Elevated levels of σ^S inhibit biofilm formation in *Escherichia coli*: a role for the Rcs phosphorelay

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The Rcs phosphorelay is composed of RcsC, RcsD and the response regulator RcsB, and this signalling pathway has been implicated in virulence and biofilm formation in many enteric bacteria. It was previously shown that a mutation in *rscC* resulted in defective biofilm formation in *Escherichia coli* [Ferrières, L. & Clarke, D. J. (2003) *Mol Microbiol* **50**, 1665–1682]. To identify the molecular mechanisms underlying the observed biofilm defect we carried out a screen looking for suppressor mutants that restored biofilm formation in the *rscC* mutant background. One of the mutants was identified to be in *rprA*, a gene encoding a small RNA molecule that is involved in the post-transcriptional control of the alternative sigma factor, σ^S . The expression of *rprA* is regulated by the Rcs phosphorelay, and there are elevated σ^S levels present in the *rscC* mutant due to the overexpression of *rprA* in this background. Using different approaches, we have established that the increase in σ^S levels is responsible for the biofilm defect. Therefore, the Rcs phosphorelay is involved in maintaining appropriate levels of σ^S during biofilm formation in *E. coli*.

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-

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Abbreviations: CV, crystal violet; HPT domain, histidine-phosphotransfer domain; RcsB-P, phospho-RcsB.

Figures demonstrating that mutations in *rscC* and *rscD* reduce expression of *flhDC* and motility, colanic acid production is not responsible for the biofilm defect observed in the *rscC* mutant background, the deletion of *rprA* restores σ^S levels in the *rscC* mutant, and the RprA-mediated increase in the level of σ^S is not responsible for the decrease in motility, are available with the online version of this paper.

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 biosynthesis (Ferrières & Clarke, 2003; Francez-Charlot *et al.*, 2003; Vianney *et al.*, 2005). Therefore, the activation of the Rcs phosphorelay would be expected to have a negative effect on the attachment of cells to a surface. Together, these reports suggest that the Rcs phosphorelay might 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, σ^S , through the activation of RprA, a small non-coding RNA that positively controls the level of σ^S in the cell by base-pairing with the 5' end of the *rpoS* mRNA (Majdalani *et al.*, 2001, 2002). σ^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 σ^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 σ^S present in this background, suggesting that the temporal control of σ^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-lacU*)169] (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 concentration ($\mu\text{g ml}^{-1}$), 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 λ Red recombinase 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'-GTGTAGGCTGGAGCTGCTTC-3' (forward primer); 5'-CATATGAATATCCTCCTTAG-3' (reverse primer). After purification, the PCR-amplified cassettes were introduced by electroporation into ZK2686 cells expressing λ Red recombinase from pKD46 (Datsenko & Wanner, 2000). The transformants were selected on plates containing appropriate antibiotics at the following concentrations ($\mu\text{g ml}^{-1}$): 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'-CATGCCATGGTCAGAATACGCTGAAAG-3') and LF169 (5'-GCTCTAGAGCTTACTTACTCGCGGAACAG-3'). The purified PCR amplicon was digested with *NcoI* and *XbaI* and ligated into the corresponding sites in pTRC99a. The ligation mix was electroporated into strain EC100 and positive clones were selected by colony PCR (using the primers DC160, 5'-CTTGCATGCCTGCAGGTCG-3', and DC164, 5'-CAATTAATCATCCGGCTCG-3'). One clone was selected, pRpoS, and sequenced to confirm the integrity 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 $\text{OD}_{600}=0.01$ 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 λ p/acMu53. A random insertion library of BMM533 was constructed using λ p/acMu53, 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 λ p/acMu53 (10^8 p.f.u.) and λ pMu507 (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 λ p/acMu53 integrants were finally selected by plating 100 μl of 10^{-1} to 10^{-4} 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 λ p/acMu53 insertion was present in the genome and to confirm that the mutation itself did not affect biofilm formation. The site of

