Virulence attenuation in *Salmonella enterica* rcsC mutants with constitutive activation of the Rcs system

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Mutations in *rcsC* that result in constitutive colanic acid capsule synthesis were obtained in *Salmonella enterica* serovar Typhimurium. Most *rcsC* alleles were dominant; however, recessive *rcsC* alleles were also found, in agreement with the postulated double role (positive and negative) of RcsC on the activation of the RcsB/C phosphorelay system. *Salmonella rcsC* mutants with constitutive activation of the Rcs system are severely attenuated for virulence in BALB/c mice and their degree of attenuation correlates with the level of Rcs activation. Partial relief of attenuation by a gmm mutation indicates that capsule overproduction is one of the factors leading to avirulence in constitutively activated *rcsC* mutants.

**INTRODUCTION**

The Rcs phosphorelay system was initially characterized in *Escherichia coli* as a two-component positive regulator of colanic acid capsule synthesis encoded by *rcsC* and *rcsB* (Brill et al., 1988; Gottesman et al., 1985; Stout & Gottesman, 1990). The sensor protein RcsC, a hybrid histidine kinase, and the transcriptional activator RcsB are the main components of the system, which also includes a second transcriptional activator, RcsA (Stout et al., 1991) and an intermediate phosphotransmitter, YojN (Takeda et al., 2001). The Rcs system is also a negative regulator of the flagellar master operon *flhDC* (Francez-Charlot et al., 2003), whose products are necessary for the expression of genes involved in flagellum synthesis, motility and chemotaxis.

The first environmental signal shown to strongly and rapidly induce colanic acid synthesis was osmotic upshift (Sledjeski & Gottesman, 1996). This effect was mediated by the Rcs system. More recently it has been shown that the Rcs phosphorelay can be activated when wild-type cells are grown at 20°C in the presence of glucose and a high concentration of Zn^{2+} (Hagiwara et al., 2003). Furthermore, several genetic conditions that result in alterations in envelope composition or integrity are known to activate the Rcs system. For example, a mutation in the *mdoH* gene involved in the biosynthesis of membrane-derived oligosaccharides (Ebel et al., 1997) or overproduction of the DnaJ-like transmembrane protein DjlA (Kelley & Georgopoulos, 1997) has been shown to stimulate the expression of genes regulated by the *E. coli* Rcs system. In

*Salmonella enterica* serovar Typhimurium, mutations in the essential gene igaA (Canò et al., 2001) [also known as *yffF* or *mucM* (Costa & Antón, 2001)] result in mucoidy and reduced motility (Canò et al., 2002). A recent report has shown that viable igaA mutations also result in virulence attenuation in the mouse model (Dominguez-Bernal et al., 2004). Like the other phenotypes associated with igaA mutations (Canò et al., 2002), attenuation is suppressed by null mutations in *rcsC*, *yojN* or *rcsB* (Dominguez-Bernal et al., 2004), suggesting that overactivation of the Rcs phosphorelay causes loss of virulence. This hypothesis is addressed in this study.

Here, we describe the isolation of *Salmonella rcsC* mutants with constitutive activation of the Rcs system, and the characterization of amino acid substitutions that lead to different degrees of activation of the system. Virulence trials with constitutively activated *rcsC* mutants provide a direct demonstration of the link between activation of the Rcs system and avirulence in mice. We also show that virulence attenuation associated with activation of the RcsB/C system is partially due to colanic acid overproduction.

**METHODS**

Bacterial strains, bacteriophages and strain construction. *S. enterica* serovar Typhimurium strains used in this study are described in Table 1. Unless otherwise indicated, the strains derive from the mouse-virulent strain 14028. Transductional crosses using phage P22 HT 105/1 int201 (Schmieder, 1972) were used for strain construction operations. The transduction protocol was described by Maloy (1990). To obtain phage-free isolates, transductants were purified by streaking on green plates (Chan et al., 1972). Phage sensitivity was tested by cross-streaking with the clear-plaque mutant P22 H5.
Table 1. Strains of S. enterica serovar Typhimurium used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
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<td>14028</td>
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<td>flhDC5213::MudA</td>
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<td>Duplication obtained from SV3193 (Camacho &amp; Casadesús, 2001)</td>
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</table>

* gmm-21::MudI, flhDC5213::MudA, rcsB::KmR, rcsA::MudI and rcsA::MudQ derivatives of these strains were also used where appropriate, as indicated in the text.

