INTRODUCTION

Biofilm formation is a complex process that can be divided into several stages, from initial attachment of the cells to the surface to the development of mature biofilms, characterized by the formation of complex, three-dimensional structures (O'Toole et al., 2000; Stoodley et al., 2002). Genetic studies suggest that gene expression during biofilm formation is temporally regulated and, as a consequence, cells will have different expression profiles at different times during the biofilm formation process (Beloin et al., 2004; Domka et al., 2007; Sauer et al., 2002). In particular, a variety of cell-surface appendages, including flagella and pili, are required for the initial attachment to the surface, whilst the transition to a mature biofilm involves the production of an exopolysaccharide-rich matrix by the bacteria (Danese et al., 2000a, b; O'Toole & Kolter, 1998; Pratt & Kolter, 1998; Prigent-Combaret et al., 2000).

Over the past few years, a role for the Rcs phosphorelay in biofilm formation by Escherichia coli has been proposed (Danese et al., 2000b; Ferrières & Clarke, 2003). The Rcs phosphorelay is composed of the sensor kinase, RcsC, the histidine-phosphotransfer (HPt)-containing protein RcsD and the cytoplasmic RcsB response regulator (Huang et al., 2006; Majdalani & Gottesman, 2005). RcsC is a hybrid kinase that has both a transmitter domain and a receiver domain. Upon activation, His463 in the transmitter domain of RcsC is autophosphorylated and the phosphoryl group is transferred to Asp859 in the receiver domain. Subsequently, the phosphoryl group is transferred to the HPt domain of RcsD and finally to the receiver domain of RcsB. Phospho-RcsB (RcsB-P) then binds to DNA and affects the expression of an extensive Rcs regulon (Chen et al., 2001; Ferrières & Clarke, 2003; Hagiwara et al., 2003; Takeda et al., 2001). In addition, three accessory proteins, RcsA, RcsF and YrfF (IgaA), participate in the modulation of the inputs and outputs of this phosphorelay (Majdalani et al., 2005; Meberg et al., 2001; Stout et al., 1991; Tierrez & Garcia-del Portillo, 2004).

Activation of the Rcs phosphorelay occurs under a variety of conditions, including growth on a solid surface (reviewed by Huang et al., 2006; Majdalani & Gottesman, 2005). The activation of the Rcs phosphorelay results in an increase in the expression of the extracellular polysacchar-
ide colanic acid (Gottesman et al., 1985). As the expression of colanic acid is required for the maturation of biofilms formed by E. coli, this suggests a positive role for the Rcs phosphorelay in the regulation of biofilm maturation (Danese et al., 2000b). On the other hand, activation of the Rcs phosphorelay has been shown to repress the expression of genes encoding surface adhesins, such as antigen 43 (Ag43) and curli, and it has been reported that overproduction of RcsB (a situation that mimics the activation of the Rcs phosphorelay) inhibits the expression of the flhDC operon, encoding the master regulators of flagella function during biofilm formation (Collet et al., 2008). We have previously reported that the Rcs phosphorelay may play an important role in regulating the transition from attached cell to mature biofilm by reciprocally regulating the expression of genes required for attachment and maturation (Huang et al., 2006).

Consistent with this role, the Rcs phosphorelay has been shown to regulate the general stress response-associated sigma factor, $\sigma^S$, through the activation of RprA, a small non-coding RNA that positively controls the level of $\sigma^S$ in the cell by base-pairing with the 5’ end of the $rpoS$ mRNA (Majdalani et al., 2001, 2002). $\sigma^S$ is involved in the adaptation of the bacteria to starvation, hyperosmotic shock, pH downshift and low temperature, and controls many genes during the transition from the exponential to the stationary phase of growth (Patten et al., 2004; Weber et al., 2005; White-Ziegler et al., 2008). Recent studies have reported that $\sigma^S$ influences global gene expression and protein production within E. coli biofilms, suggesting that this alternative sigma factor may have an important function during biofilm formation (Collet et al., 2008; Ito et al., 2009). We have previously reported that the inactivation of $rcsC$ resulted in a biofilm-defective phenotype in E. coli (Ferrières & Clarke, 2003). In the present study we show that the biofilm defect observed in the $rcsC$ mutant background is largely associated with increased levels of $\sigma^S$ present in this background, suggesting that the temporal control of $\sigma^S$ levels is important during biofilm formation.

