expression of *ompF* is not reduced (Inoue *et al.*, 1997). The results suggest that EnvZ-OmpR is not activated by the *pgsA3* mutation. In due time, lack of phosphatidylglycerol has been found to activate another phosphorelay regulatory system, Rcs, and cause thermosensitive growth (Shiba *et al.*, 2004; Nagahama *et al.*, 2006). Examination of the second phenomenon, the impairment of flagellar formation, has revealed that it is due to the repression of transcription of the flagellar master operon *flhDC* in the *pgsA3* cells (Kitamura *et al.*, 1994). However, the molecular mechanism of *flhDC* repression that responds to the acidic phospholipid deficiency has so far not been clarified.

In the present work, in order to elucidate the factors involved in the repression, we looked for genes that, if present in multicopy, would counteract the repression caused by the *pgsA3* mutation of *flhDC* expression, and found that *gadW*, *metE* and *yeaB* were such multicopy suppressors of the repression of *flhDC* expression. The most effective among these was *gadW*, the gene product of which is involved in the negative regulation of the production of σ^S (Ma *et al.*, 2003), the general stress sigma factor that plays a central role in the stress response under many stress conditions and in cells entering the stationary phase (Hengge-Aronis, 2002). Taking into account the action of *gadW*, the content of σ^S in *pgsA3* cells was examined and they turned out to have a higher level of σ^S than wild-type cells. Introduction of a *gadW* plasmid into *pgsA3* cells reduced the σ^S level. These results indicate that the accumulation of σ^S is one of the causes of the repression of transcription of *flhDC*. We further describe the observation that the transcription of *rpoS* is increased in *pgsA3* mutant cells and that the mutant cells are in a condition of envelope disorder that facilitates post-transcriptional σ^S accumulation.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
MG1655	<i>rph-1</i>	Laboratory stock
ST001	$\Delta(lac-pro) ara thi lpp-2 uvrC279::Tn10$	Inoue <i>et al.</i> (1997)
ST002	ST001 <i>pgsA3</i>	Inoue <i>et al.</i> (1997)
FS401	ST001 λ lysogen [<i>flhDC</i> upstream (−305 to +290)-operon fusion]	Inoue <i>et al.</i> (1997)
FS501	FS401 <i>pgsA3</i>	Inoue <i>et al.</i> (1997)
JM109	<i>recA1</i> $\Delta(lac-proAB)$ <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>hsdR17</i> <i>supE44</i> <i>relA1</i> <i>F'</i> [<i>traD36</i> <i>proAB</i> ⁺ <i>lacF</i> ⁺ <i>lacZ</i> Δ M15]	Laboratory stock
JWK3483	BW25113 Δ <i>gadW</i> :: <i>kan</i>	KO Collection
JWK5437	BW25113 Δ <i>rpoS</i> :: <i>kan</i>	KO Collection
JU01	ST001 λ lysogen [<i>flhDC</i> upstream (−88 to +26)-operon fusion]	This work
JU02	JU01 <i>pgsA3</i>	This work
JU03	JU01 Δ <i>rpoS</i> ::FRT	This work
JU04	JU02 Δ <i>rpoS</i> ::FRT	This work
JU05	JU01 <i>rcsC</i> :: <i>cat</i>	This work
JU06	JU02 <i>rcsC</i> :: <i>cat</i>	This work
JU07	ST001 Δ <i>rpoS</i> ::FRT	This work
JU08	ST002 Δ <i>rpoS</i> ::FRT	This work
JU09	ST001 Δ <i>rpoS</i> ::FRT:: <i>lacZ</i>	This work
JU10	ST002 Δ <i>rpoS</i> ::FRT:: <i>lacZ</i>	This work
JU75	JU01 Δ <i>gadW</i> :: <i>kan</i>	This work
Plasmids		
pWKS30	pSC101ori Ap ^R	Wang and Kushner (1991)
pMetE	pWKS30 <i>metE</i>	This work
pGadW	pWKS30 <i>gadW</i>	This work
pYeaB	pWKS30 <i>yeaB</i>	This work
pDSW204	pBR322 <i>lacI</i> ^q <i>P</i> <i>trc</i> Ap ^R	Weiss <i>et al.</i> (1999)
pDSW204-rpoS	pDSW204 <i>rpoS</i>	This work
pCE37	R6Kori <i>lacZ</i> <i>lacY</i> Km ^R	Ellermeier <i>et al.</i> (2002)
pKG137	Improved version of pCE37	M. Garsha
pCP20	λ P _R -FLP d857 Rep ^{ts} Ap ^R Cm ^R	Datsenko and Wanner (2000)

METHODS

Bacterial strains. The *E. coli* derivatives and plasmids used in this study are listed in Table 1. New strains were constructed by P1 phage transduction and the methods described below, and their genotypes were verified by determining drug resistance, PCR amplification, nucleotide sequencing, and determination of phospholipid composition, where applicable.

