Escherichia coli tol and rcs genes participate in the complex network affecting curli synthesis

Anne Vianney, Grégory Jubelin, Sophie Renault, Corine Dorel, Philippe Lejeune and Jean Claude Lazzaroni

Curli are necessary for the adherence of Escherichia coli to surfaces, and to each other, during biofilm formation, and the csgBA and csgDEFG operons are both required for their synthesis. A recent survey of gene expression in Pseudomonas aeruginosa biofilms has identified tolA as a gene activated in biofilms. The tol genes play a fundamental role in maintaining the outer-membrane integrity of Gram-negative bacteria. RcsC, the sensor of the RcsBCD phosphorelay, is involved, together with RcsA, in colanic acid capsule synthesis, and also modulates the expression of tolQRA and csgDEFG. In addition, the RcsBCD phosphorelay is activated in tol mutants or when Tol proteins are overexpressed. These results led the authors to investigate the role of the tol genes in biofilm formation in laboratory and clinical isolates of E. coli. It was shown that the adherence of cells was lowered in the tol mutants. This could be the result of a drastic decrease in the expression of the csgBA operon, even though the expression of csgDEFG was slightly increased under such conditions. It was also shown that the Rcs system negatively controls the expression of the two csg operons in an RcsA-dependent manner. In the tol mutants, activation of csgDEFG occurred via OmpR and was dominant upon repression by RcsB and RcsA, while these two regulatory proteins repressed csgBA through a dominant effect on the activator protein CsgD, thus affecting curli synthesis. The results demonstrate that the Rcs system, previously known to control the synthesis of the capsule and the flagella, is an additional component involved in the regulation of curli. Furthermore, it is shown that the defect in cell motility observed in the tol mutants depends on RcsB and RcsA.

INTRODUCTION

The ability of bacteria to recognize and adhere to a specific surface is a fundamental aspect of microbial ecology and pathogenesis. Bacterial adhesins and fimbriae confer specific recognition and adhesion to diverse target molecules, such as mammalian host tissue components or inorganic materials. Curli are highly adhesive proteinaceous fibres involved in this process that are produced by environmental conditions such as osmolarity, temperature and starvation. The ability to adapt rapidly to changing environmental conditions is crucial for the growth and pathogenicity of bacteria in their natural environments. In E. coli K-12, this complex regulatory network controls initial adhesion and biofilm formation via regulation of the csgD gene (Prigent-Combaret et al., 2001). It involves H-NS (Arqvist et al., 1992, 1993), IHF (Gerstel et al., 1993, 1994), Crl (Arqvist et al., 1994, 1995, 2001), and MlrA (Brown et al., 2001). The OmpR/EnvZ (Prigent-Combaret et al., 2001), CpxR/A (Dorel et al., 1999, 2002), and CpxR/A (Dorel et al., 1999, 2002) phosphorelays also control curli expression. Using microarray analysis, the RcsC sensor kinase of the RcsB/CD phosphorelay has recently also been found to control csgD expression (Ferrieres & Clarke, 2003).

