Polysialic and colanic acids metabolism in *Escherichia coli* K92 is regulated by RcsA and RcsB

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**Synopsis**

We have shown previously that *Escherichia coli* K92 produces two different capsular polymers known as CA (colanic acid) and PA (polysialic acid) in a thermoregulated manner. The complex Rcs phosphorelay is largely related to the regulation of CA synthesis. Through deletion of rcsA and rcsB genes, we show that the Rcs system is involved in the regulation of both CA and PA synthesis in *E. coli* K92. Deletion of either rcsA or rcsB genes resulted in decreased expression of cps (CA biosynthesis cluster) at 19 °C and 37 °C, but only CA production was reduced at 19 °C. Concerning PA, both deletions enhanced its synthesis at 37 °C, which does not correlate with the reduced kps (PA biosynthesis cluster) expression observed in the rcsB mutant. Under this condition, expression of the nan operon responsible for PA catabolism was greatly reduced. Although RcsA and RcsB acted as negative regulators of PA synthesis at 37 °C, their absence did not reestablish PA expression at low temperatures, despite the deletion of rcsB resulting in enhanced kps expression. Finally, our results revealed that RcsB controlled the expression of several genes (dsrA, rfaH, h-ns and slyA) involved in the thermoregulation of CA and PA synthesis, indicating that RcsB is part of a complex regulatory mechanism governing the surface appearance in *E. coli*.

**Key words:** Capsular polysialic acid, colanic acid, qRT–PCR, RcsA and RcsB regulation

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**INTRODUCTION**

Over 80 distinct capsular or K antigens have been described in *Escherichia coli*, which are classified into four groups [1]. Group II K antigens exhibit capsule expression at 37 °C but not at low temperatures (18 °C) and their regulation is temperature-dependent [1,2]. The group II capsule gene cluster (kps) consists of a central serotype-specific region 2, encoding proteins for synthesis and polymerization of the specific K antigen that is flanked by conserved regions 1 and 3 (Figure 1A) [1,3]. Transcription of the kps cluster is driven by two convergent temperature-regulated promoters located upstream of regions 1 and 3 [4]. Transcription of regions 2 and 3 is driven by the PR3 promoter [4] and is dependent on RfaH for transcription elongation [5]. In addition, H-NS plays an unusual dual role, not only being required for maximal transcription at 37 °C but also contributing to transcriptional repression at low temperatures (≤20 °C) [4,6]. Transcription of region 1 is driven by promoter PR1, and its maximal expression at 37 °C requires both H-NS and SlyA, whereas a reduced SlyA expression at 20 °C results in repressed transcription from PR1 [4,6,7].

*E. coli* K92 synthesizes a type II capsule known as PA (polysialic acid), which is responsible for bacterial virulence [8,9]. As with the group II K antigens, PA is mainly generated at 37 °C and is negligible at low temperatures (below 20 °C) [10]. We previously showed that *E. coli* K92 is able to synthesize a different capsular polysaccharide known as CA (colanic acid) [11]. CA is associated with bacterial protection against desiccation, extreme temperatures and acidic environmental conditions [12,13].
as well as against osmotic and oxidative stress [14]. In contrast to PA, CA is predominantly synthesized at low temperatures [11,15] and does not seem to play a role in bacterial virulence [1,16].

A complex signal transduction pathway, namely the Rcs phosphorelay, controls the expression of the *cps* operon (Figure 1B) responsible for CA production. This pathway can be activated at low temperatures and involves a histidine kinase (RcsC), a response regulator (RcsB), a phospho-transfer protein (RcsD), a signal transductor (RcsF) and an auxiliary activator protein (RcsA) [15,17]. The transcriptional activator RcsB is the principal regulator of this system and it forms homodimers which activate *cps* operon transcription. In addition, RcsB may form heterodimers with the auxiliary activator RcsA, enhancing *cps* operon transcription. It has been described that RcsB is required for *cps* gene expression, whereas the absence of RcsA only decreases it [15]. Under normal conditions, the amount of RcsA protein is limited by its low synthesis level and because it is rapidly degraded by a Lon protease. However, synthesis of RcsA is increased at low temperatures, leading to a mucoid phenotype as a consequence of the expression of *cps* genes [15]. In addition, other regulatory molecules are implicated, either directly or indirectly, in control of CA synthesis. Thus, the anti-termination factor RfaH is required for transcription of genes downstream of *wzc* [5,18], while DsrA is a small molecule of RNA which increases *cps* transcription through its negative regulation of H-NS synthesis [19].