Media and chemicals. The culture medium for bacteria was Luria–Bertani (LB) broth. Solid media contained agar at 1.5% final concentration. Antibiotics were used at the following concentrations: mecinllam, 1 μg ml⁻¹; tetracycline, 20 μg ml⁻¹; kanamycin, 50 μg ml⁻¹; chloramphenicol, 20 μg ml⁻¹; ampicillin, 30 μg ml⁻¹. Motility assays were carried out in LB prepared without yeast extract (Gillen & Hughes, 1991). Solid motility medium contained agar at 0.25% final concentration.

Isolation of mucoid mutants in mecinllam plates. The initial isolation of mucoid mutants was performed with mecinllam plates, as previously described (Costa & Antón, 2001). Stationary cultures (100 μl) of S. enterica serovar Typhimurium strain 14028 were plated on LB agar containing 1 μg mecinllam ml⁻¹ at 37°C. Most mecinllam-resistant colonies were mucoid.

Construction of rcsB mutants. Disruption and replacement of rcsB with a kanamycin (Km)-resistance gene in selected rcsC mutants (SV4756, SV4757, SV4758 and SV4949) were performed by the method described by Datsenko & Wanner (2000). Briefly, the Km-resistance gene from plasmid pKD4 was PCR-amplified with primers 5'-ATT ATT GCC GAC CAC CCG ATT GTA CGT TTC GGT ATT CGT GTA GCC TGG AGC TGA GTC TTC-3' and 5'-AGA GAG ATA GTT GAG CAC GCG GAT ATC ATT CTC TAC GCC CCA TAT GAA TAT CTT CCT GAG-3'. The PCR product was used to transform rcsC mutant strains carrying the Red recombinase expression plasmid pKD46. Insertion of the Km-resistance gene was verified by PCR with primers flanking the rcsB locus (5'-AGC GGA ATT CAG GAG GAA TAT AGC AAC AAT ATG AAC G-3' and 5'-GGT AAA GCT TGT CTA CGA CAA CGG ATT TAT TCT TGT G-3').

β-Galactosidase assays. Levels of β-galactosidase activity were assayed for exponential cultures in LB medium as described by Miller (1972), using the CHCl₃/SDS permeabilization procedure.

Motility assays. Liquid cultures were prepared in motility medium and incubated at 37°C with shaking. At the stage of mid-exponential growth, a sterile toothpick was soaked in the culture and used to inoculate a motility agar plate. The plate was incubated at 37°C. The diameter of the bacterial growth halo was measured every hour.

DNA amplification with PCR and DNA sequencing. Amplification reactions were carried out in a Perkin Elmer Gene-Amp PCR System 2400 (Perkin Elmer Cetus). The final volume of reactions was 50 μl and the final concentration of MgCl₂ was 1 mM. Reagents were used at the following concentrations: dNTPs, 200 μM; primers, 1 μM; and Taq polymerase (Expand High Fidelity PCR System, Roche Diagnostics), 1 unit per reaction. Amplification of the rcsC coding sequence was carried out with primers 5'-GTA ATG TTA TTG CTC GTG TGG GAG-3' and 5'-TAT GTT ACC CAG CCT GAT GG-3'. The products of amplification were sequenced with an automated DNA sequencer (Sistemas Genómicos) using the primers 5'-GTA ATG TTA TTG CTC GTG TGG GAG-3', 5'-CAT GAG CCG ATT ATG AAA TAC-3', 5'-TGA GTA ACG AAC TGC CGC AC-3', 5'-CTG TTG AGC AAC GCC ATT AAG-3' and 5'-GAT CCC GTT GGA AAG AGC-3'. Mutations were confirmed by sequencing at least two independent PCR products.