Strain JU01 was lysogenic for λ prophage and carries an operon fusion of *lacZ* with an *flhD* upstream sequence from nucleotide -88 to +26, taking the putative transcriptional start site of Shin & Park (1995) as +1. This single-copy chromosomal fusion was constructed using a method described elsewhere (Simons *et al.*, 1987). The fusion strain showed a comparable activity and response to the *pgsA3* mutation with a fusion strain FS401, which contains a larger upstream sequence of nucleotides -305 to +290 (K. Busujima and others, unpublished data). JU02 is a derivative of *pgsA3* harbouring the same fusion prophage as JU01.

The $\Delta rpoS$ strains (JU03, JU04, JU07 and JU08) were constructed by P1 transduction of the $\Delta rpoS::kan$ allele from JWK5437, followed by removal of the *kan* cassette using pCP20, as described by Datsenko & Wanner (2000). Chromosomal *rpoS-lacZ* transcriptional fusions (JU09 and JU10) were constructed by FLP-mediated site-specific recombination, as described by Ellermeier *et al.* (2002).

Cloning of *rpoS* into vector pDSW204 was conducted as follows. The fragment containing *rpoS* was obtained by PCR amplification from MG1655 chromosomal DNA using the sense primer prpoScloingup starting at 209 nt upstream from the initiation codon and the antisense primer prpoScloingdown starting at 9 nt downstream from the stop codon (Table 2). The PCR fragment obtained was digested with *EcoRI* and *HindIII* and then inserted into the *EcoRI-HindIII* site of the vector. The plasmid thus constructed was designated pDSW204-rpoS.

Growth conditions. Luria-Bertani (LB) medium, containing 1 % Tryptone (Difco), 0.5 % yeast extract (Difco) and 1 % NaCl (Miller,

1972), was supplemented with 0.1 M sodium phosphate buffer (pH 7.2) or with 20 mM Tris/HCl (pH 7.2). In some cases, LB media were adjusted to pH 7.2 with NaOH. For solid media, 1.5 % agar (Difco) was included and 0.6 % agar for top agar. When required, ampicillin (Sigma), kanamycin (Sigma) and spectinomycin (Sigma) were added to obtain final concentrations of 50, 25 and 50 mg l⁻¹, respectively. Cells were grown at 37 °C and the growth was monitored using a Spectomonitor BACT-2000 (Nissho electronics).

Isolation of the multicopy suppressor. The fragments (2–9 kb) of chromosomal DNA of *E. coli* JU02 *pgsA3* obtained after partial digestion with *Sau3AI* were inserted into the *BamHI* site of the pWKS30 (pSC101 *ori*) plasmid and then used to transform JM109. The plasmid DNAs from a library of about 6000 transformants were extracted. The DNAs prepared were used to transform the *pgsA3* strain JU02, which harbours a *flhD-lacZ* transcriptional fusion, and transformants were plated on MacConkey lactose plates containing 50 µg ampicillin ml⁻¹ and incubated at 37 °C. Nearly 6000 colonies were screened and three red (positive) clones were isolated. The plasmid DNA of the clones was purified, and insert junctions were sequenced using a pair of primers for the vector (see Table 2).

Motility assay. From overnight cultures, 5 µl aliquots with approximately equal numbers of bacterial cells were inoculated on the surface of tryptone motility plates [tryptone broth supplemented with 0.25 % (w/v) agar] and incubated at 37 °C for 6 h. The motility plates were handled as described by Wolfe & Berg (1989). Images were taken with a Sharp JX-230 scanner and the diameters of swarms were measured.

SDS-PAGE and Western blot analysis. Samples (1.5 ml) were prepared from exponentially growing cells (OD₅₄₀ 0.35). Whole cells were pelleted and immediately resuspended to equal OD₅₄₀ values in SDS loading buffer (Silhavy *et al.*, 1984). After boiling for 5 min, equal volumes (25 or 5 µg protein) were loaded onto SDS-12 % polyacrylamide gels. After electrophoresis, proteins were transferred onto nitrocellulose membranes and probed with a 1 : 5000 dilution of anti- σ^S antibody (Tanaka *et al.*, 1993). As the secondary antibody,