**METHODS**

**Strains, plasmids, phages and growth conditions.** The strains used in this study are derivatives of E. coli K-12 ZK2686 [W3110 Δ(argF-lacU169)] (Danese et al., 2000a), except where stated, and are listed in Table 1. The bacteria were grown at 30 °C in Luria–Bertani broth (LB) with antibiotics at the following concentrations (µg ml$^{-1}$), where appropriate: ampicillin (Ap), 100; chloramphenicol (Cm), 20; kanamycin (Km), 30; tetracycline (Tc), 15. All the mutations and the deletions constructed during this study were transferred from strain to strain by phage transduction using P1vir or P1cml. The plasmids were introduced into the different backgrounds by chemical transformation with CaCl$_2$ (Sambrook et al., 1989).

**Construction of deletion mutants.** Gene replacement in E. coli was carried out using the λRed recombination system (Datsenko & Wanner, 2000). Briefly, DNA cassettes carrying the genes encoding Cm or Km resistance were amplified from either pKD3 or pKD4, respectively, by PCR, using the high fidelity KOD HiFi DNA polymerase (Novagen). The primers used were 60 bp long and were designed to contain a 5’-end sequence homologous to the beginning (forward primer) or the end (reverse primer) of the gene to be deleted. The 3’-end sequences of the primers were as follows: 5’-GTGTAAGCTGAGCTGTCCTC-3’ (forward primer); 5’-CATATGAATATCCTGCTTAG-3’ (reverse primer). After purification, the PCR-amplified cassettes were introduced by electroporation into ZK2686 cells expressing λRed recombine from pKD46 (Datsenko & Wanner, 2000). The transformants were selected on plates containing appropriate antibiotics at the following concentrations (µg ml$^{-1}$): Km, 20; Cm, 10. The mutations were confirmed by PCR, using two primers flanking the point of insertion of the cassette, and were transferred into clean backgrounds by P1vir transduction.

**Cloning rpoS.** The rpoS gene was cloned from ZK2686 genomic DNA using PCR and the primers LF168 (5’-CATGCCATGGTCGAGATACGCGGAAAG-3’) and LF169 (5’-GCTCTAGACTTTACTGCGGAAAC-3’). The purified PCR amplicon was digested with Ncol and XbaI and ligated into the corresponding sites in pTREC99a. The ligation mix was electroporated into strain EC100 and positive clones were selected by colony PCR (using the primers DC160, 5’-CTTGCATGGCGCAAGTCG-3’, and DC164, 5’-CAATTAATCATCGCGTCG-3’). One clone was selected, pRpoS, and confirmed by PCR amplification of the rpoS gene. In addition, the functionality of the cloned gene was verified by its ability to restore catalase production in ZK1000 (rpoS::Km).

**Biofilm formation assay.** The capacity of E. coli to form a biofilm was assayed as described previously with a few modifications (Ferrières & Clarke, 2003). Briefly, 150 µl of an overnight culture, diluted to OD$_{600}$=0.01 in LB, was inoculated into a well of a PVC 96-well microtitre plate and the plate was incubated at 30 °C for 48 h, unless otherwise stated. The planktonic cells were then removed by aspiration and the well was rinsed three times with PBS. To stain the attached cells, 200 µl 0.1 % crystal violet (CV) was added and left in the well for 10 min at room temperature. After three washes with PBS, the well was carefully dried and the CV entrapped in the cells was dissolved in 200 µl ethanol. The amount of biofilm formed was quantified by measuring the optical density of 100 µl of the CV solution at 595 nm with a microtitre plate reader (GENios; Tecan UK Ltd).

