Instability of the *Salmonella* RcsCDB signalling system in the absence of the attenuator IgaA

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IgaA is a *Salmonella enterica* membrane protein that attenuates the response of the RcsCDB signalling system to envelope stress. This protein is essential unless the RcsCDB system is inactivated, suggesting that IgaA may constantly adjust the magnitude of the response. Such a functional link is also supported by the concurrence of the *igaA* and *rscD-rscB-rscC* loci in genomes of enteric bacteria and the selection of spontaneous mutations in the RcsCDB system following IgaA deprivation. However, the exact nature of the spontaneous mutations rendering IgaA dispensable remains undefined. In this work, we examined how the transduction of an *igaA* null allele affects the status of the RcsCDB system. Loss of RcsCDB response was registered in ~90 % of the IgaA-defective clones, which failed to produce the capsule material positively regulated by this system. About half of these non-mucoid clones suppressed the loss of IgaA with large deletions encompassing variable regions of the *rscD-rscB-rscC* locus. Unexpectedly, mucoid transductants were also reproducibly obtained and indicated the capacity of *S. enterica* to retain a functional RcsCDB system in the absence of IgaA. Decreased levels of either RcsC or RcsD were shown in 'mucoid' clones lacking IgaA and displaying low responsiveness to stimuli. Taken together, these data demonstrate that the stability and responsiveness of the RcsCDB system relies on its attenuator IgaA. The type of suppressions found also support a model with IgaA controlling the level of signal flowing through RcsC and RcsD.

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INTRODUCTION

Two-component systems (TCSs) are widely used by micro-organisms to continuously monitor changes in the niche they inhabit (Stock *et al.*, 2000; West & Stock, 2001). These systems transduce external signals into gene expression changes required to adapt to the new environmental cue (Mascher *et al.*, 2006). TCSs are implicated in regulation of a large variety of processes including chemotaxis, motility, biofilm formation, nutrient acquisition, adaptation to diverse stress conditions, and virulence (Beier & Gross, 2006; Calva & Oropeza, 2006; Francez-Charlot *et al.*, 2005). The simplest scheme of TCS consists of a sensor histidine kinase (HK) and a cytosolic response regulator (RR) (Mascher *et al.*, 2006; Stock *et al.*, 2000; West & Stock, 2001). These systems can also have a more complex design, including phosphotransfer between modules of the same protein and/or intermediate proteins between the HK and the RR (Mascher *et al.*, 2006).

The RcsCDB signalling pathway exemplifies one of the most complex TCSs known. It was initially identified in *Escherichia coli* based on its requirement for the production

of colanic acid capsule (Stout & Gottesman, 1990). The RcsCDB system is restricted to enterobacteria and controls the production of flagella and exopolysaccharides that promote biofilm formation and virulence in diverse plant and mammalian bacterial pathogens (Ferrieres & Clarke, 2003; Huang *et al.*, 2006; Majdalani & Gottesman, 2005, 2007; Page *et al.*, 2001; Vianney *et al.*, 2005). The RcsCDB system also modulates responses such as the adaptation to osmotic shock (Zhou *et al.*, 2003) and is activated upon exposure to antibiotics or antimicrobial peptides (Conter *et al.*, 2002; Erickson & Detweiler, 2006; Kaldalu *et al.*, 2004; Sailer *et al.*, 2003), or by defects in the synthesis of lipopolysaccharide, membrane phospholipids or membrane-derived oligosaccharides, as well as by loss of envelope integrity (Clavel *et al.*, 1996; Ebel *et al.*, 1997; Ize *et al.*, 2004; Parker *et al.*, 1992; Shiba *et al.*, 2004). Other activating conditions include the accumulation of TDP-glucose (El-Kazzaz *et al.*, 2004), or the overproduction of certain proteins such as DjlA, LolA or OmpG (Chen *et al.*, 2001; Kelley & Georgopoulos, 1997). Two membrane proteins, the hybrid sensor RcsC and the phosphotransmitter RcsD, together with the cytosolic response regulator RcsB, constitute the RcsCDB system (Huang *et al.*, 2006; Majdalani & Gottesman, 2005). Despite no stimulus specifically recognized by the system being known, all activation conditions reported to date have in

Abbreviation: TCS, two-component system.