Table 2. Primers used in this study

Primer	Sequence (5'→3')	Source
pflhDup	CAGCGTTTGATTGTTTCAGGA	This work
pflhDdown	GATGCCGGTATGAATTTGCT	This work
pgapAup	GTGATCCGGCTAACCTGAAA	This work
pgapAdown	GTCCTGGCCAGCATATTTGT	This work
prpoSup	TGGACATCCTGGCCGATGAAAA	This work
prpoSdown	TGCCGCTTCGTACCCCAGCAAA	This work
pkatEup	CAATTCCACAAGGGCAAAGT	This work
pkatEdown	TTTACCTGCCAGTGGTTTCC	This work
pecnBup	GGTGAAGAAGACAATTGC	This work
pecnBdown	CCAGAAATCGCGTTACCGCC	This work
prpoScloingup	GTCGAATTCGGAACAACAAGAAGTTAAGG	This work
prpoScloingdown	GACAAGCTTTTACTCGCGGAACAGCGCTTCG	This work
pmetEup	AGCTAGAAGCTTCTCCCGGGGATGAATAAACT	This work
pmetEdown	AGCTACGGATCCTGCTAATGCGAAAGCAGAGA	This work
pgadWup	AGCTAGAAGCTTCAAAATCTGGGCAGTTTTTGC	This work
pgadWdown	AGCTGGATCCAAGAGAACTAGCCGGTAGC	This work
pyeaBup	AGCTAGAAGCTTGCTGACTGCCATTAACGG	This work
pyeaBdown	AGCTGGATCCGCGATGATTGTCTCAAGTAGTG	This work
pWKS30cloning1	CTCACTAAAGGGAACAAAAG	This work
pWKS30cloning2	TAATACGACTCACTATAGGGC	This work

peroxidase-conjugated affinity-pure goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch) was used at a dilution of 1:2000. The bands were detected using the ECL antibody detection kit (GE Healthcare Bioscience) and X-ray film (Fuji Medical X-ray film RX).

β -Galactosidase assay. The activity of β -galactosidase was assayed using ONPG as substrate and was recorded as $\mu\text{mol } o\text{-nitrophenol produced min}^{-1} (\text{mg cellular protein})^{-1}$ (Miller, 1972).

Real-time PCR. Cultures were grown as described above, and aliquots were removed from exponential- and stationary-phase cultures (OD_{540} 0.35 and 0.9, respectively). RNA was extracted using an RNeasy Protect Bacteria Mini kit (Qiagen) and an RNase-Free DNase set (Qiagen) according to the manufacturer's recommendations. The DNase-treated RNA was reverse-transcribed using the SuperScript VILO cDNA Synthesis kit (Invitrogen) according to the manufacturer's recommendations. Real-time PCR with SYBR Green I was performed using SYBR Premix EX Taq (Perfect Real-Time) (Takara). Real-time PCR was carried out according to the manufacturer's instructions, using the pairs of primers for the *gapA*, *flhD*, *rpoS*, *katE* and *ecnB* genes listed in Table 2. The 25 μl reaction mix contained 1 $\times$ SYBR Premix EX Taq (Perfect Real-Time), 0.2 μM of each primer and 1 μl template. The following temperature profile was used for amplification: denaturation for one cycle at 95 $^{\circ}\text{C}$ for 10 s, and 30 cycles at 95 $^{\circ}\text{C}$ for 5 s, 60 $^{\circ}\text{C}$ for 20 s, 72 $^{\circ}\text{C}$ for 30 s with fluorescence acquisition at 63 $^{\circ}\text{C}$ for 1 s. PCR cycling was followed by melting curve analysis at 72–95 $^{\circ}\text{C}$ with stepwise fluorescence acquisition. Each real-time PCR was performed in triplicate with the *gapA* gene as the internal standard (Nagahama *et al.*, 2007).

RESULTS

GadW in multicopy suppresses *flhD* repression in the *pgsA3* mutant cells independently of the Rcs system

In order to elucidate the mechanism of repression of *flhD* by acidic phospholipid deficiency in *pgsA3* mutant cells, we set out to obtain genes that suppress the repression when they are supplied in a high number of copies. Cells of strain

JU02 [a *pgsA3* mutant harbouring λ lysogen (*flhD-lacZ*)] were transformed with an *E. coli* chromosomal DNA library constructed with the pWKS30 vector, a pSC101 *ori* plasmid which has a partially *Sau3AI*-digested fragment (2–9 kb) of *pgsA3* chromosomal DNA inserted into its *Bam*HI site. Transformed ampicillin-resistant cells, a total of about 6000 colonies, were screened for red colonies on MacConkey plates, where red indicates expression of the *flhD-lacZ* fusion. Three plasmid clones were isolated, and sequencing of the inserts revealed that three genes, *gadW*, *metE* and *yeaB*, functioned as suppressors when present in multicopy. The *flhD-lacZ* activity of *pgsA3* cells, 2.5 % of that of JU01 (*pgsA*⁺) cells, was restored with multicopy plasmids bearing *gadW*, *metE* or *yeaB* to 48, 39 or 43 %, respectively, of the wild-type level (the value of 87 units for *pgsA*⁺/pWKS30 was taken as 100 %) (Fig. 1). With improved *flhD* activity, swarming of the cells that had the plasmid bearing each gene was also markedly recovered; the relative swarm distance was 30–60 % of that of JU01 (*pgsA*⁺) cells.

