Involvement of $\sigma^S$ 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 $\sigma^S$ production, the level of $\sigma^S$ in the pgSA3 mutant was examined. We found that pgSA3 cells had a high level of $\sigma^S$ and that introduction of a gadW plasmid into pgSA3 cells did reduce the $\sigma^S$ level. The pgSA3 cells exhibited a sharp increase in $\sigma^S$ 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 $\sigma^S$. GadW in multicopy exerts its effect by post-transcriptionally downregulating $\sigma^S$. YeaB and MetE in multicopy also exert their effect via $\sigma^S$. Disruption of rpoS caused an increase of the flhD mRNA level, and induction from P_{Ac}-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 $\sigma^S$.

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 pgSA3::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 lipoprotein, the modification cannot be carried out in the mutant cells, hampering processing for maturation, and the thus unmodified lipoproteins 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 phosphatic 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 $\omicF$ RNA level, but the level of transcriptional expression of $\omicF$ 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 $\flhD$ 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 multicity, would counteract the repression caused by the pgsA3 mutation of $\flhD$, and found that $gadW$, $metE$ and $yeaB$ were such multicity suppressors of the repression of $\flhD$. The most effective among these was $gadW$, the gene product of which is involved in the negative regulation of the production of $\sigma^S$ (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 $\sigma^S$ in pgsA3 cells was examined and they turned out to have a higher level of $\sigma^S$ than wild-type cells. Introduction of a $gadW$ plasmid into pgsA3 cells reduced the $\sigma^S$ level. These results indicate that the accumulation of $\sigma^S$ is one of the causes of the repression of transcription of $\flhD$. 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 $\sigma^S$ accumulation.

| Table 1. Bacterial strains and plasmids used in this study |
|---------------------|----------------------|-----------------------------|
| **Strains**         | **Relevant characteristics** | **Source or reference**    |
| MG1655              | rph-1                | Laboratory stock            |
| ST001               | Δ(lac-pro) ara thi lpp-2 uvrC279::Tn10 | Inoue et al. (1997)         |
| ST002               | ST001 pgsA3          | Inoue et al. (1997)         |
| FS401               | ST001 λ lysogen [flhD upstream (−305 to +290)-operon fusion] | Inoue et al. (1997)         |
| FS501               | FS401 pgsA3          | Inoue et al. (1997)         |
| JM109               | recA1 Δ(lac-proAB) endA1 gyrA96 thi-1 hsdR17 supE44 relA1 F’ [traD36 proAB" lacF" lacZ ΔM15] | Laboratory stock            |
| JWK3483             | BW25113 AgadW::kan   | KO Collection               |
| JWK5437             | BW25113 ΔrpoS::kan   | KO Collection               |
| JU01                | ST001 λ lysogen [flhD upstream (−88 to +26)-operon fusion] | This work                   |
| JU02                | JU01 pgsA3           | This work                   |
| JU03                | JU01 ΔrpoS::FRT      | This work                   |
| JU04                | JU02 ΔrpoS::FRT      | This work                   |
| JU05                | JU01 rcsC::cat       | This work                   |
| JU06                | JU02 rcsC::cat       | This work                   |
| JU07                | ST001 ΔrpoS::FRT     | This work                   |
| JU08                | ST002 ΔrpoS::FRT     | This work                   |
| JU09                | ST001 ΔrpoS::FRT::lacZ | This work                   |
| JU10                | ST002 ΔrpoS::FRT::lacZ | This work                   |
| JU75                | JU01 ΔgadW::kan      | This work                   |
| **Plasmids**        |                      |                             |
| pMetE               | pWKS30 metE           | This work                   |
| pGadW               | pWKS30 gadW           | This work                   |
| pYeaB               | pWKS30 yeaB           | This work                   |
| pDSW204 prpS        | pDSW204 prpS          | This work                   |
| pCE37               | R6Kori lacZ lacY Km⁸ | Ellermeier et al. (2002)    |
| pKG137              | Improved version of pCE37 | M. Garsha                 |
| pCP20               | $\delta_{1857}^R$ Rep⁸ Ap⁸ Cm⁸ | 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 PI 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 \( \lambda \) 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 PI transduction of the \( \Delta rpoS: \lambda \) kan allele from JW5437, 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 prpoScloningup starting at 9 nt downstream from the stop codon (Table 2). The PCR fragment obtained was digested with *Eco*RI and *Hind*III and then inserted into the *Eco*RI–*Hind*III 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 plasmid 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 \( \mu \)g 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 \( \mu \)l 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 (OD\(_{540}\) 0.35). Whole cells were pelleted and immediately resuspended to equal OD\(_{540}\) values in SDS loading buffer (Silhavy et al., 1984). After boiling for 5 min, equal volumes (25 or 5 \( \mu \)g 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-\( \alpha^5 \) antibody (Tanaka et al., 1993). As the secondary antibody,
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 BamHI 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 σ5 production in the mechanism involved in acid resistance (Ma et al., 2003), although the level at which GadW affects σ5 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-Charlott et al., 2003), a possible effect of an rcs mutation on the suppression with gadW was also investigated. The repressed flhD–lacZ activity in pgsA3 cells was recovered with disrupted rcsC, which causes a lack of activated RcsB, to 16% of that of the