Mouse mixed infections. Eight-week-old female BALB/c mice (Charles River Laboratories) were subjected to mixed infections. Groups of three or four animals were inoculated with a 1:1 ratio of the mutant and the wild-type. Bacteria were grown overnight at 37°C in LB with shaking, diluted into fresh medium (1:100), and grown to an OD₆₀₀ of 0.3–0.5. Intraperitoneal inoculation was performed with 0.2 ml physiological saline containing 10⁵ c.f.u. Bacteria were recovered from spleen 48 h after inoculation and c.f.u. were enumerated on LB and on selective medium (LB with mecinllam for rcsC constitutive mutants). A competitive index (CI) for each mutant was calculated as the ratio between the mutant and the
wild-type strain in the output (bacteria recovered from the host after infection) divided by their ratio in the input (initial inoculum) (Beuzo´n & Holden, 2001; Freter et al., 1981; Taylor et al., 1987).

**In vitro CI calculation.** Overnight cultures of the wild-type and mucoid mutant strains were diluted and mixed in LB in order to get an OD600 of about 0.05. The mixed culture was incubated in a shaker at 37°C for 24 h; c.f.u. for both strains were enumerated on LB at the beginning and at the end of the experiment. Strains were distinguished by the mucoidy of the mutant colonies. A CI for each mutant was calculated as the ratio between the mutant and the wild-type strain in the output (24 h) divided by their ratio in the input (initial mix).

**RESULTS**

**Isolation of mucoid rcs mutants**

Mucoid mutants were isolated on mecillinam plates as described by Costa & Antón (2001). Out of 300 independent mucoid mutants of spontaneous origin, 27 carried mutations 50% co-transducible with ompC396::Tn10dTc. This insertion is linked to rcsC, yojN and rcsB (McClelland et al., 2001). An analogous gene arrangement is found in E. coli (Gottesman et al., 1985). Reconstruction experiments confirmed that the mutations responsible for mucoidy and mecillinam resistance were linked to ompC.

**DNA sequence analysis of rcsC mutants**

To identify mutations located in rcsC, and to characterize them at the DNA sequence level, PCR amplification products of the rcsC coding region from the 27 mucoid mutants were sequenced. For 24 mutants, a mutation was found in rcsC. The nature and position of the mutations are indicated on the diagrammatic representation of the predicted RcsC product in Fig. 1 and Table 2. Eleven

**Table 2. Summary of features of rcsC constitutively activated mutants compared to wild-type**

Values are means from representative experiments. β-Galactosidase activities were calculated for appropriate derivatives of the strains indicated. CI was calculated for representative rcsC mutants with different degrees of activation after mouse mixed infections with the wild-type strain (mouse) or after growing together with the wild-type strain in LB for 24 h, as described in Methods. D, dominant; R, recessive; −, not applicable or not determined.

<table>
<thead>
<tr>
<th>Strain</th>
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**Fig. 1.** Schematic representation of the Salmonella RcsC protein. TM1 and TM2 represent the predicted transmembrane domains. H479 and D875 are the histidine and the aspartic residues phosphorylatable in the transmitter and the receiver domain, respectively. The amino acid changes found in the mutants described in this work are indicated.
different mutations were found among the 24 rcsC mutants sequenced. The following mutations were independently isolated more than once: V438I, F473Y, P484L, P484S and N877S. When the same mutation was isolated several times, only one mutant was chosen for further study.

All the mutations were located in the putative main cytoplasmic portion of RcsC. E354K, H355R, V438F and V438I are in the putative input domain; F473I, F473Y, P484L, P484S and I531S in the putative histidine kinase domain; N877S and T903A in the putative receiver domain. In silico analysis of the predicted RcsC structure, based on its homology with well-characterized sensors or response regulators, has permitted predictions of the putative effect of some of the mutations on the activity of the RcsC protein (see Discussion).

**Dominance/recessivity of constitutively activated rcsC alleles**

To examine whether the rcsC mutations under study were dominant or recessive for the mucoid phenotype, they were introduced into a merodiploid strain carrying a chromosomal duplication of the hisH–cysA region, which encompasses centisomes 44 to 53 and includes rcsC (Camacho & Casadesús, 2001). For dominance/recessivity analysis, strain SV4985 was transduced with a P22 HT lysate grown on derivatives of the rcsC mutant strains that carried the ompC396::Tn5 dTc mutation. Because this insertion is 50% linked to the rcsC mutations, dominance can be expected to result in about 50% of mucoid transductants, while recessivity will result in an absence of mucoid transductants. Most mutations were dominant: only two mutations in the putative RcsC receiver domain were found to be recessive (Table 2). Since recessive mutations usually correspond to loss-of-function mutations, this result supports a negative role for this domain in the activation of the Rcs phosphorelay (see Discussion).