Among the three genes (*gadW*, *metE* and *yeaB*) identified, the product of *gadW* has been reported to be an AraC-like transcriptional regulator that negatively regulates σ^S production in the mechanism involved in acid resistance (Ma *et al.*, 2003), although the level at which GadW affects σ^S production has not been exactly determined. Thus, we first focused our study on *gadW*. We noted that addition to the culture of glutamate or γ -aminobutyric acid, the substrate and product, respectively, of glutamate decarboxylase, the gene product of *gadA*, which is repressed by *gadW*, had no effect on *flhD-lacZ* activity (data not shown). Since the RcsCDB phosphorelay system negatively regulates the *flhDC* operon (Francez-Charlot *et al.*, 2003), a possible effect of an *rsc* mutation on the suppression with *gadW* was also investigated. The repressed *flhD-lacZ* activity in *pgsA3* cells was recovered with disrupted *rscC*, which causes a lack of activated RcsB, to 16 % of that of the

Fig. 1. Effect of the multicopy suppressors *gadW*, *metE* and *yeaB* on *flhD* transcription in cells deficient in acidic phospholipids. JU02 (*pgsA3*) cells transformed with pWKS30 (empty plasmid) and with plasmids containing *gadW*, *metE* and *yeaB* (pGadW, pMetE and pYeaB, respectively) were cultivated to OD_{540} 0.35 and assayed for the β -galactosidase activity of the *flhD* transcriptional fusion. JU01 (*pgsA*⁺) with pWKS30 was included as a control. JU06 (*pgsA3 rcsC::cat*) cells transformed with pWKS30 and pGadW were also assayed in the same way for β -galactosidase activity. The data represent mean activities in units [1 unit = 1 $\mu\text{mol } o\text{-nitrophenol produced min}^{-1} (\text{mg cellular protein})^{-1}$] of three independent culture samples; error bars, SD.

wild-type level (Fig. 1). Recovery of *flhD-lacZ* activity of *pgsA3* cells with *gadW* in multicopy was approximately additive to that (16%) with *rscC* disruption, resulting in 79% of that of *pgsA*⁺ in *pgsA3 rscC/pGadW* (Fig. 1). *GadW* is thus concluded to exert its effect on *flhD* expression independently of the Rcs system.

The *flhD* mRNA level, measured by real-time PCR, in *pgsA3* cells was quite low, 1.1% of that of *pgsA*⁺ in the mid-exponential growth phase (*pgsA*⁺ cells; filled bar in Fig. 2), consistent with the *flhD-lacZ* fusion experiment (Fig. 1), but plasmid-borne *gadW* recovered it to 62% of that of *pgsA*⁺ in mid-exponential phase. The *flhD* mRNA level became lower, 11% of that of the mid-exponential phase, when cell growth entered into stationary phase (*pgsA*⁺ cells; open bar), suggesting that the low *flhD* mRNA level is related to entry into stationary phase. The low level (0.4%) of transcription on entry into stationary phase of *pgsA3* (indicated by an asterisk in Fig. 2) also recovered to 61% with the plasmid-borne *gadW* (*pgsA3* cells; open bar). The low level (11%) of transcription in the *pgsA*⁺ cells on entry into stationary phase also recovered to 66% with the *gadW* plasmid (*pgsA*⁺ cells; open bar). These results suggest that the *flhD* repression in the *pgsA3* mutant cells may occur via an increase in the stress condition- and stationary phase-induced sigma factor σ^S .

***rpoS* transcription and σ^S levels are elevated in *pgsA3* cells; *GadW* overproduction counteracts this effect by post-transcriptionally downregulating σ^S**

First we examined the level of transcription of *rpoS* in *pgsA3* cells. The activity of the *rpoS-lacZ* transcriptional fusion in *pgsA3* cells (JU10/pWSK30) was 2.6 times higher

than in *pgsA*⁺ cells (JU09/pWKS30); the values were 58.4 ± 9.1 and 22.5 ± 6.0 units, respectively (Fig. 3a). In addition, the results of real-time PCR analysis indicated that the *rpoS* mRNA level in *pgsA3* cells (JU02) was 2.7 times higher than in *pgsA*⁺ cells (JU01) (data not shown). Next, we checked whether or not the content of σ^S is actually increased in *pgsA3* cells. Western blotting with anti- σ^S antiserum indicated a significant increase of σ^S in *pgsA3* cells even in mid-exponential phase, although it was at quite a low level in *pgsA*⁺ cells in the same growth phase (Fig. 3b). Introduction of the plasmid-borne *gadW* substantially reduced the content of σ^S in *pgsA3* cells, with no reduction at all of the activity of *rpoS-lacZ* transcription (Fig. 3a, b).

