Deletion analysis of RcsC reveals a novel signalling pathway controlling poly-N-acetylglucosamine synthesis and biofilm formation in *Escherichia coli*

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INTRODUCTION

The Rcs system was first described in *Escherichia coli* based on its role in regulating colanic acid synthesis (directed by the *cps* genes), the capsular polysaccharide conferring a mucoid phenotype when overproduced. Its basic design corresponds to the two-component family system: nevertheless, RcsC is a hybrid sensor kinase that upon receiving a signal hydrolyses ATP and autophosphorylates, and it then transfers the phosphoryl group from a conserved histidine residue to a conserved aspartate in its receiver domain. This domain functions as a phosphoryl donor to RcsD, a histidine phosphotransferase, which in turn transfers the phosphoryl group to RcsB, the cognate response regulator, able to function as a DNA-binding protein. In addition, RcsB can function as a homodimer or forms heterodimers with either RcsA or BglJ. When unstimulated, RcsC functions as a net phosphatase (Fredericks *et al.*, 2006). Furthermore, RcsA is a substrate of the Lon protease (Brill *et al.*, 1988; Chen *et al.*, 2001; Fredericks *et al.*, 2006; Gottesman *et al.*, 1985; Salscheider *et al.*, 2014; Takeda *et al.*, 2001; Torres-Cabassa & Gottesman, 1987); for a review see Majdalani & Gottesman (2005).

It has been reported that the Rcs system is activated by osmotic shock or when cells are grown at low temperature (20 °C), in the presence of (0.4 %) glucose and 1 mM external zinc (El-Kazzaz *et al.*, 2004; Francez-Charlot *et al.*, 2005; Hagiwara *et al.*, 2003; Stout & Gottesman, 1990). It has also been proposed that it responds to peptidoglycan damage (Laubacher & Ades, 2008).

There are a large number of Rcs regulon members. Under particular growth conditions, 15 new Rcs-regulated genes were reported besides the 17 *cps* genes already known (Hagiwara *et al.*, 2003), and the overexpression of *djlA*, coding for an upstream signalling protein, rendered more than 150 genes putatively controlled by the Rcs system (Ferrières & Clarke, 2003). These authors showed the involvement of this system in normal biofilm formation in *E. coli*, suggesting that it plays an important role in remodelling the bacterial surface, as 50 % of the Rcs-regulated genes encode proteins predicted to be located in the envelope or to have activities that affect it.

Several molecules and surface structures have been implicated in biofilm formation in *E. coli*. Among these are the flagella, fimbriae, autotransporter proteins, curli, colanic acid and conjugative pili [for a review see Van Houdt & Michiels (2005), and references therein]. For biofilm formation, the following developmental stages have been proposed: surface contact and reversible attachment, irreversible attachment, microcolony formation and early development of biofilm structure, maturation and dispersal. Importantly, it has been shown that genes involved in these different stages are reciprocally regulated by Rcs, with repression of flagella,
curli and the autotransporter adhesin antigen 43, and activation of colanic acid synthesis. In addition, it has been proposed that the Rcs phosphorelay regulates temporal development of biofilm by controlling the transition from attached cells to mature biofilm [for reviews see Huang et al. (2006) and Dorel et al. (2006), and references therein].

More recently, a genetic locus promoting surface binding, intracellular adhesion and biofilm formation was identified. It contains the genes *pgaABCD* coding for envelope proteins involved in synthesis, translocation and possibly surface docking of a particular polysaccharide (unbranched β-1,6-N-acetyl-β-d-glucosamine, polyglucosamine or PGA), previously unknown for Gram-negative bacteria but present in staphylococci (Wang et al., 2004). It has been reported that transcription of the *pgaABCD* operon requires NhaR, a LysR family member of DNA-binding proteins. In an *nhaR* background, PGA production is undetectable (Cerca & Jefferson, 2008; Goller et al., 2006).

Furthermore, PGA formation is repressed at the RNA level: *pga* transcript availability is regulated by the CsrA RNA-binding protein. Binding of CsrA to *pga* inhibits 30S ribosomal subunit binding, preventing translation and promoting transcript degradation (Wang et al., 2005); it also prevents 5′ *pga* mRNA secondary structure formation, thus facilitating transcription termination by Rho (Figueroa-Bossi et al., 2014).

An analysis of the role played by the RcsC hybrid sensor kinase domains is presented here, which unveils the existence of a signalling pathway independent of and alternative to the RcsD and RcsB phosphorelay proteins, involving the OmpR response regulator, which acts by repressing the *pgaABCD* operon and can be phosphorylated by acetyl phosphate. Moreover, an epistasis test indicates a complex regulatory network involving further unknown players and a wider role for acetyl phosphate.