**Different degrees of activation of the RcsB/C system caused by rcsC mutations as measured by increase in wca expression**

The phenotype of a representative mucoid mutant is compared with the wild-type in Fig. 2(a, b). Mucoidy is a consequence of activation of the RcsB/C system, which leads to an increase in colanic acid capsule synthesis (reviewed by Gottesman, 1995). The colanic acid polysaccharide capsule biosynthetic genes (wca genes) are at least 19 clustered genes, likely arranged in a single long operon, in E. coli and S. enterica (Stevenson et al., 1996, 2000; Stout, 1996). Therefore, the activity of a wca::lacZ fusion can be used as a reliable estimation of the degree of activation of the system in the different mutants. gmm (also called wcaH) was used as a representative of the whole wca cluster. Analysis of β-galactosidase activity from exponential cultures in LB medium showed that a gmm::lacZ fusion was repressed in the wild-type (<1 Miller units), while the β-galactosidase activities found in the mutants ranged from 53 to 2200 units (Fig. 3a and

**Fig. 2.** Constitutively activated rcsC mutants are mucoid and non-motile. Phenotypes of colonies growing in LB plates for the wild-type strain (a) and one representative rcsC mutant (b). Motility of the wild-type strain (c) and the rcsC mutant SV4758 (d). Bacteria were spotted onto motility agar and incubated for 6 h at 37°C.
A previous study showed that igA mutants, which are also mucoid, have a defect in motility that can be suppressed by null mutations in rcsC or rcsB (Cano et al., 2002). This result suggested that activation of the RcsB/C system could cause both mucoidy and loss of motility. Microscopic observation of exponential cultures of the mucoid rcsC mutants showed that all were non-motile. This result was confirmed by comparing the wild-type and the mutant strains in motility agar plates (see Methods). While the wild-type was able to grow, forming a halo whose diameter increased progressively, the mutants grew only in the area of inoculation. A representative experiment is shown in Fig. 2(c, d).

Constitutive rcsC mutations decrease flhDC expression

Since all the rcs mutants described above were non-motile, the expression of flagellar genes in these mutants was monitored using a transcriptional lac fusion in the flagellar master operon flhDC. This operon encodes transcription factors necessary to trigger the genetic cascade that controls flagellar synthesis (Claret & Hughes, 2000; Furness et al., 1997; Gygi et al., 1995; Macnab, 1996). The results are shown in Fig. 3(b). As expected, expression of the flhDC fusion was significantly decreased in all the rcsC mutants under study. In addition, expression of flhDC negatively correlates with gmm expression. This correlation was anticipated by the fact that the Rcs system activates wca genes but represses flagella synthesis through flhDC.

An rcsB null mutation suppresses both mucoidy, mecillinam resistance and motility defects in constitutively activated rcsC mutants

RcsB is a downstream element in the signal transduction pathway that leads from RcsC to transcriptional regulation of the wca cluster (and of other genes). Hence, an rcsB null mutation can be expected to suppress the phenotypes of constitutively activated rcsC mutants. This prediction was tested by replacing rcsB through transformation with a Km resistance gene (see Methods) in the rcsC mutants. These rcsB rcsC double mutants were non-mucoid, motile and sensitive to mecillinam. These results confirmed that mecillinam resistance, lack of motility and mucoidy resulted from canonical activation of the Rcs system via RcsB.