A supplementary table of oligonucleotides and two supplementary figures are available with the online version of this paper.

common some type of envelope stress or contact with surfaces. RcsCDB system activation has been proposed to facilitate adaptation to envelope stress mediated by membrane remodelling. Mechanistically, the phosphorelay in the RcsCDB system follows the sequence RcsC→RcsD→RcsB (Chen *et al.*, 2001). Once phosphorylated, RcsB modulates expression of target genes. RcsA is a co-regulatory protein that together with RcsB controls expression of a subset of genes, such as those involved in the production of the colanic acid capsule (Majdalani & Gottesman, 2005). Auxiliary proteins also proposed to act with RcsB are TviA and RmpA, which regulate production of other capsules (Majdalani & Gottesman, 2005). RcsF is an outer-membrane protein that acts as an upstream sensor involved in activating RcsC (Castanie-Cornet *et al.*, 2006; Majdalani *et al.*, 2005). In contrast to the numerous reports describing activation of the system, there is no clear picture of how the response could be downregulated once the bacteria adapt to the new niche.

Intracellular growth attenuator-A (IgaA) was identified in *Salmonella enterica* serovar Typhimurium (hereinafter *S. Typhimurium*) as a membrane protein that represses the RcsCDB system (Cano *et al.*, 2002). Mutations causing instability of IgaA activate the RcsCDB system, leading to overproduction of colanic acid capsule (mucoidy phenotype) and a severe decrease in the synthesis of flagella (Cano *et al.*, 2002). Unlike the studies showing activation of the RcsCDB system concomitantly with envelope stress (Huang *et al.*, 2006; Majdalani & Gottesman, 2005), no such evidence has been found in *S. Typhimurium* producing unstable IgaA variants (Dominguez-Bernal *et al.*, 2004). The role of IgaA as repressor of the RcsCDB system influences the pathogenic potential of *S. Typhimurium* in acute infection models. Thus, over-activation of the RcsCDB system has been shown to attenuate virulence (Dominguez-Bernal *et al.*, 2004; Garcia-Calderon *et al.*, 2005; Mouslim *et al.*, 2004). IgaA is an essential protein in *S. Typhimurium* but dispensable in mutants lacking any of the three main components of the RcsCDB system (Cano *et al.*, 2002; Costa *et al.*, 2003). This genetic evidence establishes a functional link between IgaA and the RcsCDB system. Costa *et al.* (2003) reported that transduction of a null allele in *yrfF* (*mucM*), as the *igaA* locus was originally named, selected mutations mapping in *rcsC*, *rcsB* or *rcsD*. This observation suggested that only a full inactivation of the RcsCDB system suppresses the loss of IgaA. However, neither the exact type of mutations nor the underlying suppression mechanisms were defined.

In this work, we further explored the essentiality of IgaA by determining the extent to which the transduction of an *igaA* null allele affects the status of the RcsCDB system. Inactivation of the system was found in most of the clones analysed, with large deletions encompassing the *rcsD-rcsB-rcsC* region as a common type of mutation. In addition, characterization of mucoid clones obtained in these experiments revealed, for the first time, that a partially

functional RcsCDB system having lower amounts of either RcsC or RcsD is sufficient to make IgaA dispensable.

METHODS

Bacterial strains and growth conditions. The *S. Typhimurium* strains used in this work are listed in Table 1. All are isogenic derivatives of the mouse-virulent strain SL1344 (Hoiseh & Stocker, 1981). Bacteria were grown at 37 °C in Luria–Bertani (LB) broth, on LB agar plates or in minimal intracellular-salt (ISM) medium as indicated (Cano *et al.*, 2002; Wilson *et al.*, 1997). The ISM medium, of high osmolality (403 mosM), provides an optimal stimulus for induction of the RcsCDB system in strains with defects in IgaA (J. Mariscotti & F. Garcia-del Portillo, unpublished). Glycerol (38 mM) was used as carbon source in ISM medium. When appropriate, kanamycin (30 µg ml⁻¹) or chloramphenicol (10 µg ml⁻¹) was added to select for transductants in LB agar plates.

Phage transductions. The transductional crosses were made using the P22 HT 105/1 *int201* phage (Schmieger, 1972), as described by Maloy (1990). Phage-free transductants were screened on green plates as described by Chan *et al.* (1972) and further tested for sensitivity to the clear-plaque mutant P22 H5.