It is important to mention that several studies with RcsC have been done by cloning *rcsC* on multicopy plasmids under the control of inducible promoters to enhance RcsC-associated phenotypes (Fredericks et al., 2006; Huang et al., 2006, 2009), which was the approach followed here; further efforts to study the phenotype evidenced in this report, under more physiological conditions, are under way.

**METHODS**

**Bacterial strains and plasmids used.** *E. coli* K-12 strain BW25113 and isogenic deletion mutants of the Keio collection or previously reported isogenic mutants supplied by the *E. coli* Genetic Stock Center at Yale University were used (Baba et al., 2006; Zhou et al., 2005). When a mutant was not available, it was generated by the one-step inactivation procedure described by Datsenko & Wanner (2000). Double mutants were made by eliminating the kanamycin resistance cassette of the single mutant using plasmid pCP20 (Datsenko & Wanner, 2000), and then moving the second mutation, still carrying the kanamycin gene, by P1 transduction as suggested on the OpenWetWare website (http://openwetware.org/wiki/Sauer:P1vir_phage_transduction). Triple mutants were generated following a similar reiterative procedure. Plasmids used as cloning vectors were pMAL from New England Biolabs, pMMP-T6O (Mayer, 1995) and pKK232-8 from Pharmacia. Other plasmids used to generate mutants were pKD46 and pKD4 (Datsenko & Wanner, 2000). *E. coli* AJW678 and the AJW1939 (*ackA*) and AJW2013 (*pta-ackA*) mutant strains were previously described (Fredericks et al., 2006; Kumari et al., 2000). For the complete list of strains used in this study see Table S1 (available in the online Supplementary Material).

**PCR for gene amplification, cloning and subcloning.** Primers used for gene amplification are reported in Table S2. Fragments of *rcsC* amplified by PCR were cloned in pMAL to generate fusion proteins tagged at the N terminus with full-length MBP. Then, taking advantage of an Ncol restriction site present 155 nt upstream of the *m63E* stop codon, the *rcsC* fragments were subcloned into pMMP-T6O, under control of the stricter pBAD promoter, to obtain the pMMP derivatives tagged with 50 MBP amino acids at the N terminus.

**Mucoidy assay.** To monitor mucoidy, *E. coli* strain AJW678 alone or transformed with plasmids pMMP-T6O or pMMPABD was grown on M63 minimal salts agar with 0.4 % (w/v) glucose, 2 mM ZnCl2 and 0.025 % L-arabinose. Plates were incubated at 25 °C for 48 h and images were taken with the Alphaimager mini device (Cell Biosciences) using epillumination visible light.

**Assay for biofilm formation.** The procedure of Stepanovic et al. (2000) to quantify biofilm formation, using 96-well polystyrene microtitre plates (Costar cat. no. 3599, flat bottom with lid), was followed with slight modifications. Briefly, bacterial cells were grown overnight in Luria–Bertani medium (LB) supplemented with 0.025 % (w/v) glucose and the appropriate antibiotic for plasmid maintenance. Cells were diluted 1:100 in 200 μl LB + water (1:1 ratio) containing antibiotic, and glucose (0.025 %) or L-arabinose (0.025 %) for the controls and biofilm-producing cells, respectively. Four to six wells contained the same bacterial dilution. Three microtitre plates were prepared for each experiment to quantify biofilm formation every 4 h. Plates were incubated at 30 °C. At the indicated times, total cell growth was measured at OD570 using a Bio–Tek Elx808 microplate reader, with the KC4 software, and the planktonic cells were then discarded and the plate treated with 200 μl of the following reagents: rinsed three times with 1 × PBS; and the remaining biofilm was fixed with methanol (100 %), stained with crystal violet (2 %), rinsed with water three times and the dye was solubilized with acetic acid (33 %). Finally, the OD570 was determined with the microplate reader. The amount of biofilm formed is reported as the ratio of the OD570/OD420 values, which corresponds to a simplified expression of that used by Niba et al. (2007).

**Construction of cat fusions.** Reporter *pgaA–cat* fusions were constructed by PCR amplification using *E. coli* BW25113 chromosomal DNA as template and pGaBAmHF as the forward primer and either pgaAHinD1R or pgaAHinD2R as the reverse primers. The first pair of forward and reverse primers amplifies a DNA fragment of 508 bp containing four NhaR-binding boxes, the *pga* promoter region and its transcription start site, while the second set amplifies an extended 734 bp fragment containing further downstream sequence including the *pgaA* leader mRNA where six CsrA-binding boxes have been identified. Both PCR products were digested with BamHI and HindIII and cloned into the pK232-8 vector digested with the same enzymes, rendering plasmids pKK232pgaA1 and pKK232pga2N, respectively. Construction of the *ompC-cat* fusion was as previously reported (Villarreal et al., 2014).