Although most of the targets present in the Rcs regulon are positively regulated, it has been suggested that the Rcs regulon also may repress the synthesis of type II capsules in *E. coli* [20]. Through deletion of *rcsB* and *rcsA* genes, in this report we show that RcsA and RcsB act as negative regulators of PA synthesis at 37 °C in *E. coli* K92, although the mechanism remains unclear. Moreover, both proteins enhance the expression of the *cps* operon, which is required for maximal CA production at low temperatures. However, they barely play a role in CA synthesis at high temperatures (37 °C). More importantly, neither *rcsA* nor *rcsB* deletion completely abolished CA synthesis under any of the conditions tested. In sum, these data suggest that the Rcs system is a critical regulator of the adaptation response of *E. coli* K92 to different environmental conditions through the expression of PA and CA capsules.

### MATERIALS AND METHODS

**Strains, culture media and growth conditions**

The strains and plasmids used in the present study are shown in Table 1. Bacterial cultures were inoculated and grown at 37 or 19 °C as previously described in Navasa et al. [21]. Bacterial cultures were grown in LB (Luria–Bertani) complex medium, LA [LB supplemented with 2% (w/v) agar] and Xyl–Asn (xylose–asparagine) or Glc–Pro (glucose–proline) MM (minimal media) for *E. coli* [22]. Where indicated, Glc–Pro MM was supplemented with agar 2% (w/v). We chose Xyl–Asn or Glc–Pro MM because they induce maximal PA and CA production in *E. coli* K92, respectively [2,14]. During the gene allelic exchange experiments, LA medium supplemented with 5% (w/v) sucrose and without NaCl was used to select plasmid excision from the chromosome [23]. When required, the following supplements were added to the culture media: rifampicin (25 and 10 μg/ml for liquid and solid media, respectively), kanamycin (25 and 12.5 μg/ml for liquid and solid media, respectively), ampicillin (100 μg/ml) and chloramphenicol (60 μg/ml).
**Table 1** Strains and plasmids used in the present study

| Strain       | Description                                                                 | Reference or source      |
|--------------|------------------------------------------------------------------------------|--------------------------|
| DH5α        | F− ΔlacU169 Str803::lacZ1M15 hsdR17 recA1 endA1 gpyA96 thy−1 relA1 supE44 deoR | [46]                     |
| S17-1λpir    | RP4-2-Tc::Mu-Km::Tn7prothiromeA/SmR− M+ λpir                                | [29]                     |
| K92         | Wild-type                                                                   | ATCC 35860               |
| K92ΔrcsA    | K92 ΔrcsA; constructed using pDS132-WX                                      | The present study         |
| K92ΔrcsB    | K92 ΔrcsB; constructed using pDS132-YZ                                       | The present study         |