We then asked whether the mRNA level of the σ^S -dependent promoters *kate* and *ecnB* (Vijayakumar *et al.*, 2004) was increased in *pgsA3* cells, since the result described above is suggestive of an increased level of σ^S . The level of *kate* mRNA in *pgsA3* cells (JU02) was 33 times and that of *ecnB* mRNA four times higher than that in the *pgsA*⁺ cells (JU01) according to real-time PCR analysis (Fig. 3c), giving further evidence that the level of σ^S in *pgsA3* cells is increased. We were thus led to the working hypothesis that the expression of *flhD* is repressed by an effect of the accumulated σ^S in *pgsA3* mutant cells. Note that this significant increase of the σ^S level in *pgsA3* cells cannot be fully explained by the small increase of *rpoS* mRNA (to 2.6-fold over *pgsA*⁺ cells, as determined by the activity of the *rpoS-lacZ* fusion).

To control the transcription level of *rpoS*, we constructed the $\Delta rpoS$ mutant strains JU03 (*pgsA*⁺ $\Delta rpoS$) and JU04 (*pgsA3* $\Delta rpoS$) and a plasmid, pDSW204-*rpoS*, in which the expression of *rpoS* was under the control of P_{trc} and examined the effect of *rpoS* expression on the expression

Fig. 2. Growth phase-dependent change in *flhD* transcription and the effect of the multicopy suppressor *gadW*. JU02 (*pgsA3*) and JU01 (*pgsA*⁺) cells transformed with pWKS30 (empty plasmid) and a plasmid containing *gadW* (pGadW) were cultivated to OD₅₄₀ 0.35 (mid-exponential growth phase; filled bars) or to OD₅₄₀ 0.90 (entry into stationary phase; open bars) and total RNA was extracted. The RNA preparations were used to prepare cDNA samples. The cDNAs were then subjected to real-time PCR analysis for mRNA of *flhD* as well as *gapA* as an internal standard. Mean values (arbitrary units) relative to the mRNA level of JU01 (*pgsA*⁺) cells in mid-exponential growth phase are presented; error bars, SD. The level for *pgsA3* cells on entry into stationary phase (indicated by an asterisk) was 0.4% of that for the control (*pgsA*⁺ cells in mid-exponential growth phase).

Fig. 3. *RpoS* transcription and σ^S levels are elevated in *pgsA3* cells; GadW overproduction counteracts this effect by post-transcriptionally downregulating σ^S . (a) JU09 (*pgsA*⁺) and JU10 (*pgsA3*) cells, both harbouring *rpoS* transcriptional fusion $\Delta rpoS::FRT::lacZ$, transformed with plasmid pWKS30 and plasmids containing *gadW* (pGadW), *metE* (pMetE) and *yeaB* (pYeaB), were cultivated in LB medium to OD₅₄₀ 0.35 (mid-exponential growth phase) and assayed for the β -galactosidase activity of the *rpoS* transcriptional fusion. The *rpoS* transcription activity data represent the mean values in units of three independent culture samples; error bars, SD. (b) JU02 (*pgsA3*) cells transformed with pWKS30, pGadW, pMetE and pYeaB were cultivated in LB medium to OD₅₄₀ 0.35 (mid-exponential growth phase) and assayed for σ^S content by Western blotting analysis. Protein samples (25 μ g) were applied to each slot of the SDS-polyacrylamide gel for blotting. JU01 (*pgsA*⁺) with pWKS30 and JU03 ($\Delta rpoS$) were also included as controls. (c) JU02 (*pgsA3*) and JU01 (*pgsA*⁺) cells were cultivated in LB medium to OD₅₄₀ 0.35 (mid-exponential growth phase) and total RNAs were extracted. The extracted RNA preparations were subjected to real-time PCR analysis for mRNA of *katE* and *ecnB* according to the method described in the legend to Fig. 2. Mean values (arbitrary units) relative to the mRNA level of JU01 (*pgsA*⁺) cells are presented; error bars, SD.

level of *flhD* mRNA. Although the levels of *rpoS* mRNA from *P_{trc}-rpoS* were almost the same for the two strains, *pgsA3* (JU04/pDSW204-*rpoS*) and *pgsA*⁺ (JU03/pDSW204-*rpoS*), incubated both with and without 0.5 mM IPTG (Fig. 4a), the σ^S content in *pgsA3* cells was higher than that in *pgsA*⁺ cells, that is, the ratio of densitometer readings of *pgsA3*/*pgsA*⁺ at 0.5 mM IPTG was 2.1 and that at 0 mM was 7.3 (Fig. 4b). As the level of *rpoS* mRNA in *pgsA3* and *pgsA*⁺ was the same, the significantly higher σ^S content in *pgsA3* cells implies that *pgsA3* cells possess a post-transcriptional mechanism, not effective in *pgsA*⁺ cells, that facilitates σ^S accumulation by an increase in translation or an obstruction of degradation of the protein.