**Gene reporter activity assays.** To evaluate the *pgaA–cat* reporter fusions, the pKK232pgaA1 or pKK232pga2N plasmids were transformed into strain JW1007-2, a BW25113 *pgaC* ORF deletion
derivative carrying a kanamycin cassette; this strain was used to avoid bacterial clumping while sampling, which allows more accurate OD determination. The pgaC mutation was combined with all other genetic backgrounds tested. CAT-specific activity was calculated as reported by Martinez-Laguna et al. (1999), with minor modifications: strains were grown in LB diluted (1:1) with sterile water, at 37 °C and 220 r.p.m. Glucose or L-arabinose was added at 0.025% when cultures reached an OD600 of 0.4 and samples were taken at the indicated OD.

OmpR-6xHis-tagged protein purification and phosphorylation in vitro. OmpR was overexpressed and purified as described previously (Oropeza & Calva, 2009). Purified OmpR protein was incubated with 500 mM acetyl phosphate dissolved in 10 mM Tris in phosphorylation buffer (50 mM Tris/HCl, 50 mM KCl and 20 mM MgCl2) for 2 h at room temperature.

Electrophoretic mobility shift assay (EMSA) and DNase I protection assays. The EMSA and DNase I protection experiments were performed as previously reported (De la Cruz et al., 2009; Oropeza et al., 1999). DNA fragments for EMSA and footprinting were PCR amplified using primers pgaABamHF and pgaAHinD2R, and plasmid pKK232pgaA2N as template, or pgaABamHF and pgaAHinD1R, and plasmid pKK232pgaA1 as template, respectively. For EMSA, 80 ng DNA per reaction was used. The amounts of OmpR protein used in the EMSA experiments are specified in the figure legend Fig. 6(b).

EnvZ F390L generation and cloning. The DNA region coding for the EnvZ cytoplasmic domain was amplified by PCR, using chromosomal DNA from E. coli strain BW25113 as a template and primers envZKpnNcoF and envZpstR. The product was digested with KpnI and PstI and cloned into plasmid pUC18 digested with the same restriction enzymes. The wild-type sequence was corroborated with the M13/pUC forward and reverse universal primers. The pUC primers and the mutagenic semi-complementary primers, envZF390L or envZF390Lr, were used to obtain two PCR products, which were purified and combined in equimolar ratios as templates for a second PCR. This PCR product was gel purified, digested with KpnI and PstI and cloned again into the pUC18 plasmid. After corroboration by sequencing that the F390L mutation had been introduced, an NcoI–PstI restriction fragment was subcloned into the pMPM-T6Ω vector digested with the same restriction enzymes.

RESULTS

Delimitation of RcsC domains

The initial aim of this study was to better understand the phosphorylation in RcsC, a 949 aa hybrid sensor kinase. Based on alignments of RcsC with other histidine kinases (data not shown) and the motif tool at www.genome.jp, this protein was divided into the following functional domains: the periplasmic domain (P) from amino acid 64 to 337, which includes the second transmembrane segment; the linker domain (L) from amino acid 338 to 455; the AB domain, from amino acid 456 to 705, where the His phosphorylation site and the ATP-binding subdomain reside; and the receiver domain (D), from amino acid 706 to 949, which contains the aspartic residue involved in phosphorylase.

Four plasmids were generated and named according to the RcsC domains carried on them: PLAB, residues 64–705; LAB, 338–705; AB, 456–705; and ABD, 456–949. Depending on the backbone vector used, they were either pMal or pMMP derivatives, as explained in Methods (Fig. 1). Thus, the resulting recombinant E. coli cells were noted for their high capacity for biofilm formation. This phenomenon was therefore explored in greater detail.