Abbreviation: A, enzyme activity.
The RcsB/CD His-Asp phosphorelay was initially identified as a positive regulator of the capsular exopolysaccharide biosynthesis gene cluster (wza–wca) in E. coli in association with RcsA (Gottesman & Stout, 1991; Stout, 1994). The response regulator RcsB is activated upon the transfer of a phosphate group from its cognate sensor, RcsC, via a histidine-containing phosphotransmitter domain protein, RcsD. RcsF, a putative outer-membrane lipoprotein, is also involved in this regulatory network (Hagiwara et al., 2003). In addition to the wza–wca gene cluster, targets regulated by the Rcs system with its cofactor RcsA include the motility master operon flhDC (Francez-Charlot et al., 2003), rcsA and, in Salmonella, the gene ugd, which is required for the incorporation of 4-aminoarabinose into the lipopolysaccharide (Mouslim & Groisman, 2003). The cell division fits genes (Carballes et al., 1999), the osmoregulated osmC gene (Davalos-Garcia et al., 2001) and rplA, encoding a small RNA which stimulates the translation of the general stress-response sigma factor RpoS (Majdalani et al., 2002), are also controlled by RcsB, but independently of RcsA. Although RcsB acts preferentially as an activator, it negatively controls flhDC expression (Francez-Charlot et al., 2003). rcsA is repressed by the global regulator H-NS, and dsrA, a gene located upstream of rcsA, encodes a small RNA that controls H-NS synthesis by interfering with the hns mRNA (Sledjeski & Gottesman, 1995). RcsA is also degraded by Lon (Stout et al., 1991) and ClpYQ (Kuo et al., 2004) proteases. The specific signal triggering the activation of the Rcs system remains unknown. However, many reports suggest that the Rcs system might sense alterations of the cell envelope. The Rcs system has been reported to be activated by environmental signals, such as desiccation, osmotic shock and Zn2+ concentration (Hagiwara et al., 2003; Ophir & Gutnick, 1994; Sledjeski & Gottesman, 1996), in the presence of mutations affecting cell envelope components, such as tol (Clavel et al., 1996; Mouslim & Groisman, 2003), tfa (Parker et al., 1992), mdoH (Ebel et al., 1997), dsbA and dsbB (El-Kazzaz et al., 2004), or after overproduction of envelope proteins, such as TolB/Pal (Majdalani et al., 2002), RcsF (Gervais & Drapeau, 1992), DjlA (Toutain et al., 2002), LoLA and OmpG (Chen et al., 2001).

The tol–pal genes play a fundamental role in maintaining outer-membrane integrity. Mutations in any of the tol–pal genes result in hypersensitivity to deleterious agents, release of periplasmic content, formation of outer-membrane vesicles at the cell surface and induction of capsule synthesis, which results in a mucoid phenotype (Lazzaroni et al., 1999). A survey of gene expression in Pseudomonas aeruginosa biofilms using DNA microarrays has identified tolA as a gene activated in biofilms (Whiteley et al., 2001). In addition, the expression of both tolQRA and csgDEFG is modulated by RcsC (Clavel et al., 1996; Ferrieres & Clarke, 2003), suggesting a potential link between Tol–Pal, Rcs and curli expression. This led us to investigate the potential roles of the tol genes in biofilm formation in laboratory and clinical isolates of E. coli, and of the Rcs system in the regulation of curli synthesis.

**METHODS**

**Strains and growth conditions.** The E. coli strains are listed in Table 1. All the relevant markers were introduced by P1 transduction into MG1655 or the clinical isolate strains PHL881 and PHL885 (Miller, 1992). Plasmid pHRcsA was used to amplify RcsA after induction by 500 μM IPTG (Francez-Charlot et al., 2003). The media used were Luria–Bertani (LB), minimal M63 medium supplemented with 0.2% glucose (Miller, 1992), and M63/2 supplemented with 0.2% glucose, a low-osmolarity medium obtained by twofold dilution of M63. Congo red indicator plates contained 1% Casamino acids (Difco), 0.15% yeast extract (Difco), 0.005% MgSO4, 0.0005% MgCl2, 20 μg ml−1 Congo red (Sigma), 10 μg ml−1 Coomassie brilliant blue G (Sigma) and 2% agar (Evans et al., 1977; Hammar et al., 1995). Curli-producing bacteria form red colonies on these plates, whereas non-curli-producing strains remain white. In addition, most clinical isolates show a lacy colony morphology, also called convoluted phenotype (Allen-Vercue et al., 1997; Hammar et al., 1995). Tryptone swarm plates contained 0.35% agar (Difco), 1% tryptone (Difco) and 0.5% NaCl (Hedblom & Adler, 1980).

**Motility assays.** Swarm plates were inoculated from single colonies using a sterile toothpick and scored, after 16 h of growth at 30°C, by measuring the diameter of the growth zone.

**Enzyme assays.** β-Glucuronidase and β-galactosidase activities were assayed as described previously (Prigent-Combaret et al., 2001). M63/2 medium supplemented with 0.2% glucose was inoculated with 7 × 105 bacteria ml−1 and grown at 30°C. Samples were recovered at intervals during the culture and used to assay enzyme activity (A).