In the exponential growth phase, the growth of *pgsA3* mutants was similar to that of *pgsA*⁺ (the generation times of *pgsA3* and *pgsA*⁺ were 38 and 34 min, respectively, in the growth between OD₅₄₀ 0.05 and 0.5 in LB medium). Therefore, the higher σ^S content in *pgsA3* cells, examined at OD₅₄₀ 0.35, is not due to an indirect effect of a low growth rate but due to a unique signal in the mutant cells.

flhDC expression is tightly linked to σ^S levels

Since σ^S accumulated in *pgsA3* mutant cells, we surmised that introduction of $\Delta rpoS$ into the *pgsA3* mutant would counteract the repressed *flhDC* expression and restore it to the wild-type *pgsA*⁺ level and that an increased level of σ^S would repress *flhDC* transcription in wild-type *pgsA*⁺ cells. Introduction of $\Delta rpoS$ into the *pgsA3* mutant restored the level of *flhD* mRNA to 50 % of the *pgsA*⁺ mRNA level, from less than 1 % (*pgsA3* mutant) (Fig. 5a). Overexpression of *rpoS* from a plasmid-borne allele (strain JU04/pDSW204-*rpoS*) reduced *flhD* mRNA to 4 %. Consistent with the changes in the level of *flhD* mRNA, strain JU04, having $\Delta rpoS$, formed a large swarm in contrast to the non-motile parental strain JU02 (*pgsA3*) (Fig. 5b). Introduction of pDSW204-*rpoS* into JU04 made the strain non-motile. These results indicate that the σ^S accumulation is a causative agent of the repression of *flhDC* and the consequent loss of motility in *pgsA3* mutant cells. Recent analysis has shown that the motility of *rpoS* mutants is enhanced relative to wild-type in minimal

Fig. 4. Post-transcriptional accumulation of σ^S in cells deficient in acidic phospholipids. (a) Cells of strains JU03 ($pgsA^+ \Delta rpoS$), and JU04 ($pgsA3 \Delta rpoS$) harbouring pDSW204 (empty plasmid) or pDSW204- $rpoS$ were cultivated in LB media to OD_{540} 0.15. The cultures were then inoculated (50 μ l aliquots) into 4.95 ml LB media containing 0.5 mM IPTG and further cultivated. At OD_{540} 0.35 (mid-exponential growth phase) the cells were harvested and assayed for $rpoS$ mRNA by real-time PCR. The data represent the mean $rpoS$ mRNA levels of three independent culture samples; error bars, SD. Mean values relative to the level in JU01 ($pgsA^+ rpoS^+$)/pDSW204 cells are presented. (b) Protein samples (5 μ g) from the harvested cells were applied to each slot of SDS-polyacrylamide gels and Western blotting analysis was conducted as described in the legend to Fig. 3.

media, in which σ^S levels are much higher than those in LB media (Dong & Schellhorn, 2009).

Note that disruption of $rpoS$ does not cause full restoration of the $flhD$ mRNA level (Fig. 5a), in spite of the tight link between $flhD$ expression and σ^S levels.

GadW, MetE and YeaB all exert their effect on $flhD$ expression by controlling σ^S levels

We examined whether the suppressive effect of $gadW$ in multicopy on the repression of $flhD$ mRNA in $pgsA$ cells would also be effective in $\Delta rpoS$ $pgsA$ cells. The level of $flhD$ mRNA in $\Delta rpoS$ $pgsA$ cells was the same as in $pgsA$ cells, showing that no change was induced after introduction of $gadW$ in multicopy into cells suppressed by $\Delta rpoS$ (Fig. 6). This indicates that the restorative effect on the $flhD$ mRNA level of $gadW$ in multicopy is achieved through the reduction of σ^S content in $pgsA3$ cells. Disruption of $gadW$ did not increase the σ^S content in $pgsA^+$ cells (data not shown). The disruption caused no decrease in $flhD$ transcription (data not shown), as expected. This might indicate that GadW in multicopy exerts its suppressive effect only on the cells with a high σ^S content, consistent with an earlier report that suppression by GadW is effective on cells with a high σ^S content caused by acid induction (Ma *et al.*, 2003).

We also examined the mechanism(s) for the suppression by $metE$ and $yeaE$ in multicopy of $flhD$ repression in $pgsA3$

mutant cells. With $metE$ and $yeaE$ in multicopy, the σ^S content was reduced, as in the case of $gadW$, without a reduction in $rpoS$ transcription (Fig. 3a, b). As for $metE$, an earlier report has shown that $metE::Tn10$ elevates σ^S levels (Goodrich-Blair & Kolter, 2000), consistent with the reduction of σ^S content with $metE$ in multicopy. These results suggest that the suppressive effect of $metE$ on a multicopy plasmid involves the reduction of σ^S levels. A similar reduction in σ^S level was observed with $yeaB$ on a multicopy plasmid. The suppressive effect on repressed $flhD$ transcription of all three genes in multicopy is thus most likely achieved through the reduction of σ^S , which otherwise accumulates in the mutant acidic phospholipid-deficient cells.