(b) Plasmids and constructions

| Plasmid          | Description                                                                 | Reference or source      |
|------------------|-----------------------------------------------------------------------------|--------------------------|
| pGEM-T Easy      | Ap'ori/ColE1 lacZu+ SP6 T7 lac promoter, direct cloning of PCR products     | Promega                  |
| pDS132           | R6K ori mobRP4 cat sacB                                                     | [23]                     |
| pGEM-W           | rcsA upstream sequences PCR amplified with primers rcsAup5' and rcsAup3 cloned into pGEMT-easy; Ap' | The present study         |
| pGEM-X           | rcsA downstream sequences PCR amplified with primers rcsAdown5' and rcsAdown3 cloned into pGEMT-easy; Ap' | The present study         |
| pGEM-Y           | rcsB upstream sequences PCR amplified with primers rcsBup5' and rcsBup3 cloned into pGEMT-easy; Ap' | The present study         |
| pGEM-Z           | rcsB downstream sequences PCR amplified with primers rcsBdown5' and rcsBdown3' cloned into pGEMT-easy; Ap' | The present study         |
| pGEM-WX          | ΔrcsA; rcsA upstream sequence from pGEM-W removed with EcoRI and ligated with rcsA downstream sequence from pGEM-X removed with EcoRI; Ap' | The present study         |
| pGEM-YZ          | ΔrcsB; rcsB upstream sequence from pGEM-Y removed with EcoRI and ligated with rcsB downstream sequence from pGEM-Z removed with EcoRI; Ap' | The present study         |
| pDS132-WX        | ΔrcsA sequences from pGEM-WX removed with SacI and Sphi inserted into pDS132 digested with the same enzymes; Cat' | The present study         |
| pDS132-YZ        | ΔrcsB sequences from pGEM-YZ removed with SacI and Sphi inserted into pDS132 digested with the same enzymes; Cat' | The present study         |

**Table 2** Primers used in the present study

| Function | Name        | Sequence (5'→3')                  |
|----------|-------------|-----------------------------------|
| rcsA deletion | rcsAup5' | CGACTAGTTAATCCGGGTATCTG            |
|           | rcsAup3'  | GTTGATTATATGAGCTTGATACG            |
|           | rcsAdown5' | CGACGTATCATCATGACGGAAC            |
|           | rcsAdown3' | CATTTGACACATTACCAGCAGTCG           |
| rcsB deletion | rcsBup5' | TAAAGCAGTTATTCGCGCGATGAC          |
|           | rcsBup3'  | GGAATGCGCTGTTGATGTCCTTG           |
|           | rcsBdown5' | CAGTGCTGGTGGTACGGTGAC             |
|           | rcsBdown3' | TTATGCTGCCGACTAAAGGTCAC           |

**DNA manipulation and RNA isolation**

Routine molecular biology techniques were performed according to standard procedures [24]. Restriction and modifying enzymes (Invitrogen S.A.) were used as recommended by the manufacturer. Plasmid DNA was isolated from *E. coli* using the Wizard R Plus SV Miniprep DNA Purification System (Promega). For deletion experiments, PCR products were generated by Taq DNA polymerase (Stratagene) using the same primers described previously [25]. Mobilization of plasmids between *E. coli* strains was accomplished as described previously [26]. Purification of total RNA was performed using an Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare). The isolated total RNA was treated with DNase I (Invitrogen S.A.) and quantified by spectrophotometry [24].

**qRT–PCR (quantitative real-time PCR)**

The DNase-treated RNA was reverse transcribed with the ThermoScript™ RT–PCR (reverse transcriptase–PCR) System (Invitrogen S.A.). For qRT–PCR, each cDNA product was used as a template for DNA amplification, using primer pairs as previously described [21], 10 μl of SYBR® Green PCR Master Mix (Applied Biosystems) and up to 20 μl of water. Primers were designed using the Oligo Primer Analysis Software [27], based on sequences retrieved from the GenBank/EMBL databases. In all cases, the oligonucleotides used in qPCR (quantitative PCR) were designed to have similar melting temperatures (60°C) and to amplify DNA fragments of similar lengths (around 100 nucleotides). Reactions were performed using an ABI Prism 7000 sequence detection system (Applied Biosystems) and applying the following conditions: 50°C for 2 min, 95°C for 5 min, 40 cycles of 94°C for 15 s and 60°C for 1 min. The results were analyzed using specific software (ABI Prism 7000 SDS software). The relative gene expression levels were calculated as...
previously described [28] using the equation: \[ \Delta C_T = \left( C_T \text{ gene at } x^\circ C, Y \text{ genotype} - C_T \text{ housekeeping gene at } x^\circ C, Y \text{ genotype} \right) - \left( C_T \text{ gene at } x^\circ C, WT \text{ (wild-type) genotype} - C_T \text{ housekeeping gene at } x^\circ C, WT \text{ genotype} \right) \] and then transformed into relative changes \((n\text{-fold})\) using \(2^{-\Delta\Delta C_T} \). \(C_T\) (threshold cycle value) is the cycle number at which the real-time amplification curve crosses the user-defined threshold, \(x^\circ C\) is the temperature at which the RNA was isolated (37°C or 19°C) and \(Y\) is the mutant strain \((E. coli K92\Delta rcsA\) or \(E. coli K92\Delta rcsB\)). The data represent the average change \((n\text{-fold})\) determined from at least three independent experiments. As a control we used the housekeeping gene \(gapdh\), which was carefully validated before its use in the quantitative mRNA assays, with 16S rRNA expression as internal control obtained under the same conditions and determined from at least three independent experiments.