**Visualization and quantification of biofilm formation.** Cells were grown at 30°C in 24-well polystyrene plates (Nunc) containing 2 ml M63/2 medium. Biofilm formation was visualized after 24 h of culture as follows: planktonic cells were discarded, and the biofilm that had developed on the bottom of the plate was washed twice with M63. For each well, the two washes were pooled with the initial supernatant and referred to as the swimming cells. The biofilm was recovered in 1 ml M63 by scraping and pipetting up and down. The number of surface-attached and swimming bacteria was estimated from the OD600 to give the percentage adherence for each bacterial strain. A minimum of three independent assays was performed and the mean calculated.

**Construction of ompR–lac and cpxR–lac fusions.** cpxR and ompR operon fusions to lac were constructed by PCR amplification of a 715 bp fragment containing a 370 bp fragment upstream of the cpxR start codon, and of a 695 bp fragment containing a 305 bp fragment upstream of the ompR start codon. The following primers were used: WB1, 5′-ATTAACAGGAGGAATTCGTTCCGCGCTTG; WB2, 5′-GCGACGATCCCGGAAATACGTGC; WB3, 5′-CCAGCCAAGAATTCAAGCTCTTGTTT; WB4, 5′-CCGACAGGATCCGCGCAGCA. The PCR products were then restricted with EcoRI and BamHI, recognition sites of which had been introduced into the primers (underlined), and ligated into the EcoRI/BamHI sites of pRS551 (Simons et al., 1987). The corresponding plasmids were linearized and used to transform the strain TE2680 (Elliott, 1992). This generated the insertion of a single-copy fusion in the trp region of the chromosome, creating a merodiploid. The fusion was then transferred to AV11814 by P1 transduction.

**Electron microscopy.** Bacteria were grown at 30°C on M63/2 plates. Bacteria were suspended in 0.2 M cacodylate and allowed to adhere to a carbon-coated copper grid for 5 min. Negative staining was carried out for 30 s with 0.7% ammonium molybdate.
### Table 1. Bacterial strains used in this study

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<td>C. Beloin &amp; J. M. Ghigo, Institut Pasteur</td>
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*The Δtol mutation corresponded to a Δ(ybgC tolQ tolR tolA)-cm deletion (Germon et al., 1998).
†The csgA::uidA–kan and csgD::uidA–kan fusions resulted in the inactivation of the corresponding csg genes.
RESULTS

Biofilm formation in tol–pal mutants

To characterize the effect of the Tol–Pal system on biofilm formation, we tested the adherence properties of various tol–pal mutants in strain MG1655. A csgA::kan derivative was used as an internal control. The MG1655 Δtol derivative had a lower capacity to form biofilms, since its adherence was reduced by 50% compared with the parental strain (Fig. 1a). Under the same experimental conditions, the adherence of the csgA derivative was 24% that of MG1655, showing that strains deprived of curli could still adhere to surfaces. The adherence of a double csgA–Δtol mutant was similar to that of the Δtol mutant, suggesting that adhesion mediated by curli was not efficient in the Δtol background. This property was also found in all the tol–pal mutants tested (data not shown). The Δtol mutation was introduced into the clinical E. coli isolates. The effect of the mutation on adherence was more dramatic in PHL885 than in PHL881, which behaved like MG1655 (Fig. 1b). The same results were obtained with strains carrying point mutations in the tolA, tolB and pal genes (data not shown). We can conclude that the tol mutations impaired adherence and therefore biofilm formation in E. coli.

Curli formation in the Δtol mutant

As the tol–pal strains are altered in their outer-membrane integrity, a defect in biofilm formation in such mutants could be explained by a modification of their cell surface properties, leading to incorrect curli formation. An electron microscopy study confirmed the altered morphology of the Δtol mutants (Fig. 2a). At low magnification, the wild-type MG1655 cells were cylindrical, and most of them were aggregated. The Δtol derivatives were smaller and had a more spherical shape than MG1655. In addition, while MG1655 synthesized a large number of curli, many Δtol derivatives lacked curli or had a limited number of them.