**Random mutagenesis with iplacMu53.** A random insertion library of BMM533 was constructed using iplacMu53, as described previously (Ferrières & Clarke, 2003). Briefly, BMM533 was grown overnight in LB medium supplemented with 10 mM MgSO$_4$ and 0.2 % (w/v) maltose, pelleted and resuspended in LB containing 10 mM MgSO$_4$ and co-infected with iplacMu53 (10$^8$ p.f.u.) and λMu507 (10$^9$ p.f.u.) for 30 min at 30 °C. The unabsorbed phage particles were removed by centrifugation and, after three washes, the cells were incubated at 30 °C for 1 h in 1 ml LB to allow phenotypic expression. The iplacMu53 integrants were finally selected by plating 100 µl of 10$^{-4}$ to 10$^{-1}$ serial dilutions on LB medium containing Km and the plates were incubated at 30 °C overnight. Mutations that restored biofilm formation in BMM533 were identified by screening independent mutants in PVC 96-well microtitre plates, as described above, using BMM533 and ZK2686 as negative and positive controls, respectively. The mutations of interest were finally moved into clean genetic backgrounds by P1vir transduction to ensure that a single iplacMu53 insertion was present in the genome and to confirm that the mutation itself did not affect biofilm formation. The site of
cells were lysed by heating to 100 °C. Bacteria were grown at 30 °C with agitation, centrifuged and resuspended to OD 600 = 0.2 OD600 units of the crude extract was loaded and separated on 12.5% acrylamide gel (running buffer; 25 mM Tris, pH 6.8, 0.192 M glycine, 1% SDS). The proteins were then transferred onto a nitrocellulose membrane (0.45 μm; BDH) using a mini-Protein transfer system (Bio-Rad), and successful transfer was verified by staining with Ponceau S (Sigma; P-7170). Following extensive washes with PBS, the membrane was blocked overnight at 4 °C in PBS containing 5% (w/v) milk powder and 0.05% (v/v) Tween 20. To visualize the αS protein, the membrane was incubated for 4 h at 4 °C with a 1/1000 dilution of ECL peroxidase-linked anti-rabbit IgG (Amersham Biosciences). The membrane was then washed three times in PBS containing 0.05% Tween 20 before detection by ECL Western blotting detection reagents (Amersham Biosciences). To quantify the intensity of the bands, the membrane was visualized by ECL Western blotting detection reagents (Amersham Biosciences). To quantify the intensity of the bands, the membrane was.

**Western blotting.** The amount of αS present was determined by arbitrary-primed PCR, as described previously (Ferrières & Clarke, 2003).

### Table 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Strain/plasmid/phage</th>
<th>Relevant characteristics</th>
<th>Source</th>
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<tbody>
<tr>
<td>ZK2686</td>
<td>W3110 Δ(argF-lac)U169</td>
<td></td>
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<tr>
<td>BMM520</td>
<td>ZK2686 rcsC52::Tn10</td>
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<tr>
<td>BMM526</td>
<td>ZK2686 gmd::iplacMu53</td>
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<tr>
<td>BMM532</td>
<td>ZK2686 rcsB::Cm</td>
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<tr>
<td>BMM533</td>
<td>ZK2686 rcsC::Cm</td>
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<tr>
<td>BMM534</td>
<td>ZK2686 rcsF::Cm</td>
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<tr>
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<tr>
<td>AB012</td>
<td>MG1655 rssB::Tc</td>
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**Plasmid**

- pTRC99a: ori colE1, ApR
- pBMM102: pBMM101, rcsC+
- pBMM624: pBMM101, rcsCH339D (encodes a constitutive RcsC protein)
- pRpoS: pTRC99a derivative carrying rpoS
- pKD3: Template plasmid carrying CmR cassette
- pKD4: Template plasmid carrying KmR cassette