DISCUSSION

The flagellar master operon $flhDC$ shows a dynamic response to a variety of physiological changes, and so it is no surprise that many factors involved in its transcriptional regulation have been reported; RcsAB (Francez-Charlot *et al.*, 2003), as well as the classical transcription factor OmpR (Shin & Park, 1995), negatively regulate the operon, whereas cAMP-CAP and H-NS (Soutourina *et al.*, 1999), and QseBC (Sperandio *et al.*, 2002), positively regulate it. Activation of the Rcs phosphorelay system has been observed in acidic phospholipid-deficient cells (Shiba *et al.*, 2004; Nagahama *et al.*, 2006). RcsB, upon activation

Fig. 5. Repression of *flhD* transcription with induced *rpoS*. (a) Cells of strains JU01 (*pgsA*⁺), JU03 (*pgsA*⁺ Δ *rpoS*), JU02 (*pgsA3*) and JU04 (*pgsA3* Δ *rpoS*) harbouring pDSW204 (empty plasmid) or pDSW204-*rpoS* were cultivated in LB media to OD₅₄₀ 0.15. The cultures were then inoculated (50 μ l aliquots) into 4.95 ml LB media containing 0.5 mM IPTG and further cultivated. At OD₅₄₀ 0.35 (mid-exponential growth phase) the cells were harvested and assayed for *flhD* mRNA by real-time PCR. The data represent the mean *flhD* mRNA level of three independent culture samples; error bars, SD. Mean values relative to the level of JU03 (*pgsA*⁺ *rpoS*⁺)/pDSW204 cells are presented. (b) The cultures (5 μ l samples) described above were tested for motility on motility plates incubated at 30 °C for 6 h.

of the phosphorelay, seems to bind with RcsA to form a heterodimer that prevents transcription of the *flhDC* operon (Francez-Charlot *et al.*, 2003). Hence, the repression of the *flhD* operon in cells with acidic phospholipid deficiency seemed to have its cause in the activated Rcs phosphorelay system. However, introduction of *rscC::cat*, which cannot activate RcsB (Shiba *et al.*, 2004), restores the *flhD*-*lacZ* activity of *pgsA3* cells to a level that is only about

17% of the activity in *pgsA*⁺ (Fig. 1), indicating that the Rcs phosphorelay system cannot fully account for the repressed transcription of *flhD* in cells deficient in acidic phospholipids.

Thus, in order to clarify the mechanism of the repression of *flhD* in acidic phospholipid-deficient cells, we screened for genes that would suppress the repression when supplied in

Fig. 6. Effect of the multicopy suppressors *gadW*, *metE* and *yeaB* on the repressed level of *flhD* mRNA in Δ *rpoS* *pgsA* cells. JU01 (*pgsA*⁺), JU02 (*pgsA3*) and JU04 (*pgsA3* Δ *rpoS*) transformed with pWKS30, pGadW, pMetE and pYeaB were cultivated in LB medium to OD₅₄₀ 0.35 (mid-exponential growth phase) and total RNAs were extracted. The extracted RNA preparations were subjected to real-time PCR analysis for the level of *flhD* mRNA according to the method described in the legend to Fig. 2. Mean values relative to the mRNA level of JU01/pWKS30 cells are presented; error bars, SD.

multicopy, and identified three such genes: *gadW*, *metE* and *yeaB*. Since GadW negatively regulates σ^S production (Ma *et al.*, 2003), we decided to examine the content of σ^S in acidic phospholipid-deficient cells and found that they have a higher σ^S level than wild-type cells (Fig. 3). In fact, transcription of *rpoS* in acidic phospholipid-deficient cells, examined through the activity of an *rpoS-lacZ* fusion and real-time PCR, was more than doubled in the exponential growth phase with respect to the wild-type (Fig. 3), and the σ^S -dependent promoters *katE* and *ecnB* (Vijayakumar *et al.*, 2004) were highly induced (Fig. 3c). As for the effect of MetE in multicopy, an earlier report has shown that a *metE::Tn10* mutant, which is deficient for conversion of homocysteine to methionine and therefore accumulates homocysteine thiolactone, has an elevated σ^S level, and although the underlying molecular mechanism is as yet unknown (Goodrich-Blair & Kolter, 2000), this effect is apparently consistent with the results of the reduction of σ^S content with *metE* in multicopy. YeaB, a putative nucleoside diphosphate hydrolase, may be concerned indirectly with a change in the level of cyclic AMP that is involved in the regulation of σ^S content (Lange & Hengge-Aronis, 1994).