Expression of some RcsC domains produced biofilm formation either in a wild-type or an rcsCDB background

The four pMMP derivatives, carrying different RcsC domains (Fig. 1), were transformed into strain BW25113 and its ΔrcsCDB (BW27557) derivative, and biofilm formation was quantified as described in Methods (Fig. 2a). Under control growth conditions (glucose at 0.025%), the amount of biofilm produced was very low, less than 0.5 relative units (biofilm/total growth). Conversely, when L-arabinose was added at two concentrations (0.025 and 0.1%), different amounts of biofilm were produced depending on the RcsC construct expressed from the plasmid, but independently of the genetic background and the arabinose concentration (Fig. 2a and data not shown). Remarkably, biofilm was produced at relatively high levels, in times as short as 4 h, and kept increasing during the next 8 h. Plasmids expressing the RcsC ABD and LAB domains induced the highest amount of biofilm (with around 16 and 13 relative units at 8 h), while the strains carrying the AB or the PLAB plasmids produced moderate (with about 5 units at 8 h) or the lowest (0.5 units at 8 h) amount of biofilm, respectively, but still above the background levels. The same profile of biofilm production was observed when the pMAL derivatives were used (data not shown). Immunological detection of the MBP tag, carried by each recombinant protein, indicated that all of the RcsC protein constructs were produced in equivalent amounts (data not shown).

Nature of the biofilm produced by the RcsC signalling pathway

Plasmid pMPMABD, which codes for the RcsC histidine kinase and receiver domains (amino acids 456–949), was used in the experiments described hereafter, because it was the plasmid causing highest biofilm production. To determine the nature of the biofilm made in the parental and rcsCDB backgrounds, deletion mutants in genes known to be involved in biofilm formation, such as csgA, fliC, wcaA, wcaF, fliC, motA, fimH and bcsA, were tested to find a mutant that abolished biofilm production. None of them decreased biofilm production (Fig. 2b and data not shown).

Interestingly, mutations in genes involved in the synthesis of PGA, i.e. pgaA, pgaB, pgaC, pgaD and nhaR, abolished biofilm formation by the otherwise parental BW25113 and rcsCDB backgrounds (RO17123 and RO17135, respectively) (Fig. 2b and data not shown). This observation suggests that RcsC signalling is turning on the biosynthetic pathway encoded by the pgaABCD operon, a regulatory link not described previously.
**Fig. 1.** Schematic representation of the RcsC histidine kinase and the deletions generated in this work. RcsC is represented at the top, divided into several domains as described in the text. Below are the four constructs generated either as MBP fusions in the pMAL vector (New England Biolabs) or as RcsC truncations cloned in the pMPM-T6 vector. Numbers at the beginning or the end of each rectangle represent the corresponding RcsC position in amino acids where the construct begins or ends.

**Fig. 2.** (a) Biofilm production in the parental and rcsCDB backgrounds is equivalent but depends on the particular fragment of RcsC expressed. *E. coli* BW25113 or rcsCDB (BW27557) transformed with the indicated pMPM plasmid derivatives expressing different truncations of RcsC under control of pBAD was grown for 8 h as indicated in Methods. RcsC was induced by adding L-arabinose (0.025%); similar concentrations of glucose were used as a negative control. Relative amounts of biofilm are reported, assessed by crystal violet staining in microtitre dishes as described in Methods. (b) Biofilm production is abolished in the pgaA, pgaB and nhaR backgrounds in the presence and absence of the rcsCDB genes. Mutants in different genes coding for structures or biosynthetic pathways involved in biofilm formation were transformed with the pMPMABD plasmid and evaluated for biofilm formation.
pgaA–cat fusions corroborated the control of the pga operon by RcsC

To validate the results obtained by the phenotypic analysis, two pgaA–cat fusions were constructed, as described in Methods. One of these, on plasmid pKK232pgaA1, carries nucleotides −508 to +1, and thus the pgaA transcription start site, the pgaA promoter region and the NhaR-binding boxes for transcriptional regulation. The other pgaA–cat fusion, on plasmid pKK232pgaA2N, starts at the same nucleotide at the 5’ end but continues further downstream of the transcription start site to nucleotide +226, hence containing several CsrA-binding boxes (Fig. 3a).

Expression of both fusions was monitored at different times, starting at an OD_{600} of 0.6 up to 1.8 (15 h of growth) (Fig. 3b and data not shown). As expected, both fusions were active upon RcsC ABD domain expression. The fusion containing only the NhaR-binding boxes had a twofold higher expression than the one also containing the CsrA-binding boxes. Although the shorter fusion was leaky, while the other had a stricter regulation, expression levels for each fusion were similar in the pgaC (JW1007-2) and the ΔpgaC–rcsCDB (RO17135) strains (Fig. 3b), validating that biofilm formation was enhanced by the RcsC ABD domain-dependent activation of the pga operon independently of the genetic background.