**Phage**

- P1vir: Strictly lytic phage
- P1col: Lysogenic P1 derivative, CmR
- ΔiplacMu53: Δ-Mu hybrid phage carrying lacZY, KmR
- ΔpMu507: cts857 Sam7 MuA+ B+

ΔiplacMu53 insertion in each mutant was determined by arbitrary-primed PCR, as described previously (Ferrières & Clarke, 2003).
RESULTS

Mutations in rcsC and rcsD affect biofilm formation

Using an rcsC insertion mutant, rcsC::Tn10, we have previously demonstrated that the RcsC protein is required for normal biofilm formation in E. coli K-12 (Ferrières & Clarke, 2003). In order to better understand the role of the Rcs phosphorelay during biofilm formation, we decided to systematically construct deletion mutants of each of the genes encoding components of this phosphorelay (rcsC, rcsD, rcsB, rcsF and rcsA). We also constructed selected double mutants (rcsC rcsB and rcsD rcsB), and analysed the behaviour of each strain during biofilm formation using the microtitre plate assay (Pratt & Kolter, 1998; Fig. 1). The data clearly confirmed that the defect previously observed in the rcsC::Tn10 mutant is due to the inactivation of the rcsC gene and not to a side-effect of the Tn10 insertion, since a clean rcsC::Cm mutation results in a similar phenotype. Moreover, while both rcsC and rcsD appeared to be required for normal biofilm development, deletion of either rcsF or rcsA had no effect on biofilm formation, suggesting that the accessory proteins encoded by these genes do not have any important role in this process. Surprisingly, a mutation in rcsB alone has no effect on biofilm formation whilst, in contrast, the same mutation restores biofilm formation in the rcsC and rcsD mutants, suggesting that the defect in biofilm formation observed in these mutants may be due to the activation of RcsB (i.e. phosphorylation) in these backgrounds. It has been proposed that RcsB-P accumulates in the cell in the absence of RcsC or RcsD, presumably due to the lack of an RcsCD-dependent RcsB-P phosphatase activity (Majdalani & Gottesman, 2005). Therefore, it is possible that the biofilm defect observed in the rcsC and rcsD mutant backgrounds is due to an accumulation of RcsB-P in the cell.

Suppressor analysis reveals that the inactivation of either barA or rpaA increases biofilm formation in rcsC mutant strains

The Rcs phosphorelay controls the expression of >150 genes and an increase in the level of active RcsB would be expected to result in the altered expression of at least some of these genes (Ferrières & Clarke, 2003). Our working hypothesis was that, in the rcsC background, the increased level of RcsB-P in the cell resulted in the inappropriate regulation of one (or more) of the genes in the Rcs regulon and this resulted in the defect in biofilm formation. Several Rcs-regulated genes have already been shown to be involved in biofilm formation, in particular the genes involved in flagella and colanic acid production. We first checked whether one of those could be responsible for the rcsC phenotype.

Active RcsB represses the expression of the class I regulator of flagella biogenesis (FlhD2C2) by binding to the promoter region of the flhDC operon, and motility was shown to be important for biofilm formation (Francez-Charlot et al., 2003; Pratt & Kolter, 1998). Using an flhD-lacZ fusion, we observed a clear correlation between strains that are deficient in biofilm formation and strains for which flhD is repressed (Supplementary Fig. S1a). Consistent with this, the same strains displayed reduced motility on plates (Supplementary Fig. S1b). Although it was possible to restore motility in the rcsC mutant strain to wild-type levels by expressing flhDC from a plasmid, the ectopic expression of flhDC blocked biofilm formation in both the wild-type and the rcsC mutant (data not shown). Therefore, it was not possible, at this point, to determine whether the relatively small effect on motility observed in the rcsC mutant background was also responsible for the biofilm defect.