Table 1. Strains and plasmids

Strain/plasmid/phage	Relevant characteristics	Source
Strain		
ZK2686	W3110 $\Delta(\text{argF-lac})U169$	Danese <i>et al.</i> (2000a)
BMM520	ZK2686 <i>rcsC52::Tn10</i>	Ferrières & Clarke (2003)
BMM526	ZK2686 <i>gmd::λplacMu53</i>	Ferrières & Clarke (2003)
BMM532	ZK2686 <i>rcsB::Cm</i>	This work
BMM533	ZK2686 <i>rcsC::Cm</i>	This work
BMM534	ZK2686 <i>rcsF::Cm</i>	This work
BMM551	ZK2686 <i>rcsA::Cm</i>	This work
BMM552	BMM520 <i>rcsB::Cm</i>	This work
BMM555	BMM526 <i>rcsC52::Tn10</i>	This work
BMM562	BMM533 <i>gmd::λplacMu53</i>	This work
BMM564	ZK2686 <i>rcsD::Cm</i>	This work
BMM586	BMM533 <i>barA::λplacMu53</i>	This work
BMM588	ZK2686 <i>barA::λplacMu53</i>	This work
BMM589	ZK2686 <i>rprA::λplacMu53</i>	This work
BMM591	BMM533 <i>rprA::λplacMu53</i>	This work
BMM910	ZK2686 <i>rpoS::Km</i>	This work
BMM911	BMM520 <i>rpoS::Km</i>	This work
BMM921	ZK2686 (<i>rcsD-rcsB</i>)::Cm	This work
BMM938	ZK2686 <i>rprA::Km</i>	This work
BMM939	ZK2686 <i>rprA::Cm</i>	This work
BMM940	BMM533 <i>rprA::Km</i>	This work
BMM941	BMM520 <i>rprA::Cm</i>	This work
BMM942	BMM911 <i>rprA::Cm</i>	This work
CMM100	ZK2686 <i>rssB::Tc</i>	This work
CMM102	BMM941 <i>barA::λplacMu53</i>	This work
ZK1000	W3110 $\Delta\text{lac}U169$ <i>tna-2 rpoS::Km</i>	Bohannon <i>et al.</i> (1991)
AB012	MG1655 <i>rssB::Tc</i>	Bougdour <i>et al.</i> (2006)
Plasmid		
pTRC99a	<i>ori colEI</i> , Ap ^R	Laboratory stock
pBMM101	pTRC99a-derivative plasmid carrying a <i>c-myc</i> epitope tag, Ap ^R	Clarke <i>et al.</i> (2002)
pBMM102	pBMM101, <i>rcsC</i> ⁺	Clarke <i>et al.</i> (2002)
pBMM624	pBMM101, <i>rcsCH339D</i> (encodes a constitutive RcsC protein)	Y. Huang
pRpoS	pTRC99a derivative carrying <i>rpoS</i>	This work
pKD3	Template plasmid carrying Cm ^R cassette	Datsenko & Wanner (2000)
pKD4	Template plasmid carrying Km ^R cassette	Datsenko & Wanner (2000)
pKD46	Thermosensitive, Ap ^R , encodes the λ Red recombinase	Datsenko & Wanner (2000)
Phage		
P1 _{vir}	Strictly lytic phage	Laboratory stock
P1 _{cml}	Lysogenic P1 derivative, Cm ^R	Laboratory stock
λ placMu53	λ -Mu hybrid phage carrying <i>lacZY</i> , Km ^R	Bremer <i>et al.</i> (1985)
λ pMu507	c1s857 Sam7 MuA ⁺ B ⁺	Magazin <i>et al.</i> (1977)

λ placMu53 insertion in each mutant was determined by arbitrary-primed PCR, as described previously (Ferrières & Clarke, 2003).

Western blotting. The amount of σ^S present was determined by SDS-PAGE and immunoblotting. Bacteria were grown at 30 °C with agitation, centrifuged and resuspended to OD₆₀₀=20 in loading buffer (62.5 mM Tris/HCl, pH 6.8, 10 %, v/v, glycerol, 2 %, w/v, SDS, 5 %, v/v, β -mercaptoethanol, 0.025 %, w/v, Bromophenol Blue). The cells were lysed by heating to 100 °C for 5 min, and 10 μ l (equivalent to 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 μ m; 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 σ^S protein, the membrane was incubated for 4 h at 4 °C in a 1/2000 dilution of anti- σ^S antiserum (a kind gift from Regine Hengge) in hybridization solution (0.5 % milk powder, 0.05 % Tween 20 in PBS), rinsed three times with the hybridization solution and incubated for 1 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 incubated for 5 min in ECL Advance Western blotting detection reagents (Amersham Biosciences) and exposed to a CCD camera

(Hamamatsu). Data analysis was carried out using Labworks 3.02 software (UVP Inc).

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

Fig. 1. RcsC and RcsD are required for normal biofilm formation in the presence of RcsB. Strains from an overnight culture were inoculated at an OD₆₀₀ of 0.01 into the wells of PVC microtitre plates and incubated at 30 °C, without agitation, for 48 h. The attached cells were stained with 0.1 % CV and biofilm formation was quantified by dissolving the CV in ethanol and measuring A₅₉₅. Strains: ZK2686, WT; BMM520, *rcsC::Tn10*; BMM533, *rcsC::Cm*; BMM552, *rcsC::Tn10 rcsB::Cm*; BMM564, *rcsD::Cm*; BMM921, *(rcsD-rcsB)::Cm*; BMM532, *rcsB::Cm*; BMM551, *rcsA::Cm*; BMM534, *rcsF::Cm*. Relative biofilm formation was determined by calculating the A₅₉₅ mutant/A₅₉₅ WT ratio. The values shown are the means of at least three independent experiments and the error bars represent SD.