RcsC ABD domains show phosphatase activity

Previous research has established that when E. coli is grown on minimal plates with glucose as a carbon source it accumulates acetyl phosphate, which acts as phosphodonor for RcsB, leading to activation of cps genes, and thus producing mucoid colonies. Moreover, RcsC expression from a constitutive promoter can suppress mucoidy caused by colanic acid production because of the RcsC basal phosphatase activity on RcsB-P (Fredericks et al., 2006). Thus, to explore the enzymic activity of the RcsC ABD domains, the following in vivo phenotypic approach was used: mucoidy was evaluated in the E. coli AJW678 strain per se or transformed with the pMPM-T6 vector or pMPMABD plasmids. To exacerbate mucoidy, ZnCl₂ was added to the plates while L-arabinose was used to induce expression of the RcsC ABD cytoplasmic domain. E. coli AJW678 alone or transformed with the vector was mucoid, while the strain producing RcsC ABD domains was not (Fig. 4), suggesting that the basal activity of the RcsC ABD domains is as a phosphatase, in agreement with an earlier report on the whole RcsC protein (Fredericks et al., 2006).

Acetyl phosphate could be acting as the phosho-donor in the regulatory pathway controlling PGA-dependent biofilm formation

The participation of acetyl phosphate on biofilm formation was evaluated by transforming mutant strains lacking genes involved in acetyl phosphate degradation and synthesis [ackA (AJW1939) and pta–ackA (AJW2013)] with plasmid pMPMABD. The resulting strains, as well as the parental strain (AJW678) and wild-type BW25113 also carrying pMPMABD, were grown in the presence of glucose or L-arabinose to repress or induce RcsC ABD expression, respectively. When grown in the presence of glucose, the pta–ackA strain and its parent (AJW678) produced about four units of biofilm, while the ackA and the BW25113

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Fig. 3. Schematic representation of the two pgaA–cat reporter fusions generated and cat-specific activity obtained from strains transformed with each fusion. (a) The scheme at the top represents the pga locus (not drawn at scale); the next depicts the mRNA produced, which can be sequestered by CsrA. Plasmid pKK232pgaA1 carries a DNA fragment containing the pgaA promoter region, from the NhaR-binding sites to the transcription start site (pgaA1–cat). Another plasmid, named pKK232pgaA2N, includes sequences downstream of the transcription start site where several CsrA-binding boxes are present (pgaA2N–cat). (b) CAT-specific activity from either parental or rcsCDB strains, both deficient in PGA production (pgaC mutants), transformed with the pgaA reporter fusions. Strains were grown and sampled as described in Methods.
strains only made basal amounts (<0.5 units) (Fig. 5). Under induction conditions, strains BW25113 and AJW678 produced similar amounts of biofilm (14 and 12 relative biofilm units at 8 h), suggesting that expression of the RcsC ABD domains has the same outcome in both laboratory strains, thus validating the use of the AJW678 derivative mutants (Fig. 5). The ackA mutant, unable to transform acetyl phosphate to acetate and thus presumably accumulating large amounts of acetyl phosphate, produced six times less biofilm than the parental strain; while the pta–ackA mutant, unable to produce acetyl phosphate, made 1.5 times more biofilm than the control. However, even under these conditions, it appears that high levels of expression of RcsC ABD are required for maximum biofilm formation. Hence, acetyl phosphate appears to inhibit biofilm formation in an RcsC-dependent manner.

OmpR is one of the response regulators that could be modified by acetyl phosphate to affect biofilm formation

To determine whether a response regulator affected by acetyl phosphate levels could be involved in the regulatory pathway studied here, deletion mutants in some of the corresponding genes (i.e. cpxR, ompR, phoR, uvrY) from the Keio collection were analysed for biofilm formation (Fredericks et al., 2006; Matsubara & Mizuno, 1999; McCleary & Stock, 1994; Tomenius et al., 2005; Wolfe et al., 2008). Each mutant was transformed with the pMPMABD plasmid, grown in the presence of glucose or L-arabinose and evaluated in the microtitre dish assay. Only the ompR mutant (JW3368-1) was affected (Fig. 6a), producing more biofilm, from 1.3-fold (at 4 h) to about 2-fold (at 8 and 12 h), than the parental strain (BW25113) under inducing conditions. Remarkably, the envZ mutant (JW3367-3) produced similar amounts of biofilm to the wild-type (BW25113) at early sampling times (4 and 8 h) or half at 12 h (Fig. 6a). The results obtained with these two mutants indicate that OmpR has a role in this signalling pathway, acting as a repressor; while its partner, the EnvZ histidine kinase, seems to have no role.