Studies on Salmonella enteritidis and pathogenic E. coli have shown that a lacy colony morphology (also called convoluted phenotype) generated by culture in low-osmolarity medium at low temperature is indicative of the elaboration of curli (Allen-Vercoe et al., 1997; Hammar et al., 1995). PHL885 was used to test this phenotype. After growth at 30°C on Congo red indicator plates, this strain produced a convoluted phenotype, while its csgA derivative was phenotypically smooth, confirming, under our experimental conditions, the role of curli formation in the convoluted phenotype (Fig. 2b). PHL885 Δtol had an intermediate phenotype, with colonies showing only a few peripheral convolutions, the centre of the colony being almost smooth (Fig. 2b). Curli are known to bind Congo red quantitatively (Hammar et al., 1995). However, attempts to evaluate the amount of curli in the Δtol strains using Congo red binding were unsuccessful, since their altered cell permeability led to the entry of the dye into the cytoplasm, producing artefacts. Nevertheless, our results are globally consistent with a decrease in the amount of curli in the Δtol mutants.

The tol–pal mutations affect the expression of the two csg operons independently of CpxR

To check at what level the Δtol mutation influenced curli formation, we introduced the mutation into MG1655 carrying csgD–uidA or csgA–uidA transcriptional fusions. The expression of a csgD–uidA fusion was slightly increased (exponential phase) or increased threefold (stationary phase) (Fig. 3a). Under the same conditions, the synthesis of β-glucuronidase was lowered by a factor of 2 (stationary phase) to 4 (exponential phase) in Δtol strains carrying a csgA–uidA fusion (Fig. 3b). Despite the upshift observed in csgD–uidA expression, the consequence was a decrease in curli expression, leading to a lower adhesion capacity of the Δtol mutant. Therefore, it appears that the tol mutation results in the uncoupling of the two csg operon transcriptions. For the subsequent studies, β-glucuronidase activities were assayed in exponential (csgA::uidA–kan) or stationary (csgD::uidA–kan) phases of growth, where the differences in enzyme activities between wild-type and tol mutants were optimal.
The CpxR, OmpR and RcsB–RcsA proteins are good candidates for the control of curli gene expression in response to cell envelope alterations. First, we attempted to evaluate the impact of the \( \Delta tol \) mutations on the activity of the response regulator CpxR. A \( cpxR-lacZ \) transcriptional fusion was constructed in MG1655 (see Methods) and its expression was tested in the \( \Delta tol \) derivative. The synthesis of \( \beta \)-galactosidase was slightly, but significantly, increased under such conditions (data not shown). To investigate further whether CpxR is involved in \( csgDEFG \) activation and/or \( csgBA \) repression in response to cell envelope alterations, we constructed a \( \Delta tol-cpxR \) derivative of MG1655. The impact of these mutations on the expression of the two \( csg \) operons was determined. The \( \beta \)-glucuronidase activities of the \( csgD-uidA \) fusion were equivalent in the \( \Delta tol \) and \( \Delta tol-\Delta cpxR \) mutants, indicating that CpxR did not influence the expression of \( csgDEFG \) in this context (Fig. 3a). Expression of the \( csgA-uidA \) fusion was also measured in these backgrounds (Fig. 3b). The repressor effect of CpxR was alleviated in the \( \Delta cpxR \) derivative. In the \( \Delta cpxR \Delta tol \) mutant, the repressor effect due to the \( tol \) mutation was still present, and even increased in the presence of the \( \Delta cpxR \) mutation. Thus, the \( tol \) impact on \( csgAB \) expression did not involve CpxR.
RcsB/CD and RcsA control the expression of the two csg operons

The expression of both tolQRA and csgDEFG is modulated by RcsC (Clavel et al., 1996; Ferrieres & Clarke, 2003). It has been suggested that the Rcs system is able to sense cell envelope alterations (Clavel et al., 1996). The expression of the two csg operons was tested in MG1655 using different rcs backgrounds. We used an rcsB11::Tn10 mutation, in which RcsB is not functional, an rcsC338 mutation postulated to confer an enhanced activation of RcsB (Clavel et al., 1996), and a Δlon-510 mutation, leading to the presence of higher concentrations of RcsA, which is normally degraded by the Lon protease (Stout et al., 1991). Plasmid pHrCsA was also used to increase RcsA synthesis after induction by IPTG (see Methods). Under our experimental conditions, RcsB and RcsA were moderate repressors of the csgDEFG operon (Fig. 4a) but strong repressors of the csgBA operon (Fig. 4b). Thus, we confirmed earlier results obtained with macroarrays that show that RcsB represses the csgDEFG operon (Ferrieres & Clarke, 2003), and demonstrated that RcsA is involved in this mechanism. We also

Fig. 3. Effect of the Δtol and ΔcpxR mutations on the expression of the csg operons. β-Glucuronidase activity [U (mg bacterial dry weight)^–1] expressed by MG1655 derivatives, grown at 30 °C in M63/2 medium, was determined for strains carrying uidA transcriptional fusions to csgA and csgD. Exp., mid-exponential phase; Stat., early stationary phase. Values shown are the mean of at least three independent experiments. Error bars correspond to the standard deviation. WT, wild-type.