On the other hand, the Rcs phosphorelay positively controls the expression of the cps (wca) operon, encoding the proteins required for the production and secretion of colanic acid, and previous studies indicate that the constitutive expression of colanic acid can prevent bacterial
attachment to solid surfaces (Hanna et al., 2003). Nevertheless, the introduction of a mutation that blocked colanic acid production, gmd::pLacMu53 (Ferrières & Clarke, 2003), into the rcsC::Tn10 background had no effect on biofilm formation, confirming that the promiscuous expression of the cps operon was not responsible for the biofilm defect of this strain (Supplementary Fig. S2).

Since the rcsC-mediated biofilm defect could not be explained by the most obvious RcsB targets, we decided to carry out an unbiased suppressor analysis. Using pLacMu53, we generated a mutant library in the rcsC::Cm background, and individual mutants were screened for improved ability to form biofilms in the wells of microtitre plates when compared to the rcsC::Cm parental strain. In total, >5000 independent mutants were screened and this resulted in the identification of insertions in three genes: rcsD, barA and rprA. The localization of pLacMu53 in these mutants was determined: five independent insertions were found in rcsD, seven independent insertions were found in barA, and 1 insertion was found in rprA.

The rcsD gene is located upstream of rcsB and it has been proposed that rcsD and rcsB may be transcribed as an operon (Takeda et al., 2001). Therefore, it is likely that the suppression observed with the mutations in rcsD is due to polar effects on rcsB expression, and we decided not to study these mutants any further. The barA gene encodes the sensor kinase of the two-component system BarA-UvrY and this signalling pathway is involved in the regulation of several cellular processes, including cell division, motility, and carbon and iron metabolism (for a recent review see: Sahu et al., 2003). BarA is a hybrid kinase that is predicted to contain a transmitter domain, a receiver domain and an HPt domain. One of the insertions was mapped after codon 745 of the BarA protein (918 aa) and was expected to be in the receiver domain; this mutant was chosen for further analysis. The rprA gene encodes a small non-encoding RNA that post-transcriptionally controls the level of σ5 in the cell. Interestingly, the expression of rprA is regulated by the Rcs phosphorelay and the insertion identified in this study was mapped between the proposed RcsB binding box and the –35 box of the promoter of rprA. Therefore, Rcs-dependent regulation of rprA expression would not be expected to occur in this mutant. The selected insertions in rprA and barA were transduced into clean wild-type and rcsC::Cm backgrounds, and the effect of these mutations on biofilm formation in PVC wells was re-analysed. As shown in Fig. 2, mutations in either barA or rprA significantly improved biofilm formation in the rcsC mutant background.

Our hypothesis was based on the premise that the defect in biofilm formation was due to the inappropriate regulation of an Rcs-regulated gene. The expression of barA is not thought to be affected by the Rcs phosphorelay, and we confirmed this by comparing barA expression in the WT and rcsC background using a barA-lacZ fusion (data not shown). In addition, we have shown that the activity of BarA (as measured using a glgC-lacZ fusion) is also unaffected in the rcsC background (data not shown). On the other hand, the expression of rprA is regulated by the Rcs phosphorelay and it has been shown that there is a 10-fold increase in the level of the RprA transcript in the rcsC mutant background (Majdalani et al., 2002). Therefore, we decided to undertake further analysis on the role of rprA in the observed suppression of the rcsC mutation. As a starting point we constructed clean deletion mutants of rprA, i.e. rprA::Cm and rprA::Km, and showed that these alleles also improved biofilm formation in the rcsC::Tn10 and rcsC::Cm strains, respectively (Fig. 2, and data not shown). Moreover, the growth rate of each of the mutants was identical to the wild-type (data not shown). Therefore, we confirmed that deletion of rprA significantly suppressed the defect in biofilm formation observed in an rcsC mutant background.