The response regulator OmpR-P represses biofilm production by directly interacting with the pga operon promoter region

To evaluate whether the effect of OmpR was direct on the pga operon, an EMSA was set up (Fig. 6b). Increasing amounts of purified OmpR or OmpR-P were incubated with a PCR-amplified 735 bp DNA fragment spanning the pgaA regulatory region. Upon interaction of the DNA with the protein, the reaction was resolved by PAGE. When OmpR-P was used, a delayed complex was formed with the pga regulatory region starting at 400 nM: most of the pga DNA was bound at 600 nM. In contrast, most of the pgaA regulatory region remained free with non-phosphorylated OmpR, even at the highest protein concentration used in the experiment (600 nM) (Fig. 6b). As expected, neither OmpR-P nor OmpR bound to the ler fragment, an unrelated DNA region used as a negative control.

To further determine the precise location where OmpR binds to the pgaA regulatory region, a DNase I protection

Fig. 4. RcsC ABD domains act as a phosphatase, as evidenced by suppression of mucoidy. E. coli strain AJW678 carrying no plasmids (bottom streak) or transformed with the pMPM-T60 vector (upper right streak) showed mucoidy, whereas that carrying the pMPMABD plasmid (middle left streak) was not mucoid. The assay is described in Methods.

Fig. 5. Accumulation of acetyl phosphate has a negative effect on biofilm formation. Strains carrying mutations in the acetyl phosphate biosynthetic pathway were evaluated for their ability to produce biofilm when transformed with plasmid pMPMABD. All the strains were grown in the presence of glucose or L-arabinose (0.025 %). Further details are given in Methods. Columns: 1 and 5, E. coli BW25113; 2 and 6, E. coli AJW678; 3 and 7, E. coli ackA (AJW1939); 4 and 8, E. coli pta–ackA (AJW2013).
pgaA and ackA–ompR mutants as compared ompC envZ390
2+
vector. We next evaluated the effect of the pgaA operon by direct binding.

Additionaly, to perform an epistasis test, an ackA–ompR mutant was generated in the pgaC background (strain RO135155151) and was then transformed with the pKK232pgaA1 and pKK232pgaA2N plasmids. Both fusions were expressed at higher levels: the shorter fusion was expressed 2.4–3 times more in this background as compared with the ompR background, while the fusion containing the NhaR and the CsrA-binding boxes was expressed around three times above the ompR background. These results indicate that acetyl phosphate accumulation affects pga regulation not only by acting through OmpR but also by modifying a different protein.

Analysis of pgaA–cat expression in ackA, ompR and ackA–ompR backgrounds

To further study the role played by the phospho-donor acetyl phosphate and the response regulator OmpR in the RcsC- pga signalling pathway, plasmids pKK232pgaA1 and pKK232pgaA2N were transformed into the ackA and ompR mutants, carrying pPMAB. Previously, the pgaC mutation was combined with both genetic backgrounds, giving strains RO135151 and RO135155, respectively. When bacteria were grown in the presence of L-arabinose, the reporter activity of the pgaA–cat fusion containing only the NhaR-binding boxes was between one-quarter and one-third in the ackA mutant, and between 1.5 and two times higher in the ompR mutant as compared with the parental BW25113 strain. The same trend was observed for the strains carrying the fusion with the NhaR and the CsrA-binding boxes in the ackA and ompR mutants as compared with BW25113; in particular, the ratio was five to three times less in the ackA mutant and 1.4–1.8 higher for the ompR mutant (Fig. 7). These results are in accordance with those observed in the crystal violet staining assay (Figs 5 and 6a).

An EnvZ mutant with constitutive phosphatase activity is not able to elicit biofilm formation

To further explore any role of EnvZ in this signalling pathway, a mutant in the cytoplasmic domain of EnvZ that abolishes the kinase activity and preserves the phosphatase activity was generated, as EnvZ is the cognate sensor for OmpR. Hence, the gene encoding the EnvZ F390L mutant (envZ390) (Hsing et al., 1998) was cloned into the pMPM-T6Ω vector. We next evaluated the effect of the envZ390 allele on biofilm formation. Interestingly, the BW25113 strain carrying the pMPMEnvZ390 plasmid grown under induction conditions did not form any more biofilm than the negative controls (grown in the presence of glucose), and presented between 18- and 27-fold less biofilm than the strain expressing the RcsC ABD domains (Fig. 8). Use of an ompC–cat reporter fusion validated the effect of the F390L mutation on porin expression (Fig. S2). As expected, the F390L mutation showed a phenotype consistent with a constitutive phosphatase activity negatively affecting ompC expression. These results reinforce the idea that EnvZ has no role in biofilm formation comprising PGA.