Fig. 4. Effect of the Rcs system on the expression of the csg operons. β-Glucuronidase activity [U (mg bacterial dry weight)^–1] of MG1655 derivatives carrying uidA transcriptional fusions to csgD (a) and csgA (b) was determined in the presence of mutations rcsB::Tn10 (no RcsB), Δlon (increase of RcsA), rcsC338 (gain of function of RcsB) and in the presence of pHrCsA (increase of RcsA). See Fig. 3 for the legend. Activities were determined in the exponential (csgA::uidA–kan) or stationary (csgD::uidA–kan) phases of growth. The expected amounts of RcsB and RcsA are indicated (+, expression of the protein; –, no expression; *, gain of function). WT, wild-type.
demonstrated for the first time that RcsB and RcsA are strong repressors of the csgBA operon.

**Stimulation of OmpR is responsible for the activation of csgDEFG in the Δtol mutant**

In the Δtol mutants, the Rcs phosphorelay is activated (Clavel et al., 1996). OmpR activity might also be stimulated, leading to an increase in ompC (Bernadac et al., 1998; Lazzaroni et al., 1986) and csgDEFG expression. The role of RcsB and OmpR in csgDEFG activation was therefore investigated.

In the Δtol–rcsB::Tn10 background, we observed a cumulative effect of Δtol and rcsB::Tn10 mutations on the expression of the csgDEFG operon (Fig. 5). In addition, the activator effect of the Δtol mutation was more important than the repressor activity of RcsB (Fig. 5). These data demonstrate that the activator effect of a Δtol mutation on csgDEFG was independent of the Rcs system.

ompR::Tn10 Δtol and ompR234 Δtol mutants were used to investigate the effect of OmpR on the activity of a csgD–uidA fusion. The ompR234 mutation corresponded to an L43R mutation near the phosphorylation site of OmpR (D55), leading to an increase in csgDEFG transcription (Vidal et al., 1998). The activator effect of Δtol disappeared in the ompR::Tn10 Δtol mutant, while the activator effects of the Δtol and ompR334 mutations were cumulative (Fig. 5).

These results were consistent with the hypothesis of an increase in the active form of OmpR or OmpR234 in the Δtol mutant, leading to an overexpression of csgD.

**Stimulation of RcsB is responsible for csgBA repression in the Δtol mutant**

Expression of the csgA–uidA fusion was measured in a Δtol rcsB::Tn10 double mutant. The repressor effect of the Δtol mutation on the expression of the csgBA operon (A = 378±111, compared to 1511±299 for the wild-type) was abolished in the Δtol rcsB::Tn10 double mutant (A = 9238±2500), showing that the decrease in CsgA expression in the Δtol mutant could be explained by an increase in the repressor activity of RcsB. Hence, the Rcs system was activated in the Δtol mutants, leading to a strong repression of csgBA, the result being a decrease in curli synthesis. This repressor effect on csgBA synthesis was dominant following activation by CsgD. CsgA was expressed to a greater extent in the Δtol rcsB::Tn10 double mutant (A = 9238±2500) than in the rcsB::Tn10 mutant (A = 5685±1000). This could be explained by the increase in csgD expression under such conditions (Fig. 5).

**Activation of the Rcs system in the Δtol mutants is responsible for their mucoid phenotype, lack of motility and poor adherence**

The Rcs system is stimulated in the tol mutants (Clavel et al., 1996), and this results in a mucoid phenotype due to an RcsB-dependent activation of exopolysaccharide gene expression. We show, in this paper, that the activation of the Rcs system leads to a decreased expression of csgA. The possibility that the Rcs system plays a role in the decrease in adherence of the Δtol mutant was investigated. As expected, the adherence of a rcsB::Tn10 derivative (67±8±3·9%) was higher than that of the wild-type strain (39·8±6·3%). Under such conditions, the percentage adherence of the Δtol derivative was 20·4±5·6%. The adherence of the Δtol rcsB::Tn10 double mutant was similar to that of the rcsB::Tn10 derivative (75·2±11·0%), indicating that stimulation of the Rcs system in the tol mutants was responsible for their lowered adherence.