**A deletion in rprA improves biofilm formation when RcsC is constitutively active**

The increase in RcsB-P levels in the rcsC mutant background would be expected to be quite modest since there is no significant induction in the production of colanic acid (our unpublished observations; Brill et al., 1988). Nonetheless, if the increased level of RcsB-P is...
directly responsible for the observed defect in biofilm formation, we would expect that full activation of the Rcs phosphorelay would have the same phenotype. We previously identified an rcsC allele encoding a constitutively active RcsC protein, i.e. this allele drives high levels of cps expression in the absence of any signal (Y. H. Huang, L. Ferrieres & D. J. Clarke, unpublished). This mutation was predicted to change the histidine at position 339 to aspartate (rcsCH339D). We took advantage of rcsCH339D to analyse the effect of constitutive activation of the Rcs system on biofilm formation. The plasmid carrying the rcsCH339D allele was introduced into the rcsC::Cm background and, as expected, cells producing RcsCH339D were impaired in biofilm formation compared to cells carrying a plasmid expressing rcsC+ (see Fig. 3). This is consistent with our hypothesis that the deficiency in biofilm formation observed in cells deleted for rcsC is due to accumulation of RcsB-P. Cells expressing rcsCH339D produce large amounts of colanic acid and this could inhibit biofilm formation by hiding short adhesins and changing the ability of the cell to adhere to the surface (Hanna et al., 2003). We therefore analysed the effect of RcsCH339D in a strain unable to produce capsule, BMM562 (rcsC::Cm gmd::iPlacMu53), and we found that the absence of capsule did not improve biofilm formation, suggesting that the deficiency in biofilm formation is not due to capsule overproduction (see Fig. 3). We also expressed rcsCH339D in the rcsC::Cm rprA::Km background and observed that biofilm formation was restored to 60% of the level obtained with the wild-type rcsC allele. This confirms that the constitutive activation of the Rcs phosphorelay, whether it is low-level activation (as observed in rcsC mutant strains) or high-level activation (by expressing an allele of rcsC that encodes a constitutively active sensor protein), impairs normal biofilm development via the increased expression of rprA.

Biofilm formation is inhibited if σ5 levels are increased independently of RprA

A previous study demonstrated that a mutation in rcsC resulted in a twofold increase in the level of σ5 in E. coli and this increase was largely dependent on rprA (Majdalani et al., 2002). Therefore, it was possible that the suppression that we observed with the rprA mutant could be explained by an effect on the level of σ5. We confirmed that there was a twofold increase in the level of σ5 in the rcsC::Cm cells, and this increase was largely due to rprA (Supplementary Fig. S3). Therefore, increased levels of σ5 may result in decreased biofilm formation. To test this we examined biofilm formation in strains where the level of σ5 was increased independently of RprA. It has been shown that a mutation in rscB results in an increase in σ5 levels and this is due to an increase in the post-translational stability of the sigma factor (Muffler et al., 1996; Pratt & Silhavy, 1996). Therefore, we transduced the rscB::Tc allele from strain AB012 (a gift from Susan Gottesman) into our wild-type background and compared the level of biofilm formation with the rcsC::Cm and wild-type strains (see Fig. 4a). Clearly, the mutation in rscB is equally affected in biofilm formation as the rcsC::Cm mutant, confirming that increased σ5 levels do affect the level of biofilm formation in E. coli. We also cloned the rpoS gene from ZK2686 into the expression vector pTRC99a, where expression is under the control of the Ptrc promoter and inducible with IPTG. Although we did not directly measure the levels of σ5 in these cells, it is clear that biofilm formation is affected by the presence of a plasmid that overexpresses rpoS, even in the absence of added inducer (see Fig. 4b). Therefore, increased levels of σ5 have a negative impact on biofilm formation in E. coli.

The defect in biofilm formation observed in rcsC mutant strains is dependent on rpoS

We reasoned that if the defect in biofilm formation in rcsC mutant strains resulted from the presence of an increased amount of σ5 in the cell, then deleting the rpoS gene should also restore biofilm formation to these cells. To test this hypothesis we transduced the rpoS::Km allele, from ZK1000 (Bohannon et al., 1991), into the wild-type and rcsC::Tn10 strains, and these strains were assayed for biofilm formation. In agreement with other studies (Adams & McLean, 1999; Collet et al., 2008) the deletion of rpoS had a severe impact on biofilm formation (Fig. 5). Nonetheless, we did observe an increase in biofilm formation.