β-Galactosidase assay. The activity of β-galactosidase was assayed using ONPG as substrate and was recorded as μmol o-nitrophenol produced min⁻¹ (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 (OD₅₄₀ 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 ccoB genes listed in Table 2. The 25 μl reaction mix contained 1 × 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 °C for 10 s, and 30 cycles at 95 °C for 5 s, 60 °C for 20 s, 72 °C for 30 s with fluorescence acquisition at 72–95 °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).
wild-type level (Fig. 1). Recovery of flhD–lacZ activity of pgsA3 cells with gadW in multiplicity was approximately additive to that (16%) with rcsC disruption, resulting in 79% of that of pgsA+ in pgsA3 rcsC/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; open 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 σS.

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

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 σS is actually increased in pgsA3 cells. Western blotting with anti-σS antisemur indicated a significant increase of σS 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 σS 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 σS-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 σS. 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 σS 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 σS in pgsA3 mutant cells. Note that this significant increase of the σS 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 ΔrpoS mutant strains JU03 (pgsA+ ΔrpoS) and JU04 (pgsA3 ΔrpoS) and a plasmid, pDSW204–rpoS, in which the expression of rpoS was under the control of Ptrc and examined the effect of rpoS expression on the expression of rpoS–lacZ fusion.

**Fig. 2.** Growth phase-dependent change in flhD transcription and the effect of the multi-copy suppressor gadW. JU02 (pgsA3) and JU01 (pgsA+) cells transformed with pWKS30 (empty plasmid) and a plasmid containing gadW (pGadW) were cultivated to ODs40 0.35 (mid-exponential growth phase; filled bars) or to ODs40 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).
level of flhD mRNA. Although the levels of rpoS mRNA from P_\text{rpoS} were almost the same for the two strains, pgsA3 (JU04/pDSW204-rpoS) and pgsA\textsuperscript{+} (JU03/pDSW204-rpoS), incubated both with and without 0.5 mM IPTG (Fig. 4a), the \( \sigma^S \) content in pgsA3 cells was higher than that in pgsA\textsuperscript{+} cells, that is, the ratio of densitometer readings of pgsA3/ pgsA\textsuperscript{+} 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\textsuperscript{+} was the same, the significantly higher \( \sigma^S \) content in pgsA3 cells implies that pgsA3 cells possess a post-transcriptional mechanism, not effective in pgsA\textsuperscript{+} cells, that facilitates \( \sigma^S \) 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\textsuperscript{+} (the generation times of pgsA3 and pgsA\textsuperscript{+} were 38 and 34 min, respectively, in the growth between OD\textsubscript{540} 0.05 and 0.5 in LB medium). Therefore, the higher \( \sigma^S \) content in pgsA3 cells, examined at OD\textsubscript{540} 0.35, is not due to an indirect effect of a low growth rate but due to a unique signal in the mutant cells.

**flhD expression is tightly linked to \( \sigma^S \) levels**

Since \( \sigma^S \) accumulated in pgsA3 mutant cells, we surmised that introduction of \( \Delta \text{rpoS} \) into the pgsA3 mutant would counteract the repressed \( \text{flhDC} \) expression and restore it to the wild-type pgsA\textsuperscript{+} level and that an increased level of \( \sigma^S \) would repress \( \text{flhDC} \) transcription in wild-type pgsA\textsuperscript{+} cells. Introduction of \( \Delta \text{rpoS} \) into the pgsA3 mutant restored the level of flhD mRNA to 50% of the pgsA\textsuperscript{+} 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 \( \Delta \text{rpoS} \), 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 \( \sigma^S \) accumulation is a causative agent of the repression of \( \text{flhDC} \) 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 concentrations of IPTG.
media, in which $\sigma^S$ levels are much higher than those in LB media (Dong & Schellhorn, 2009).