S. enterica serovar Typhimurium rcsC mutants with constitutive activation of the Rcs system are avirulent in mice

A recent study has shown that viable igA mutants are attenuated for virulence in mice, and that attenuation is suppressed by null mutations in rcsG, yojN or rcsB (Domínguez-Bernal et al., 2004). These observations provided evidence that overactivation of the Salmonella Rcs system causes avirulence (Domínguez-Bernal et al., 2004). The rcsC mutants isolated in this work are highly suited to test this hypothesis directly, since they affect one component of the phosphorelay and exhibit different degrees of activation of the system. For virulence assays, BALB/c mice were infected intraperitoneally with a mixture of the wild-type strain and one of the mutants, and a CI was calculated for each mutant (see Methods). All the mutants were extremely attenuated and a correlation was found between the individual levels of Rcs activation and their degrees of attenuation, with CI ranging between $1 \times 10^{-3}$ and $2 \times 10^{-6}$ (Fig. 4 and Table 2). As expected, an rcsB mutation was able to completely suppress the attenuation of these mutants (Fig. 4).

Table 2). This enormous range of gmm activation (from 10-fold to 500-fold) indicated that the constitutive rcsC alleles under study were highly heterogeneous.

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A gmm mutation partially suppresses avirulence in constitutively activated rcsC mutants

A priori, attenuation in constitutively activated rcsC mutants could be caused by activation or repression of one or more genes or operons regulated by the Rcs system. Two sets of genes whose regulation by the Rcs system is well known, both in E. coli and in S. enterica serovar Typhimurium, are colanic acid genes (Brill et al., 1988; Cano et al., 2002), and genes involved in flagella synthesis and in chemotaxis (Cano et al., 2002; Francez-Charlot et al., 2003). Control of flagella and chemotaxis is exercised through the master operon flhDC (Macnab, 1996). Salmonella flhDC mutants are defective in invasion of in vitro cultured epithelial cells due to their lack of motility (Schmitt et al., 2001; Young et al., 2000) and a role in virulence for these genes has been shown in other bacteria (Givaudan & Lanois, 2000; Tans-Kersten et al., 2004). However, a S. enterica serovar Typhimurium flhDC mutant is not attenuated (Fig. 4 and Dominguez-Bernal et al., 2004), indicating that lack of motility or of expression of other genes activated by FlhDC is not responsible for the attenuation of constitutively activated rcsC mutants. Another possibility was that overproduction of colanic acid capsule could have a negative effect upon virulence. To test this possibility, we measured the CI for rcsC gmm double mutants. These are non-mucoid, since gmm is one of the structural genes necessary for colanic acid synthesis. As shown in Fig. 4, attenuation of the constitutively activated rcsC mutants was partially suppressed by the gmm mutation. However, their CIs were significantly < 1 in all cases, and correlated with the degree of activation of the system typical of each mutant, ranging from 0.22 to 7.1 × 10⁻⁴. Since the production of colanic acid capsule is also dependent on the positive regulator RcsA (Gottesman, 1995), an rcsA mutation should be able to partially suppress the attenuation of rcsC mutants. This prediction was confirmed by the CIs obtained by rcsA rcsC double mutants (Fig. 4). In addition, a gmm rcsA rcsC54 mutant was no better in competition assays than the gmm rcsC54 or the rcsA rcsC54 mutants (Fig. 4). Together these results unambiguously suggest that overproduction of colanic acid is one of the factors that contributes to the avirulence of constitutively activated rcsC mutants. However, since virulence is not fully restored, additional factors apart from capsule overproduction must exist.

**DISCUSSION**

The Rcs system was initially described as a two-component phosphorelay that controls colanic acid production in E. coli (Brill et al., 1988; Gottesman et al., 1985). Two decades later, this regulatory system has been shown to play roles in other physiological processes such as the regulation of the cell division genes ftsA and ftsZ (Carballes et al., 1999), the modulation of swarming (Takeda et al., 2001), and the control of flagella production (Francez-Charlot et al., 2003). In S. enterica serovar Typhi, the RcsB/C system modulates the expression of Vi antigen, flagellin and invasion proteins (Arricau et al., 1998; Houng et al., 1992; Virlogeux et al., 1995, 1996). A recent genome-wide analysis suggests that the Rcs regulon of E. coli may include more than 40 loci (Hagiwara et al., 2003; Oshima et al., 2002). Another study shows that the RcsC sensor kinase is required for normal biofilm development in E. coli and that more than 150 genes are under Rcs control (Ferrieres & Clarke, 2003).