![Fig. 3. The constitutive activation of RcsC alters biofilm formation in an rprA-dependent manner. A plasmid carrying the constitutively active rcsCH339D allele, pBMM624, was introduced into BMM5533 (rcsC::Cm), BMM562 (rcsC::Cm gmd::iPlacMu53) and BMM940 (rcsC::Cm rprA::Km), and the strains were tested for biofilm formation after 48 h static incubation in PVC wells at 30 °C in LB. For comparison, strain BMM533, carrying either the vector pBMM101 or a plasmid expressing wild-type rcsC (pBMM102), was included in the biofilm assay. Relative biofilm formation was determined by calculating the A595 mutant/A595 WT ratio. The values shown are the means of three independent experiments and the error bars represent SD.](http://mic.sgmjournals.org)
Increased levels of $\sigma^S$ decrease biofilm formation. (a) Strains ZK2686 (WT), BMM533 (rcsC::Cm) and CMM100 (rssB::Tc) were grown overnight and inoculated to an OD$_{600}$ of 0.01 into the wells of a PVC microtitre plate, and the plate was incubated at 30 °C, without agitation, for 48 h. Biofilm formation was quantified by staining with 0.1 % CV, and converted to relative units by dividing the $A_{595}$ obtained for each of the strains by the $A_{595}$ obtained for WT. Data shown are the means of at least three independent experiments and the error bars represent SD. (b) Strain ZK2686 was transformed with either pTRC99a (vector) or a pTRC99a derivative containing the rpoS gene under the control of the IPTG-inducible P$_{trc}$ promoter. The strains were tested for biofilm formation after 48 h static incubation in PVC wells at 30 °C in LB containing 0 mM (black bars), 0.1 mM (grey bars) or 1 mM (white bars) IPTG. For comparison, strain BMM533 carrying the vector pTRC99a was included in the biofilm assay. Biofilm formation was converted to relative units by dividing the $A_{595}$ obtained for each of the strains by the $A_{595}$ obtained for WT(pTRC99a) with no added IPTG. The values shown are the means of at least three independent experiments and the error bars represent SD.

**DISCUSSION**

In this study we have shown that a significant component of the biofilm defect in the rcsC mutant background is due to an increased level of $\sigma^S$ that is mediated by increased levels of the Rcs-regulated small RNA, RprA, present in this mutant. The inhibitory effect of inappropriately high levels of $\sigma^S$ on biofilm formation was confirmed by increasing $\sigma^S$ levels independently of RprA, namely by rssB inactivation and rpoS overexpression from a plasmid. We also identified insertion mutations in rcsD, rprA and barA as suppressors of the biofilm defect in the rcsC background. Interestingly, all of these mutations are in pathways that are involved in the regulation of $\sigma^S$ levels in the cell (Hengge, 2008). The insertion in rcsD is likely to have a polar effect on rcsB expression, and both rcsB and rprA are involved in controlling $\sigma^S$ levels through the Rcs phosphorelay. BarA, on the other hand, is required for the transcription of rpoS, confirming that the effect of rprA is exclusively due to its action on the levels of $\sigma^S$. Finally, the rcsC rpoS double mutant did not form as much biofilm as the rpoS mutant, suggesting that part of the rcsC-dependent biofilm defect remains independent of $\sigma^S$. This is consistent our observation that an rprA or a barA mutation does not completely restore biofilm formation in the rcsC background (Fig. 2).
during exponential growth, and a mutation in barA has been shown to reduce the level of $\sigma^S$ in the cell (Mukhopadhay et al., 2000). However, BarA is a sensor kinase and the BarA-UvrY two-component pathway in E. coli has been shown to regulate the expression of many genes that have a clear role in biofilm formation. Nonetheless, the suppressive effects of the barA and rprA mutations are not additive, suggesting that these mutations exert their effects through the same pathway, i.e. the level of $\sigma^S$ (see Fig. 2). Therefore, an increase in the level of $\sigma^S$ as low as twofold is sufficient to be detrimental for biofilm development in E. coli.