**Fig. 6.** (a) An ompR, but not an envZ, strain produces a higher amount of biofilm than the parental (BW25113) strain. The Keio mutant strains in ompR (JW3368-1) and envZ (JW3367-3) were transformed with the pPMABD plasmid and grown as indicated in Methods to evaluate biofilm formation. (b) OmpR-P binds with higher affinity than OmpR to the pgaA regulatory region. EMSA using the pgaA regulatory region (−482 to +259) cloned in plasmid pKK232pgaA2N as a probe after PCR amplification. The negative control was a fragment of the ler gene from enteropathogenic E. coli. OmpR-6×His was purified under native conditions, quantified by the Bradford assay and phosphorylated with acetyl phosphate. Protein concentrations added are indicated at the top of each line. The asterisk at the right-hand side indicates the protein–DNA retardation complex.
DISCUSSION

In this work, a connection between the RcsC cytoplasmic domains, pga expression and biofilm formation is presented. Several facts need to be considered from our results. (i) The amount of biofilm produced is dependent on the particular RcsC cytoplasmic domains expressed, with the ABD domains inducing more biofilm than the LAB, AB and PLAB domains (Fig. 2a). The simplest explanation is that the A domains are the minimal and essential unit for the observed effect, with the D domain strongly enhancing it and the L domain reinforcing it. It appears that the P domain plays an inhibitory role. (ii) Biofilm production occurs in the parental and the ΔrcsCDB backgrounds at similar levels (Fig. 2a), which strongly indicates that RcsC signalling or interaction is independent of the known components in the phosphorelay. (iii) Under both backgrounds, biofilm requires the presence of the pga operon and its transcriptional activator NhaR. Mutants in any of these genes are unable to produce biofilm (Fig. 2b), meaning that the main component of this particular biofilm is the PGA biopolymer. This phenotype was corroborated with a pair of pgaA–cat reporter fusions, which were induced when the ABD domains of RcsC were expressed (Fig. 3).

Thus, a current working model would involve the metabolite acetyl phosphate acting as a phospho-donor and phosphorylating the response regulator OmpR, aside from another unknown response regulator. Moreover, it has been reported that acetyl phosphate plays a role in protein acetylation by modifying hundreds of proteins in E. coli, among them OmpR (Kuhn et al., 2014). Thus, at the same time that it could be acting as a phospho-donor for at least two response regulators, it could also be acetylating different proteins besides OmpR, causing a pleiotropic effect.

Wolfe’s group has also shown the effect of acetyl phosphate as a global signal for biofilm development; remarkably, they reported that bacteria unable to produce acetyl phosphate and those defective for its degradation formed biofilms that were different from each other and from the parental strain. They also showed a link between the Rcs phospho-relay and acetyl phosphate (Fredericks et al., 2006; Wolfe, 2010; Wolfe et al., 2003). Our results are in agreement with these reports and extend the knowledge of the interplay between this molecule and signal transduction mediated by RcsC. Thus, as evidenced by the epistasis test (Fig. 7), acetyl phosphate plays an important role in this
pathway by acting through OmpR and perhaps through other proteins.

The model implicates OmpR-P acting as a direct transcriptional repressor of the pga operon, avoiding biofilm formation; as shown by experiments in vivo and in vitro (Figs 6, 7 and S1). Importantly, the source for OmpR phosphorylation under this growth condition seems to be acetyl phosphate: accumulation of acetyl phosphate in the ackA mutant has a negative effect on biofilm production, while the converse is true in the double pta–ackA mutant where no acetyl phosphate is produced (Fig. 5). Furthermore, these results were confirmed with the pgaA reporter fusions: reporter activity diminishes in the ackA background but increases in the ompR mutant (Fig. 7).

Remarkably, the OmpR protein has been implicated as an important regulator of biofilm formation in E. coli and in other bacteria such as Yersinia (Brzostek et al., 2012; Samanta et al., 2013). The phosphorylation of OmpR and some of its effects on gene regulation, either as an activator or as a repressor, have been evidenced by different groups (Prüss, 1998; Shin & Park, 1995). Our results add another member, the pga operon, to this regulatory network. To our knowledge, this is the first report showing direct regulation of the PGA biosynthetic pathway by OmpR-P. Our EMSA (Fig. 6b) and DNase protection experiments (data not shown and Fig. S1) demonstrate the binding of OmpR to the pga regulatory region and allow us to identify the sequence protected by it. Reinforcing this finding, part of this sequence shares similarity with the 20 bp consensus obtained after aligning five OmpR-binding boxes located on the E. coli ompF and ompC promoter regions (Harlocker et al., 1995). Considering the pga regulatory region as a whole, OmpR binding to this sequence could be hindering RNA polymerase or NhaR interactions with the pga promoter, as the OmpR-protected region extends from −48 to −10, where a downstream NhaR-binding box and the −35 hexamer are also located (Fig. S1).