For a long time, the only physiological stimulus known to activate the Rcs system in E. coli was osmotic shock (Sledjeski & Gottesman, 1996). Growth conditions that permit strong induction of Rcs by cold shock have recently been described for E. coli (Hagiwara et al., 2003). In normal laboratory growing conditions, the level of activation of the Rcs system is very low; as a consequence, null mutations in
the components of the phosphorelay have no appreciable effects on the phenotype. For this reason, the physiological roles of the Rcs system have often been inferred from the ability of null mutations in genes of the phosphorelay to suppress mutations in other loci. For instance, in *E. coli*, null mutations in *mdoH* lead to an increase in colanic acid production that is suppressed by null mutations in *rcsA*, *rcsB* or *rcsC* (Ebel et al., 1997). In a similar fashion, viable *igaA* alleles of *Salmonella enterica* serovar Typhimurium result in induction of *wca* genes, repression of *flhDC* and avirulence, and all these phenotypes are suppressed by null mutations in *rcsB* or *rcsC* (Canò et al., 2002; Costa & Antón, 2001; Domínguez-Bernal et al., 2004). This suppression pattern has provided evidence that the *S. enterica* *IgaA* functions as a negative regulator of the Rcs system (Canò et al., 2002; Costa & Antón, 2001; Domínguez-Bernal et al., 2004).

In this work, we describe a procedure to isolate *rcsC* mutants of *S. enterica* with constitutive activation of the Rcs system, followed by their characterization at the DNA sequence level. Among 24 independent constitutively activated *rcsC* mutants, sequencing has revealed 11 different amino acid substitutions, all located in the main cytoplasmic portion of the protein (Fig. 1). The mutations causing stronger Rcs activation map inside the putative histidine kinase domain (*F473I, F473Y, P484L, P484S* and *I531S*) or the putative receiver domain (*N877S* and *T903A*). Mutations in the input domain or in the histidine kinase (transmitter) domain are dominant, while those in the response regulatory (receiver) domain are recessive. Reces-
sive mutations are usually loss-of-function mutations, implying that, whereas most of our mutations increment a positive RcsC activity (dominant, gain-of-function muta-
tions), *N877S* and *T903A* abrogate a negative one. This is in agreement with previous results obtained in *E. coli*: (i) the *rcsCI37* allele (which causes constitutive colanic acid overproduction) is recessive (Gottesman et al., 1985); (ii) RcsC has both positive and negative regulatory effects on the expression of the *wca* operon (Brill et al., 1988; Stout & Gottesman, 1990); and (iii) the negative activity of RcsC is localized to the receiver domain (Clarke et al., 2002). It has been proposed that this negative activity is actually a phosphatase activity (Clarke et al., 2002; Gottesman, 1995), as is the case for other His-Asp phosphorelay systems (reviewed by West & Stock, 2001). In *E. coli*, this putative phosphatase activity requires the phosphorylatable Asp in the receiver domain of RcsC (Clarke et al., 2002).

A search at the Conserved Domain Database (Marchler-Bauer et al., 2003) reveals that RcsC contains two conserved domains: the histidine kinase domain and the signal receiver (response regulator) domain. The five mutations found in the histidine kinase domain are located in a subdomain called the histidine kinase A or dimerization/phosphoac-
tceptor domain. This domain contains the phosphorylatable His; in some instances, dimerization through this domain has been proved to be necessary for activation (reviewed by West & Stock, 2001). The three residues of this domain that have been replaced in some of our mutants (*Phe473, Pro484* and *Ile531*) are present in the consensus sequence of the domain. Pro484 is specially conserved and, interestingly, on the basis of the structure of the histidine kinase A dimer in the *E. coli* protein EnvZ (Tomomori et al., 1999), residues corresponding to Phe473 and Pro484 in RcsC are predicted to play a role in dimerization as part of the dimer interface.