Tests with induction from P_{trc} -*rpoS* confirmed that excess σ^S diminished *flhD* transcription (Fig. 5a). Since no σ^S recognition sequence (Lacour & Landini, 2004; Weber *et al.*, 2005) has been found upstream of *flhDC* (Lehnen *et al.*, 2002; Barker *et al.*, 2004; Regulon DB 6.3, Operon Form, <http://regulondb.ccg.unam.mx/index.jsp>), the repression caused by σ^S requires another explanation. It may be that a negative regulatory factor, which depends for its expression on σ^S , takes part in *flhDC* transcription. Possible regulatory pathways for the repression of *flhDC* are illustrated in Fig. 7. Another likely mechanism for the negative regulation in the presence of excess σ^S is that increasing σ^S contributes to the downregulation indirectly, by increasingly competing with σ^{70} , on which the transcription of *flhDC* depends (Barker *et al.*, 2004), for RNA polymerase core enzyme, as proposed elsewhere (Maeda *et al.*, 2000; Pesavento *et al.*, 2008). However, the mechanism for this increase in *rpoS* transcription which occurs in acidic phospholipid-deficient mutants (Fig. 3) has yet to be examined.

The apparently higher content of σ^S in *pgsA3* mutant cells than in *pgsA*⁺ cells, with the same levels of *rpoS* mRNA induced with the same concentration of IPTG (Fig. 4), implies a post-transcriptional accumulation of σ^S in the acidic phospholipid-deficient cells. Since translation of *rpoS* mRNA is regulated in a complicated network with many *trans*-acting factors and small regulatory RNAs (Hengge-Aronis, 2002; Peterson *et al.*, 2006), one may suppose that some members of the network play an important role in the accumulation of σ^S . In addition, degradation of σ^S is regulated by the protease complex ClpXP, whose action is under the specific control of the recognition factor RssB (Hengge-Aronis, 2002). Thus, certain steps in degradation may also be involved in the σ^S accumulation in acidic phospholipid-deficient cells. This

Fig. 7. Model summarizing accumulation of σ^S and repression of *flhDC* in acidic phospholipid-deficient mutant cells.

assumption seems to be consistent with the result of DNA microarray analysis, which indicates reductions of *clpXP* expression in cells with acidic phospholipid deficiency (Nagahama *et al.*, 2007).

How does the phospholipid deficiency trigger the σ^S accumulation in acidic phospholipid-deficient cells? The Rcs signal transduction system, which is active in the *pgsA* mutant cells (Shiba *et al.*, 2004; Nagahama *et al.*, 2006), is reported to regulate *rpoS* translation positively via the non-coding small RNA RprA (Peterson *et al.*, 2006). Thus, this positive regulation in *rpoS* translation would be responsible, at least partly, for the σ^S accumulation. The probable reduction of ClpXP due to the reduced expression of *clpXP* noted above might also be responsible for the σ^S accumulation. The expression of the *clpXP* operon involves promoters under the control of the stress sigma factors σ^H and σ^E (Regulon DB 6.4, <http://regulondb.ccg.unam.mx/index.jsp>), and the expression of these sigma factor genes is regulated by the Cpx two-component signal transduction system (De Wulf & Li, 2002). In *pgsA* mutant cells, an activation of the Cpx two-component system was observed (our unpublished results); therefore, the activated Cpx system could be a critical trigger for the impaired degradation of σ^S . Signals responsible for the increase in *rpoS* transcription (Fig. 3) are unknown at present. Although a tight link between *flhD* expression and σ^S levels has been demonstrated, disruption of *rpoS* does not cause full restoration of the *flhD* mRNA level (Fig. 5a). There must be, therefore, a σ^S -independent pathway(s) for repression of *flhD* that is activated in the acidic phospholipid-deficient cells. The extreme alteration in

phospholipid composition found in the *pgsA3* mutant cells would not occur in the wild-type cells. However, the effect of acidic phospholipid deficiency on the membranes may mimic some environmental effects, which are likely to affect flagellar gene expression in a more or less similar way to that suggested in this study.

A model of the inverse regulatory coordination between the two cascades of the master regulators σ^S and FlhDC and the different roles of cyclic-di-GMP control modules in the two cascades has been proposed by Pesavento *et al.* (2008). The expression of these master regulators is also inversely regulated by the Rcs phosphorelay system; the RcsBA heterodimer negatively regulates the *flhDC* operon (Francez-Charlot *et al.*, 2003), but RcsB activates the translation of *rpoS* by repressing LrhA synthesis and also by stimulating RprA (Peterson *et al.*, 2006). The biological significance of the inverse relationship of the two regulatory cascades between the master regulators *flhDC* (motile) and σ^S (unfavourable to growth) lies in a switch from flagella synthesis, which requires large amounts of materials and energy, to saving these when the cells encounter unfavourable circumstances, including the disorder in their membranes which accompanies acidic phospholipid deficiency.

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