The role of $\sigma^S$ in biofilm formation is unclear and appears to be dependent on the nature of the rpoS allele being tested and the conditions used for biofilm formation. For example, different deletion mutations in rpoS have been reported to have opposite effects on the ability of cells to attach to surfaces (Adams & McLean, 1999; Corona-Izquierdo & Membrillo-Hernandez, 2002). However, given that cells are likely to be exposed to a variety of stresses during the development of a biofilm and $\sigma^S$ has been shown to regulate the expression of many genes encoding proteins involved in biofilm formation, an important role for $\sigma^S$ in biofilm formation would not be surprising (Bougdour et al., 2008; Hengge, 2008; Patten et al., 2004; Weber et al., 2005). In our genetic background, the introduction of the rpoS::Km allele negatively affected biofilm formation which explains why rpoS was not picked up in our screen. Therefore, it appears that both the absence of $\sigma^S$ and the presence of increased levels of $\sigma^S$ can severely inhibit biofilm formation in E. coli. A recent study showed that rpoS is differentially expressed in different layers of the biofilm (Ito et al., 2009). Interestingly, the same study showed an overlap between the layers where rpoS is expressed and the regions where several known Rcs targets, in particular the genes of the cps operon, are induced. These observations highlight the requirement for the appropriate control of $\sigma^S$ levels during biofilm formation, and our study clearly implicates the Rcs phosphorelay as an important component of the regulatory network controlling $\sigma^S$ levels.

Several studies have reported that $\sigma^S$ controls the expression of $>500$ genes, making it very difficult to determine whether any single $\sigma^S$-controlled gene is responsible for the biofilm-defective phenotype reported here (Patten et al., 2004; Weber et al., 2005). The vast majority of $\sigma^S$-controlled genes are positively regulated by the sigma factor and, therefore, the absence of known $\sigma^S$-regulated genes in our suppressor analysis would suggest that the biofilm phenotype is not the result of changes in the expression of a single gene or locus. On the other hand, a significant number of genes are negatively regulated by $\sigma^S$, primarily as the result of competition between sigma factors (in particular $\sigma^S$ and $\sigma^D$, the housekeeping sigma factor) for the core RNA polymerase enzyme (Ferenci, 2005; Nystrom, 2004). Many of these $\sigma^S$-repressed genes are associated with motility, a trait with a key role in biofilm formation (Weber et al., 2005). Flagellar-mediated motility plays an important role in biofilm formation, and the degree of motility has been positively correlated with the ability to form biofilms (Pratt & Kolter, 1998; Wood et al., 2006). Therefore, one possibility was that the increased levels of $\sigma^S$ found in the rcsC mutant could affect biofilm formation by reducing motility. However, we did not observe any difference in the migration rates of the rcsC mutant and the migration rate of the rcsC rprA mutant, confirming that $\sigma^S$ does not affect motility under these conditions (Supplementary Fig S4). Importantly, RcsB-P also affects motility, independently of $\sigma^S$, by directly binding to, and repressing the activity of, the promoter driving flhDC expression (Supplementary Fig. S1; Francez-Charlot et al., 2003). Therefore, it remains possible that this RcsB-dependent decrease in motility does contribute to the $\sigma^S$-independent biofilm defect observed in the rprA rcsC, barA rcsC and rpoS rcsC mutants. However, we have not been able to establish whether the relatively small decrease in motility observed in the absence of rcsC does affect biofilm formation in any way. Therefore, although we have clearly shown that the biofilm defect observed in the rcsC mutant strain involves $\sigma^S$, the exact nature of the defect is complex and will involve many genes.

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REFERENCES


Bremer, E., Silhavy, T. J. & Weinstock, G. M. (1985). Transposable lambda placMu bacteriophages for creating lacZ operon fusions and


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