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 *rprA* 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::λplacMu53* (Ferrières & Clarke, 2003), into the *rscC::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 *rscC*-mediated biofilm defect could not be explained by the most obvious RcsB targets, we decided to carry out an unbiased suppressor analysis. Using *λplacMu53*, we generated a mutant library in the *rscC::Cm* background, and individual mutants were screened for improved ability to form biofilms in the wells of microtitre plates when compared to the *rscC::Cm* parental strain. In total, >5000 independent mutants were screened and this resulted in the identification of insertions in three genes: *rscD*, *barA* and *rprA*. The localization of *λplacMu53* in these mutants was determined: five independent insertions were found in *rscD*, seven independent insertions were found in *barA*, and 1 insertion was found in *rprA*.

The *rscD* gene is located upstream of *rscB* and it has been proposed that *rscD* and *rscB* may be transcribed as an operon (Takeda *et al.*, 2001). Therefore, it is likely that the suppression observed with the mutations in *rscD* is due to polar effects on *rscB* 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 σ^S 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 *rscC::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 *rscC* 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 *rscC* background using a *barA-lacZ* fusion (data not

Fig. 2. The deletion of either *barA* or *rprA* restores biofilm formation in the *rscC* mutant background. Strains ZK2686 (WT), BMM533 (*rscC::Cm*), BMM588 (*rscC::Cm barA::λplacMu53*), BMM591 (*rscC::Cm rprA::λplacMu53*), BMM586 (*barA::λplacMu53*), BMM589 (*rprA::λplacMu53*), BMM520 (*rscC52::Tn10*), BMM941 (*rscC52::Tn10 rprA::Cm*), CMM102 (*rscC52::Tn10 rprA::Cm barA::λplacMu53*) and BMM939 (*rprA::Cm*) were grown overnight and inoculated to an OD₆₀₀ 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. The values shown are the means of at least three independent experiments and the error bars represent SD.

shown). In addition, we have shown that the activity of BarA (as measured using a *glgC-lacZ* fusion) is also unaffected in the *rscC* 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 *rscC* mutant background (Majdalani *et al.*, 2002). Therefore, we decided to undertake further analysis on the role of *rprA* in the observed suppression of the *rscC* 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 *rscC::Tn10* and *rscC::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 *rscC* mutant background.

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

The increase in RcsB-P levels in the *rscC* 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 *rscC* 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. Ferrières & D. J. Clarke, unpublished). This mutation was predicted to change the histidine at position 339 to aspartate (*rscCH339D*). We took advantage of *rscCH339D* to analyse the effect of constitutive activation of the Rcs system on biofilm formation. The plasmid carrying the *rscCH339D* allele was introduced into the *rscC::Cm* background and, as expected, cells producing RcsCH339D were impaired in biofilm formation compared to cells carrying a plasmid expressing *rscC⁺* (see Fig. 3). This is consistent with our hypothesis that the deficiency in biofilm formation observed in cells deleted for *rscC* is due to accumulation of RcsB-P. Cells expressing *rscCH339D* 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 (*rscC::Cm gmd::λplacMu53*), 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 *rscCH339D* in the *rscC::Cm rprA::Km*

background and observed that biofilm formation was restored to 60 % of the level obtained with the wild-type *rscC* allele. This confirms that the constitutive activation of the Rcs phosphorelay, whether it is low-level activation (as observed in *rscC* mutant strains) or high-level activation (by expressing an allele of *rscC* that encodes a constitutively active sensor protein), impairs normal biofilm development via the increased expression of *rprA*.

Biofilm formation is inhibited if σ^S levels are increased independently of RprA

A previous study demonstrated that a mutation in *rscC* resulted in a twofold increase in the level of σ^S 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 σ^S . We confirmed that there was a twofold increase in the level of σ^S in the *rscC::Cm* cells, and this increase was largely due to *rprA* (Supplementary Fig. S3). Therefore, increased levels of σ^S may result in decreased biofilm formation. To test this we examined biofilm formation in strains where the level of σ^S was increased independently of RprA. It has been shown that a mutation in *rssB* results in an increase in σ^S 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 *rssB::Tc* allele from strain AB012 (a gift from Susan Gottesman) into our wild-type background and compared the level of biofilm formation with the *rscC::Cm* and wild-type strains (see Fig. 4a). Clearly, the mutation in *rssB* is equally affected in biofilm formation as the *rscC::Cm* mutant, confirming that increased σ^S 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 *P_{trc}* promoter and inducible with IPTG. Although we did not directly measure the levels of σ^S 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 σ^S have a negative impact on biofilm formation in *E. coli*.