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

\textbf{GadW, MetE and YeaB all exert their effect on \textit{flhD} expression by controlling $\sigma^S$ levels}

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

We also examined the mechanism(s) for the suppression by \textit{metE} and \textit{yeaE} in multicity of \textit{flhD} repression in \textit{pgsA3} mutant cells. With \textit{metE} and \textit{yeaE} in multicity, the $\sigma^S$ content was reduced, as in the case of \textit{gadW}, without a reduction in \textit{rpoS} transcription (Fig. 3a, b). As for \textit{metE}, an earlier report has shown that \textit{metE}:\textit{Tn}10 elevates $\sigma^S$ levels (Goodrich-Blair & Kolter, 2000), consistent with the reduction of $\sigma^S$ content with \textit{metE} in multicity. These results suggest that the suppressive effect of \textit{metE} on a multicity plasmid involves the reduction of $\sigma^S$ levels. A similar reduction in $\sigma^S$ level was observed with \textit{yeaE} on a multicity plasmid. The suppressive effect on repressed \textit{flhD} transcription of all three genes in multicity is thus most likely achieved through the reduction of $\sigma^S$, which otherwise accumulates in the mutant acidic phospholipid-deficient cells.

\textbf{DISCUSSION}

The flagellar master operon \textit{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
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 rcsC::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. 5. Repression of flhD transcription with induced rpoS. (a) Cells of strains JU01 (pgsA+), JU03 (pgsA+ ΔrpoS), JU02 (pgsA3) and JU04 (pgsA3 ΔrpoS) harbouring pDSW204 (empty plasmid) or pDSW204-rpoS were cultivated in LB media to OD540 0.15. The cultures were then inoculated (50 μl 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 μl samples) described above were tested for motility on motility plates incubated at 30 °C for 6 h.](http://mic.sgmjournals.org)
multi-copy, and identified three such genes: gadW, metE and yeaB. Since GadW negatively regulates σ^5 production (Ma et al., 2003), we decided to examine the content of σ^5 in acidic phospholipid-deficient cells and found that they have a higher σ^5 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^5-dependent promoters katE and ecnB (Vijayakumar et al., 2004) were highly induced (Fig. 3c). As for the effect of MetE in multi-copy, an earlier report has shown that a metE::Tn10 mutant, which is deficient for conversion of homocysteine to methionine and therefore accumulates homocysteine, has an elevated σ^5 level (Peterson et al., 2004) were highly induced (Fig. 3c). As for the effect of MetE in multi-copy, an earlier report has shown that a metE::Tn10 mutant, which is deficient for conversion of homocysteine to methionine and therefore accumulates homocysteine, has an elevated σ^5 level (Peterson et al., 2004) were highly induced (Fig. 3c).

Tests with induction from P_{nic-}rpoS confirmed that excess σ^5 diminished flhD transcription (Fig. 5a). Since no σ^5 recognition sequence (Lacour & Landini, 2004; Weber et al., 2005) has been found upstream of flhDC (Lehen 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^5 requires another explanation. It may be that a negative regulatory factor, which depends for its expression on σ^5, 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^5 is that increasing σ^5 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^5 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^5 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^5. In addition, degradation of σ^5 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^5 accumulation in acidic phospholipid-deficient cells. This 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^5 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^5 accumulation. The probable reduction of ClpXP due to the reduced expression of clpXP noted above might also be responsible for the σ^5 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^5. Signals responsible for the increase in rpoS transcription (Fig. 3) are unknown at present. Although a tight link between flhD expression and σ^5 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^5-independent pathway(s) for repression of flhD that is activated in the acidic phospholipid-deficient cells. The extreme alteration in

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**Fig. 7. Model summarizing accumulation of σ^5 and repression of flhDC in acidic phospholipid-deficient mutant cells.**
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 \( \sigma^S \) 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 (Franzé-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 \( \sigma^S \) (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 membrane phospholipid deficiency.

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