sacB-assisted allelic exchange mutagenesis

pDS132-based allelic exchange plasmids [23] were electroporated into \(E. coli\) S17-1\(^{pir}\) cells for conjugation into \(E. coli\) K92 by filter mating [29]. Transconjugants containing single crossovers of the allelic exchange plasmid integrated into the \(E. coli\) K92 genome were selected in LA supplemented with chloramphenicol and rifampicin. To force the second recombination, single-crossover strains were plated onto LA containing rifampicin and 5% (w/v) sucrose and then incubated at 37°C for 24–48 h. Sucrose-resistant colonies were placed on LA-sucrose plates and screened for a loss of chloramphenicol resistance encoded by the vector.

Deletion of rcsA and rcsB genes from \(E. coli\) K92

The 0.3-kb upstream and downstream sequences included in each \(rcsA\) or \(rcsB\) loci were PCR amplified using the primers indicated in Table 2. These 0.3-kb amplicons were individually cloned into pGEM-T Easy, yielding plasmids pGEM-W, pGEM-X, pGEM-Y and pGEM-Z, respectively. The cloned sequences were excised using EcoRI. Downstream and upstream sequences for each gene were ligated and the products were amplified using the primer pairs \(rcsAup5\) and \(rcsAdown3\) and \(rcsBup5\) and \(rcsbdown3\), and cloned again into pGEM-T Easy, yielding pGEM-WX and pGEM-YZ. The \(rcsA\) and \(rcsB\) upstream–downstream DNA sequences (\(\Delta rcsA\) and \(\Delta rcsB\)) were excised from pGEM-WX and pGEM-YZ using SacI and SphI restriction enzymes and cloned into pDS132 previously digested with the same enzymes, yielding plasmids pDS132-WX and pDS132-YZ, respectively. The suicide vectors carrying \(\Delta rcsA\) or \(\Delta rcsB\) were electroporated into \(E. coli\) S17-1\(^{pir}\) for biparental conjugation into \(E. coli\) K92. The deletions were recombined into the chromosome of \(E. coli\) K92 by using the standard two-step sucrose-resistance-assisted allelic exchange method described above. The correct allelic exchange of the WT allele for each mutant allele was confirmed by PCR using the primers \(rcsAup5\) and \(rcsAdown3\), and \(rcsBup5\) and \(rcsbdown3\). The \(\Delta rcsA\) and \(\Delta rcsB\) \(E. coli\) K92 mutants were named \(E. coli\) K92\(\Delta rcsA\) and \(E. coli\) K92\(\Delta rcsB\), respectively. For each mutant, loss of the DNA fragment was confirmed by PCR and the absence of expression of the deleted gene was confirmed by qRT–PCR.

Quantification of exopolysaccharides

Quantitative determination of CA and PA production by \(E. coli\) K92 cultures was performed as previously described [11]. Briefly, \(E. coli\) K92 cells were removed by centrifugation and the cell-free supernatant obtained was dialysed against 1000 vol of distilled water for 24 h at 4°C. Dialysed supernatant samples were used for quantitative determination of CA by the orcinol method [30], according to the amount of uronic acids. Dialysed supernatant samples were also used for quantitative determination of PA following the resorcinol protocol described by Svennerholm [31].