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

We reasoned that if the defect in biofilm formation in *rscC* mutant strains resulted from the presence of an increased amount of σ^S 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 *rscC::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

Fig. 3. The constitutive activation of RcsC alters biofilm formation in an *rprA*-dependent manner. A plasmid carrying the constitutively active *rscCH339D* allele, pBMM624, was introduced into BMM533 (*rscC::Cm*), BMM562 (*rscC::Cm gmd::λplacMu53*) and BMM940 (*rscC::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 *rscC* (pBMM102), was included in the biofilm assay. Relative biofilm formation was determined by calculating the A_{595} mutant/ A_{595} WT ratio. The values shown are the means of three independent experiments and the error bars represent SD.

Fig. 4. Increased levels of σ^S decrease biofilm formation. (a) Strains ZK2686 (WT), BMM533 (*rcsC::Cm*) and CMM100 (*rcsB::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.

formation in the *rcsC rpoS* double mutant when compared to the parental *rcsC::Tn10* mutant, suggesting that σ^S does have a negative effect on biofilm formation in this background. Furthermore, to confirm that the suppression observed by deleting *rprA* is mediated by its action on the *rpoS* mRNA, we constructed a *rcsC rpoS rprA* triple mutant. We reasoned that if *rprA* suppression involved a target other than *rpoS*, then we should observe an increase in biofilm formation in the *rcsC rpoS rprA* strain when compared to the parental *rcsC rpoS* strain. However, as shown in Fig. 5, both the *rcsC rpoS* and *rcsC rpoS rprA* strains displayed similar biofilm-forming abilities, con-

Fig. 5. Effect of *rpoS* on biofilm formation in the *rcsC* mutant background. Strains ZK2686 (WT), BMM520 (*rcsC::Tn10*), BMM911 (*rcsC::Tn10 rpoS::Km*), BMM942 (*rcsC::Tn10 rpoS::Km rprA::Cm*) and BMM910 (*rpoS::Km*) were grown overnight and inoculated to an OD_{600} of 0.01 into the wells of a PVC microtitre plate and incubated at 30 °C without agitation for 48 h. Biofilm formation was quantified by staining with 0.1 % CV and measuring A_{595} . Relative biofilm formation was determined by calculating the A_{595} mutant/ A_{595} WT ratio. The values shown are the means of three independent experiments and the error bars represent SD.

firmed that the effect of *rprA* is exclusively due to its action on the levels of σ^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 σ^S . This is consistent with our observation that an *rprA* or a *barA* mutation does not completely restore biofilm formation in the *rcsC* background (Fig. 2).

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 σ^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 σ^S on biofilm formation was confirmed by increasing σ^S levels independently of RprA, namely by *rcsB* 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 σ^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 σ^S levels through the Rcs phosphorelay. BarA, on the other hand, is required for the transcription of *rpoS*

during exponential growth, and a mutation in *barA* has been shown to reduce the level of σ^S in the cell (Mukhopadhyay *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 σ^S (see Fig. 2). Therefore, an increase in the level of σ^S as low as twofold is sufficient to be detrimental for biofilm development in *E. coli*.

The role of σ^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 σ^S has been shown to regulate the expression of many genes encoding proteins involved in biofilm formation, an important role for σ^S in biofilm formation would not be surprising (Bougourd *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 σ^S and the presence of increased levels of σ^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 σ^S levels during biofilm formation, and our study clearly implicates the Rcs phosphorelay as an important component of the regulatory network controlling σ^S levels.

Several studies have reported that σ^S controls the expression of >500 genes, making it very difficult to determine whether any single σ^S -controlled gene is responsible for the biofilm-defective phenotype reported here (Patten *et al.*, 2004; Weber *et al.*, 2005). The vast majority of σ^S -controlled genes are positively regulated by the sigma factor and, therefore, the absence of known σ^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 σ^S , primarily as the result of competition between sigma factors (in particular σ^S and σ^{70} , the housekeeping sigma factor) for the core RNA polymerase enzyme (Ferenci, 2005; Nystrom, 2004). Many of these σ^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 σ^S found in the *rscC* mutant could affect biofilm formation by reducing motility. However, we did not observe any difference in the migration rates of the *rscC* mutant and the migration rate of the *rscC rprA* mutant, confirming that σ^S does not affect motility under these conditions (Supplementary Fig S4). Importantly, RcsB-P also affects motility, independently of σ^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 σ^S -independent biofilm defect observed in the *rprA rscC*, *barA rscC* and *rpoS rscC* mutants. However, we have not been able to establish whether the relatively small decrease in motility observed in the absence of *rscC* does affect biofilm formation in any way. Therefore, although we have clearly shown that the biofilm defect observed in the *rscC* mutant strain involves σ^S , the exact nature of the defect is complex and will involve many genes.

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