Two different activating mutations were found in the receiver domain of RcsC: *N877S* and *T903A*. This domain is 38 % identical to the same domain in *E. coli* CheY. The crystallographic structure of CheY reveals the presence of five highly conserved residues in the response regulator active site: Asp57 (the site of phosphorylation) (Smith et al., 1989); Asp12 and Asp13 (involved in coordination of a Mg$^{2+}$ ion essential for phosphorylation and dephospho-
hydration) (Stock et al., 1993); Lys109 (important for the phosphorylation-induced conformational change) (Lukat et al., 1991); and Thr87 (also involved in conformational change through formation of a hydrogen bond between its OH group and the active site acceptor) (Appleby & Bourret, 1998; Cho et al., 2000). The corresponding sites in RcsC are Asp875, Asp831, Asp832, Lys925 and Thr903. Mutations equivalent to *T903A* have been described for OmpR (*T83A*) and CheY (*T87A*). In both cases, the substitution results in constitutive activation, perhaps via a conformational change which mimics the conformation of the phosphorylated domain. It has been proposed that this substitution could represent a universal activating substitution for response regulators (Smith et al., 2004). This hypothesis may receive further support from the observation that the corresponding *T903A* mutation also has an activating effect in RcsC. Finally, the T87 residue in wild-type CheY and the T83 residue in OmpR have been proposed to mediate dephospho-
hydration of acyl phosphate (Brissette et al., 1991; Sanders et al., 1989), since all similar proteins contain a hydroxy amino acid residue at this position (Stock et al., 1990). This is in agreement with the idea that *T903A*, a recessive mutation, may impair a phosphatase activity of RcsC.

The second mutation in the receiver domain is located at Asn877, a non-conserved residue in the vicinity of the active site. In CheY, the carbonyl of the corresponding Asn59 directly chelates the active site Mg$^{2+}$ in both the inactive and the active conformations (Bellslell et al., 1994; Lee et al., 2001; Stock et al., 1993). According to the structure predicted by SWISS-MODEL (Guex & Peitsch, 1997; Schwede et al., 2003), a replacement of Asn877 by Ser increases its contact with the site of phosphorylation, Asp875, through hydrogen bonding. Different substitutions at the corres-
ponding positions in VirG (*N54D*) (Han et al., 1992; Jin et al., 1993; Pazour et al., 1992; Scheeren-Groot et al., 1994), CheB (E58K) (Stewart, 1993) and CheY (N59K, N49R) (Silversmith et al., 2001) also have an activating effect, thereby outlining the importance of this non-conserved residue in the activation of response regulator domains.
Two mutations in the histidine kinase domain affecting the same residue (P484L and P484S) are of particular interest. They have been isolated independently five and three times, respectively, and exhibit the highest levels of activation of the gmm: Lac fusion: over 500 times the wild-type level (Fig. 3 and Table 2). These mutants grow more slowly than the wild-type and the remaining constitutive rcsC mutants (Table 2), and give rise at relatively high frequencies to non-mucoid revertants with normal growth rates. These features can be related to previous findings about the lethality of igaA (also called tyrF and mucM) null mutations in Salmonella (Cano et al., 2002; Costa et al., 2003). Lethality is suppressed by mutations in rcsC, yojN or rcsB, suggesting that overactivation of the Rcs system may lead either to repression of an essential gene or to derepression of a gene whose overexpression is lethal. In this sense, the rcsCP484L and rcsCP484S mutants might approach the maximum level of activation of the Rcs system still compatible with viability.

In this work, we provide direct support for the hypothesis that overactivation of the Rcs system renders Salmonella avirulent. First, we show that constitutively activated rcsC mutants are attenuated in mice. Second, we describe a nearly perfect correlation between the degree of attenuation and the level of activation of the Rcs system caused by the different mutations (Fig. 4 and Table 2). Finally, we show that overproduction of colanic acid capsule is a relevant factor in the attenuation of these mutants. In all constitutively activated rcsC mutants tested, an insertion in gmm (one of the genes necessary for colanic acid synthesis) or rcsA led to a partial but highly significant relief of attenuation in mice: up to three orders of magnitude in CI (Fig. 4). These experiments suggest that, although production of colanic acid capsule is dispensable for virulence (see CI for strain SV4609 in Fig. 4), its overproduction is detrimental for Salmonella infection in mice. Since suppression by gmm mutation is not complete, another conclusion from these experiments is that capsule overproduction is not the only cause of attenuation of the constitutively activated rcsC mutants. We thus postulate the existence of additional Rcs-regulated genes involved in Salmonella virulence.

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