The impact of the stimulation of the Rcs system on the motility of the Δtol strains was also determined. RcsB is a repressor of the master operon flhDC involved in the control of flagella expression (Francez-Charlot et al., 2003). Consistent with this finding, Δtol mutants are impaired in their cell motility (12±1 mm, compared to 64±5 mm for the wild-type strain). Under such conditions, the rcsB::Tn10 derivative was slightly more motile than the wild-type strain (80±3 mm), and the Δtol rcsB::Tn10 double mutant recovered a motility comparable to that of the wild-type (47±7 mm), taking into account its reduced growth rate. Therefore, the pleiotropic phenotype of the tol mutants results partially from the activation of the Rcs system, leading to increased capsule synthesis and decreased motility and adherence. However, other tol phenotypes, such as

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Fig. 5. Effect of ompR234, ompR::Tn10 and rcsB::Tn10 mutations on the expression of the csgDEFG operon in Δtol mutants. β-Glucuronidase activity [U (mg bacterial dry weight)−1] was determined in the stationary phase of growth for MG1655 derivatives carrying uidA transcriptional fusions to csgD, as described in Fig. 3. The absence (−) or gain of function (*) of the proteins are given at the bottom of the figure. wt, wild-type.
defects in outer-membrane integrity and the entry of biomolecules (group A colicins and filamentous phage DNA), were still present in the Δtol rcsB::Tn10 double mutant (data not shown).

**DISCUSSION**

We have demonstrated that the Tol–Pal system is required for the correct expression of curli in laboratory strains and clinical isolates of *E. coli*. This is confirmed by morphological analyses, using electron microscopy of tol–pal mutants, showing a lower content of curli and a lack of lacy colony phenotype in the clinical isolates, thus resulting in a decrease in adherence of the tol mutants. A genetic analysis of the expression of transcriptional fusions to the csg promoters in Δtol mutants led to the characterization of a decrease in the expression of the csgBA operon, while the expression of the csgDEFG operon was increased. This is an unexpected result, since until now the two operons have always been shown to be regulated in the same direction. Expression of these operons is positively regulated by OmpR (via the activation of CsgD) (Romling *et al.*, 1998), and negatively regulated by CpxR (Dorel *et al.*, 1999; Otto & Silhavy, 2002). We have investigated the possibility that the transcriptional regulation of the csg genes by tol–pal occurs through OmpR, CpxR and/or RcsB. Several lines of evidence led us to include the RcsCD/B phosphorelay in our study. RcsC negatively controls tolQRA (Clavel *et al.*, 1996) and csgD (Ferrieres & Clarke, 2003). The RcsCD/B system negatively regulates the genes involved in flagellar synthesis, motility and chemotaxis (Francez-Charlot *et al.*, 2003), and positively controls the synthesis of colanic acid, in association with the regulator RcsA and the postulated outer-membrane lipoprotein RcsF (Gottesman & Stout, 1991; Hagiwara *et al.*, 2003). In addition, the RcsCD/B phosphorelay is activated in the presence of tol mutations (Clavel *et al.*, 1996; Mouslim & Groisman, 2003). The curli, flagella and capsule are all involved in some of the steps of biofilm formation (Prigent-Combaret *et al.*, 2000).

The expression of the CpxR repressor was slightly enhanced in the tol mutant. In addition, our results suggest a decrease in the phosphorylated form of CpxR in the Δtol background. These results might explain the changes in csgA and csgD expression. However, analysis of the Δtol–ΔcpxR mutant showed that the effects of the tol mutation are dominant over ΔcpxR as regards csg expression (Fig. 3). Thus, the effect of a tol mutation on the expression of the csg genes was unlikely to involve CpxR.