Capsule staining

The presence or absence of CA surrounding the bacteria was evaluated by using a combination of negative and fuchsine staining procedures. Briefly, a very small drop of bacterial culture was placed near one end of a well-cleaned slide. Once dry, one drop of fuchsine (primary colorant) was added to the bacterial culture without spreading for 2 min. After that, the sample was washed with water and dried. Next, a drop of nigrosin (India ink) no greater than the drop of bacterial culture was added and the mixture was spread over the slide using another clean slide. Finally, a cover glass was placed on the sample, avoiding the formation of bubbles and examined under a Nikon Eclipse E600 optical microscope.

Statistical analysis

The results are presented as means ± S.E. Significant differences between means were calculated with Student’s \(t\) test. \(P\) values of 0.05 or less were considered statistically significant.

RESULTS

\(rcsA\) and \(rcsB\) gene products control CA synthesis in \(E. coli\) K92 at low temperatures

We previously showed that \(E. coli\) K92 (WT) synthesizes CA as capsular polymers in a temperature-dependent manner [11,21]. It is well known that Rcs phosphorelay and the auxiliary protein RcsA act as positive regulators of CA synthesis [15]. To investigate the role of \(RcsA\) and \(RcsB\) in CA synthesis by \(E. coli\) K92, we performed gene deletion experiments to obtain \(E. coli\) K92\(\Delta rcsA\) and \(E. coli\) K92\(\Delta rcsB\) mutant strains lacking \(rcsA\) and \(rcsB\) genes, respectively. WT and both mutant strains were grown in Glc–Pro and Xyl–Asn MM at 19°C, the optimal growth temperature for synthesis of CA by \(E. coli\) K92 [11,12], and production of this polymer was determined after 120 h. Neither mutant showed any change in growth under the conditions tested (Figure 2) and both deletions resulted in a dramatically decreased CA production at
Rcs system-regulated CA and PA synthesis in *E. coli* K92

19 °C (measured as glucuronic acid content) in the media tested (Figures 3A and 3B), but it was not completely abrogated. We ensured that all glucuronic acid detected belonged to a high molecular mass structure, such as CA polymer, through prior dialysis of supernatants using a 10 kDa membrane pore size. To assess the absence of CA capsules surrounding the bacteria, WT and *E. coli* K92ΔrcsA were grown on Glc–Pro MM agar plates at 19 °C and the Burri method using China ink was applied. This staining technique revealed large amounts of capsular polymer produced by WT in contrast to the mutant strain (Figure 4).

Next, we examined whether RcsA and RcsB control CA production at 37 °C. At this temperature, the levels of CA produced by both mutants were similar to those produced by WT (Figures 3C and 3D). Overall, these results indicate that both RcsA and RcsB positively regulate CA synthesis by *E. coli* K92 at low temperatures and suggest an Rcs phosphorelay-independent CA synthesis at high temperatures.

**Deletion of rcsA or rcsB genes down-regulates the expression of CA synthesis genes**

To determine whether RcsA and RcsB regulate the expression of the *cps* operon responsible for CA synthesis, WT and both mutant strains were grown at 19 °C to enhance CA production, and RNA samples harvested from mid-exponential phase were used to analyse the expression of several genes belonging to the *cps* operon (Figure 1B) by RT–PCR. Consistent with the reduced CA production, deletion of rcsA resulted in decreased *cps* gene expression of 1.4- and 8.5-fold (Table 3). This effect was greater in the absence of RcsB, resulting in a reduction of up to 35-fold. We also analysed expression of the *ugd* gene, which is located outside the *cps* operon but is also involved in CA synthesis [32]. Deletion of rcsB reduced *ugd* expression 2.3-fold, whereas deletion of rcsA barely increased it (1.4-fold).