RcsC could be acting on or interacting with NhaR or an unknown response regulator to reinforce their positive effects on pga transcription. This is illustrated by the experiments with the mutants in the acetyl phosphate pathway and the epistasis test with the ackA–ompR mutant (Figs 5 and 7), which suggest a complex regulatory network involving additional unknown players and a wider role for acetyl phosphate: the mutants defective in acetyl phosphate synthesis and null mutants on ompR are not constitutive for biofilm production, as a linear regulatory pathway would suggest.

We have also shown here that RcsC ABD acts predominantly as a phosphatase, by using a phenotypic assay that reveals mucoidy (Fredericks et al., 2006) when bacteria are grown on minimal medium plates plus glucose and ZnCl₂ (Fig. 4), because the strain expressing these domains eliminated mucoidy. Thus, another possibility would be that RcsC ABD is able to dephosphorylate OmpR-P and an additional phosphorylated response regulator, releasing their repressive effects on the pga operon.

Whether cross-talk or another interaction between RcsC, OmpR (and perhaps with another unknown response regulator) or NhaR occurs should be investigated further. Independently of the model considered, it has been reported that the RcsC phospho-relay plays a key role in biofilm maturation, because it represses early-expressed genes important for initial adherence and then activates genes allowing three-dimensional biofilm formation (Huang et al., 2006). Our findings are in general accordance with this view. Before our report there was no suggestion of an interaction performed by RcsC on any protein not belonging to its phosphorelay and their effects on gene regulation: however, there is evidence of a link among the RcsCDB phosphorelay and other two-component regulatory systems, such as PhoQ/P, EnvZ/OmpR and ArcA (Hagihara et al., 2003). The present study provides more detail for one of such linkages.

E. coli encodes five hybrid histidine kinases. However, only RcsC has a particular architecture with the receiver domain on its own, hence requiring the participation of an extra protein (RcsD) to complete the phosphorelay pathway to RcsB (Mizuno, 1997; Takeda et al., 2001). This particular design for the RcsCDB pathway is conserved in several members of the Enterobacteriaceae (Huang et al., 2006). This might indicate that RcsC and RcsD, encoded as independent proteins, are able to interact not only among themselves but also with other proteins, as suggested here for RcsC, conferring some advantage on signal transduction to regulate particular but complex phenotypes such as biofilm formation.

Importantly, there is evidence that when the kinase and receiver domains from hybrid histidine kinases are expressed as independent proteins, some specificity in recognition is lost, favouring detrimental cross-talk; hence, domain tethering helps to avoid this kind of interaction (Capra et al., 2012). Therefore, RcsC and RcsD acting as independent proteins, even though they belong to a particular pathway, could be designed to allow some cross-talk (or cross-communication) and have ‘promiscuous’ interactions with other proteins under particular conditions, as proposed here.

Furthermore, it was reported that RcsC possesses a new structural domain connecting the histidine kinase and receiver domains, which has been named alpha/beta/loop (ABL): this domain is conserved and is unique among RcsC-like kinases. Several hypotheses have been proposed regarding its role: it could be acting as an intermolecular binding site for additional proteins or enabling cross-talk with other two-component systems (Rogov et al., 2006), which seems to be the case reported here, considering either model. In accordance with this, the greater effect on biofilm production occurs with our ABD version of RcsC.
which is the only one that includes the ABL structural domain (Fig. 2). However, the other RcsC constructs also had an effect on biofilm production even though they lack this domain, suggesting they are still able to interact with the proposed proteins.

It is tempting to speculate why an EnvZ mutant with phosphatase activity had no effect on regulation (Fig. 8). The epistasis test suggests the existence of at least one additional protein acting on pga expression. This protein could be replacing OmpR when it is missing and not be dephosphorylated by EnvZ. Also, it is puzzling that RcsC expression is always required to activate PGA-based biofilm formation (Figs 5, 6a and 7). Clearly, some additional inputs to the pathway are required, affecting signal transduction and somehow helping biofilm formation. Even more complex scenarios, involving pleiotropic effects due to lysine acetylation on diverse proteins could be proposed, and are the subject of our current research. It will be of interest to explore how all the proteins, molecules and probable modifications identified or proposed here may contribute to our better understanding of biofilm formation in bacteria.

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REFERENCES


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