Our data suggest that OmpR orchestrates activation of curli at csgD, but the multiple repressions at csgBA offer numerous possibilities of regulation. In tol mutants, the main control occurs via RcsB/A repression of the csgBA operon. The activator effect of OmpR is absolutely necessary for csgD expression (Prigent-Combaret *et al.*, 2001). Expression of an ompR–lacZ fusion was slightly increased in a Δtol mutant, but two-dimensional electrophoretic analysis of the OmpR profile did not reveal any significant modification, since our OmpR antibody detected only one spot for OmpR (data not shown). This could be due to a low content of the phosphorylated form of OmpR (Cai & Inouye, 2002). A transcriptional fusion to csgD was inactive in both Δtol ompR::Tn10 and ompR::Tn10 backgrounds, while the effects of Δtol and ompR334, an allele that increases the activation of csgD by OmpR, were cumulative in the stationary phase. The increase in ompR expression in tol mutants is consistent with previous results that show that the porin content is modified in tol–pal mutants (Bernadac *et al.*, 1998; Lazzaroni *et al.*, 1986), and this could explain the increase in csgD expression.

Under our experimental conditions, the RcsB and RcsA proteins repressed the activity of the two csg operons. This is in agreement with previous findings on the control of csgD by RscC (Ferrieres & Clarke, 2003). The Rcs system has also been shown to be stimulated in tol mutants, as seen by the rcsB-dependent activation of capsule synthesis in such mutants (Clavel *et al.*, 1996). Here again, we were unable to detect a difference in the RcsB pattern of a Δtol mutant after two-dimensional electrophoresis (data not shown). In addition, we showed that the most dramatic effect of the Rcs phosphorelay is on csgAB and that it also involves RcsA (Fig. 4). It remains to be seen whether these two regulators are able to bind the csg promoters. Although RcsB and RcsA are mainly known as activator proteins, they are repressors of the flhCD operon that is involved in flagella synthesis (Francez-Charlot *et al.*, 2003; Wehland & Bernhard, 2000). The csg operons would be another example of negative control by these proteins, thus confirming their ambivalent character. The RcsAB box (Francez-Charlot *et al.*, 2003; Wehland & Bernhard, 2000) does not appear to be well conserved in several genes regulated by RcsAB (Hagiwara *et al.*, 2003). The minimum consensus is GA(N)3C, a sequence frequently found in the *E. coli* genome, which does not facilitate the search for a potential Rcs box in promoter sequences. The repressor effects of tol mutations on csgBA occurred via RcsB, since they were abolished in the tol–rcsB background.

We have previously shown that the tol–pal mutations affect the expression of the cps genes involved in colanic acid capsular polysaccharide synthesis (Clavel *et al.*, 1996), and we demonstrate in this study that they also affect flagella synthesis as the result of an activation of RcsB/A repressor activity. This is also in agreement with our finding that, in the tol–pal mutants, an increase in the repressor activity of RcsB/A leads to a reduction in curlin production. Our results are summarized in the model presented in Fig. 6. We propose that cell envelope alterations, like those observed in the tol–pal and rfa mutants (data not shown), lead to an increase in csgDEFG and a decrease in csgAB expression. Under such conditions, the activation of csgDEFG probably occurs via OmpR, which is dominant upon repression by RcsB, while RcsB and RcsA could repress csgAB through a dominant negative effect on CsgD.
The consequence is a reduced amount of curli at the surface of the tol–pal mutants.

The role of phosphorelay systems in surface sensing is well established. In addition to OmpR/EnvZ (Vidal et al., 1998) and CpxR/A (Dorel et al., 1999; Otto & Silhavy, 2002), we show that the Rcs system is also required for curli expression. Another example of complex transcriptional regulation involving tolB (through Rcs modulation) and three phosphorelay systems is the Salmonella enterica ugd gene involved in lipopolysaccharide synthesis (Mouslim & Groisman, 2003). After surface attachment mediated by curli, the Rcs system could repress the synthesis of flagella and activate the synthesis of colanic acid, as proposed in recent models (Lejeune, 2003; Prigent-Combaret et al., 2000). Thus, the Rcs phosphorelay is likely to be involved in the signalling network that modulates the global change in envelope composition during biofilm formation. We propose that the differential activation of these different phosphorelays allows the sequential activation/repression of the various external structures required for biofilm construction.

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