Since deletion of rcsB resulted in a higher reduction in expression of *ugd* and *cps* genes at 19 °C, we also investigated whether RcsB controls their transcription at 37 °C, even though its absence barely decreased CA production at this temperature. Deletion of rcsB resulted in a lower decrease in *cps* gene expression (between 2.1 and 4.9-fold) (Table 3) than that observed at 19 °C, and only the expression of *gmd* and *fcl* genes was reduced in a similar manner. We compared relative gene expressions for both temperatures given that temperature barely changed *cps* expression in *E. coli* K92 [21]. In addition, deletion of rcsB decreased *ugd* expression 3.3-fold.

**rcsA and rcsB gene products repress PA synthesis in *E. coli* K92**

*E. coli* K92 predominantly synthesizes PA type II capsules at 37 °C, and no production was detected below 20 °C [2,10,21]. It has been suggested that the Rcs phosphorelay system may negatively regulate bacterial group II capsule synthesis [20]. To investigate this, WT and both mutant strains were grown in Glc–Pro and Xyl–Asn MM at 37 °C, the optimal growth temperature
for synthesis of PA by *E. coli* K92 [11,21], and the production of this polymer was measured after 120 h.

Deletion of either *rcsA* or *rcsB* resulted in increased PA production under all conditions tested (Figure 5). This increment was not significant when *E. coli* K92ΔrcsA was grown in Xyl–Asn MM. Dialysis of the supernatants ensured that all sialic acid detected belonged to a high molecular mass structure. These results show that RcsA and RcsB act, directly or indirectly, to repress PA synthesis in *E. coli* K92. However, this effect was insufficient to overcome inhibition of PA capsule synthesis at low temperatures, since neither *E. coli* K92ΔrcsA nor *E. coli* K92ΔrcsB were able to generate PA synthesis at 19°C (results not shown).

### Dual role of RcsB in transcripional regulation of PA metabolism genes

We next investigated whether RcsB controls the expression of the *kps* operon responsible for PA synthesis. WT and *E. coli* K92ΔrcsB were grown at 19°C and 37°C, and RNA samples harvested from mid-exponential phase were used to analyse the expression of several genes belonging to the *kps* operon [1,10]. At 19°C, the deletion of *rcsB* resulted in an increase (between 1.4- and 7.7-fold) in the expression of all tested genes (Table 4), although no PA production was observed. In contrast, *E. coli* K92ΔrcsB showed a reduction (up to 2.3-fold) in *kps* gene expression at 37°C with respect to WT (Table 4), which does not correlate with the increased PA synthesis (see Figure 4). To determine whether increased PA production at 37°C could be due to a diminished PA catabolism, we also analysed the expression of several genes belonging to the *nan* (PA catabolism) operon (Figure 1C) and the regulator NanR. Interestingly, the expression of *nanAET* genes was greatly reduced at 37°C (up to 10-fold), suggesting a diminished PA catabolism, whereas *nanR* expression remained unchanged. Finally, deletion of *rcsB* resulted in a slight increase in the expression of *nan* genes at 19°C (Table 4).
Figure 5  PA production by E. coli K92, E. coli K92ΔrcsA and E. coli K92ΔrcsB growth in MM containing Xyl-Asn (A) or Glic-Pro (B) at 37°C  
NS, no statistically significant differences; *P< 0.05, **P = 0.005 by Student’s t-test.
RcsB as part of a complex network regulating both CA and PA synthesis

In order to gain a better understanding of the regulatory mechanism involved in the thermoregulated synthesis of CA and PA in *E. coli* K92, we further investigated whether RcsB controls the expression of several genes implicated in the regulation of CA and/or PA synthesis. Firstly, we analysed the expression of *rcsC*, *rcsF* and *rcsA* genes belonging to the Rcs pathway. Deletion of *rcsB* resulted in a greater reduction in *rcsA* expression at both temperatures compared with WT (29.5- and 128-fold at 19 and 37 °C, respectively) (Table 5), consistent with the key role of RcsB in high-level expression of *rcsA* [33]. *rcsC* gene expression was up-regulated at 19 °C (2.8-fold) but down-regulated at 37 °C (3.5-fold) in the mutant strain, whereas *rcsF* expression remained unchanged.

We also analysed the expression of *dsrA*, *rfaH*, *h-ns* and *slyA* genes, which are temperature-dependent in *E. coli* K92 [21]. DsrA RNA positively regulates *rcsA* expression [15] and the anti-terminator RfaH is required for the expression of large polysaccharide clusters, including *kps* [5,34] and presumably *cps*, whereas H-NS and SlyA are required not only for maximum transcription of the *kps* operon at 37 °C but also to repress it at 19 °C [7]. Deletion of *rcsB* resulted in an increased expression of all four genes at 19 °C, especially *dsrA* (7.6-fold), and a reduced expression at 37 °C (between 1.4- and 2.4-fold).

**DISCUSSION**

The mechanism by which the Rcs system promotes CA synthesis is well known. As a homodimer, or forming heterodimers with RcsA, RcsB binds to the RcsAB box to enhance *cps* transcription [34]. Consistent with this, we found a decreased *cps* expression in *rcsA* and *rcsB* mutants at both temperatures (Table 3). This effect was greater in the *rcsB* mutant at 19 °C, probably because deletion of *rcsB* has a double effect, abrogating both RcsB- and RcsAB-mediated *cps* transcription. Thus, it has been described that RcsB is required as the main regulator for *cps* transcription and no expression is detected in its absence, whereas RcsA merely enhances it [15]. However, we found that *cps* expression was readily detectable at both temperatures, even in *rcsB* mutants. Since RcsA alone is unable to promote *cps* expression [21], these results suggest an RcsB-independent *cps* transcription. The *cps* cluster not only codes enzymes involved in CA synthesis but also in other pathways. For example, Wzc not only participates in the polymerization and exportation of CA capsules [1] but also enhances Ugd activity through phosphorylation [35]. The *ugd* product is involved in many cellular processes such as capsular polysaccharide [36,37] and LPS (lipopolysaccharide) synthesis, polymyxin B resistance [38] and regulation of the levels of the sigma factor RpoS [39]. Thus, it would not be surprising that the regulation of Wzc was under the control of regulators other than RcsB, reflecting the variety of processes which are involved.

Several findings prompt us to believe that transcriptional regulation of the Rcs system on *cps* expression does not determine CA synthesis at high temperatures. Thus, reduced *cps* transcription in the *rcsB* mutant did not result in a reduced CA production at 37 °C (Figures 3B and 3D). Although CA production at 37 °C was lower than at 19 °C, it cannot be considered as residual since values of CA production observed at 37 °C were close to, for example, those observed in Xyl–Asn MM at 19 °C, and in this case, CA production substantially decreased to a statistically similar degree in both *rcsA* and *rcsB* mutants (Figures 3A and 3C). In addition, we might expect that a higher expression of *rcsB* would result in an increased *cps* transcription, and consequently, in enhanced CA production; however, previous results showed that the *rcsB* gene expresses 6-fold higher at 37 °C than at 19 °C in WT [21]. Furthermore, the thermoregulation of CA production is not probably explained through changes in *cps* expression, whether or not the Rcs pathway is involved. This is supported by the fact that *E. coli* K92 produced large amounts of CA at 19 °C in contrast to 37 °C, but minimal changes in *cps* transcription were detected [21], suggesting that post-transcriptional modifications take place. In this sense, phosphorylation of Ugd by Wzc was found to participate in the regulation of CA production [35].
Furthermore, the phosphorylation/dephosphorylation cycle in tyrosine kinase Wzc was shown to control the production and size distribution of CA polymer in E. coli K-12 and, more importantly, an external desiccation signal was directly linked to the phosphorylation state [40]. Whether the Rcs pathway plays a role at the post-transcriptional level remains unknown.

Unlike CA synthesis, the thermoregulation of group II capsule synthesis in E. coli occurs at a transcriptional level [4,7,41]. Consistent with this, we previously observed that kps operon expression was down-regulated by up to 500-fold in E. coli K92 at low temperatures with respect to high temperatures [21]. Russo and Singh [20] have suggested that group II K54 capsule expression is negatively regulated by RcsA, and this effect appears to be mediated through RcsB. We found that deletion of either rcsA or rcsB enhanced kps transcription by up to approximately 8-fold at 19 °C (Table 4). This effect is probably physiologically insignificant since kps expression at low temperatures is highly reduced [21]; however, it might reveal a role of the Rcs pathway negatively regulating kps transcription. We also found that deletion of either rcsA or rcsB resulted in an enhanced PA production at 37 °C (Figure 5), but we failed to explain this effect through transcriptional regulation of the kps operon, whose expression was slightly reduced (Table 4). One possibility might be that increased PA production in both mutants is due to a reduction in PA degradation rather than an increment in its synthesis, since deletion of rcsB resulted in a greater reduction of nan gene expression (Table 4); however, this hypothesis awaits confirmation. Nevertheless, although the Rcs pathway diminished PA production at high temperatures, it does not seem to be responsible for thermoregulated inhibition of group II PA capsules in E. coli at low temperatures, since no detectable PA was observed in either rcsA or rcsB mutants.

Lastly, we further investigated the possible role of RcsB controlling the expression of other molecules implicated in the transcriptional control of cps and/or kps operons. It has been shown that RcsB auto-regulates the Rcs system by modifying the expression of rcsA [33] and rcsD [42] genes. Thus, we found that RcsB greatly enhanced rcsA expression at both temperatures (Table 5). RcsB also affected rcsC expression in a temperature-dependent manner (Table 5). Since RcsC can sense external signals to trigger the action of the Rcs pathway, this result would provide a mechanism by which RcsB modifies the capacity of the bacteria to perceive changes in growth temperature. In contrast, although RcsB does not seem to regulate rcsF expression, activation of RcsF does not require increased transcription [15].

The regulatory mechanism of kps operon transcription remains unclear, although recent studies have identified a set of molecules, including H-NS and SlyA, as important factors in this regulation [4,7]. Our results show that RcsB up-regulates and down-regulates the expression of all tested genes (dsrA, rfaH, h-ns and slyA) at high and low temperatures, respectively. Although more studies are required to clarify this complex regulatory mechanism, we want to emphasize some aspects. Deletion of rcsB repressed the expression of rfaH, slyA and h-ns genes at 37 °C (Table 5), which may explain the decreased kps expression observed in the rcsB mutant, since all three proteins are necessary for maximal transcription of the group 2 capsule gene cluster at high temperatures [4,7]. On the other hand, kps transcription was repressed from PR3 at 20 °C by H-NS [7]. SlyA was able to promote kps transcription from PR3 at low temperatures in an h-ns mutant; however, there was insufficient SlyA to activate transcription in WT [7]. Thus, a critical point in this regulation seems to be the relative concentration of these two proteins. We and others previously found that slyA expression is temperature-dependent, with a reduced expression at low temperatures [4,21]. Since slyA expression was slightly enhanced at low temperatures in an rcsB mutant, we speculate that this increment may overcome H-NS repression and explain the increment in kps transcription at 19 °C in the rcsB mutant (Table 5). Furthermore, the rcsB mutant showed a great increase in dsrA expression at 19 °C. dsrA is an RNA molecule in which commencement and stability of transcription are higher at low temperatures [15]. dsrA RNA binds to h-ns RNA and blocks its translation [19], which would allow SlyA to promote kps transcription. Recently, it was found that the heterodimer RcsB-BglI strongly activates leuO transcription [43], a known antagonist of H-NS [44,45]. Thus, we propose that RcsB may repress kps transcription at low temperatures by tipping the balance in favour of SlyA.

Overall, these results suggest that the Rcs phosphorelay, and particularly the RcsB regulator, are global keys in the capacity of E. coli K92 to adapt to different environments either inside or outside host cells, and they provide a better understanding of the complex thermoregulatory network governing capsule synthesis in E. coli.

**AUTHOR CONTRIBUTION**

All authors contributed actively and similarly in the development of this work. Nicolás Navasa has been mainly responsible for the experimental development.

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