Chromosomal gene epitope tagging. The *rcsC65::3xFLAG* (Km^R) and *rcsD66::HA*(Km^R) 3'-tagged single-copy chromosomal versions have been described elsewhere (Dominguez-Bernal *et al.*, 2004). These tags were transferred to the desired strains by P22 phage-mediated transduction. The 3xFLAG-Km^R tag does not affect functionality of RcsC since an *igaA1 rcsC65::3xFLAG* strain is mucoid (Dominguez-Bernal *et al.*, 2004). Chromosome tagging at the 3'-end of the *rcsC'67* allele carried by the J4 strain (with a deletion in the 5' region of *rcsC*, see text) was done by phage transduction in the following steps: (i) replacement of the *igaA2::K1XX* allele of mutant J4 by *igaA3::Cm^R*; (ii) transfer of the 3xFLAG-Km^R tag using as donor the MD0116 (*rcsC65::3xFLAG-Km^R*) strain; and (iii) verification by PCR of the maintenance of the closely linked deletion at the 5'-end of *rcsC* (allele *rcsC'67*). The resulting strain, MD1304-J4 (*igaA3::Cm^RrcsC'67::3xFLAG-Km^R*), and all the intermediate strains retained mucoidy like the original J4 mutant (*igaA2::K1XX rcsC'67*). A similar strategy was performed for chromosomal tagging of the 3'-end of *rcsD* in the J10 strain. Unlike the case of RcsC, 3'-tagging of *rcsD* abrogates activity of the RcsCDB system as indicated by the loss of mucoidy of the MD0225 (*igaA1 rcsD66::HA*) strain (Table 1). This effect may be due to lack of function of the tagged RcsD protein or, alternatively, a polar effect in *rcsB* transcription. 3'-Tagging of *rcsD* in the J10 mutant was exclusively performed to evaluate the effect of the deletion at the 5' promoter region of *rcsD* on the protein levels (see text). The steps used for this chromosomal tagging included: (i) replacement of the *igaA2::K1XX* allele of the J10 mutant by *igaA3::Cm^R*; (ii) introduction of the HA-Km^R tag using as donor the MD0115 (*rcsD66::HA-Km^R*) strain; and (iii) verification by PCR of the closely linked Δ *ompC'-micF* deletion in the promoter region of *rcsD*. The resulting strain was MD1330-J10 [*igaA3::Cm^RΔ(ompC'-micF) rcsD66::HA-Km^R*]. Intermediate strains generated in these two genetic backgrounds (J4 or J10) and carrying the *igaA3::Cm^R* allele were used as recipient strains for the introduction of the *wcaH21::MudJ* (Km^R) transcriptional fusion (see Table 1).

PCRs. PCRs were performed in a PTC-100 Peltier thermal cycler (MJ Research) according to standard procedures. Oligonucleotide primers used in these reactions are listed in Supplementary Table S1, available with the online version of this paper. The purification of the PCR products for DNA sequencing was performed with the QIAquick PCR

Table 1. *S. enterica* serovar Typhimurium strains used in this study

Strain	Relevant genotype	Rcs phenotypes	Source or reference
SL1344	<i>hisG46</i> , <i>rpsL</i> , parental virulent isolate	—	Hoiseth & Stocker (1981)
SV4450	<i>igaA1</i>	Mucoid, low motility	Cano <i>et al.</i> (2002)
SV4255	<i>igaA</i> ⁺ <i>zhf-6311::Tn10dTet</i>	—	Cano <i>et al.</i> (2001)
MD0862	<i>igaA1 rcsB70::Tn10dCm</i>	—	This work
SV4406	<i>rcsB70::Tn10dCm</i>	—	Cano <i>et al.</i> (2002)
MD0835-J1	<i>igaA2::KIXX Δ(apbE'-rcsC')</i>	—	This work
MD0842-J4	<i>igaA2::KIXX rcsC' 67</i>	Mucoid, low motility	This work
MD0855-J10	<i>igaA2::KIXX Δ(ompC'-micF)</i>	Mucoid, low motility	This work
SV4407	<i>wcaH21::MudJ</i>	—	Cano <i>et al.</i> (2002)
MD0099	<i>igaA1 wcaH21::MudJ</i>	Low motility	Cano <i>et al.</i> (2002)
MD0893-J1	<i>igaA3::Cm^R Δ(apbE'-rcsC') wcaH21::MudJ</i>	—	This work
MD0895-J4	<i>igaA3::Cm^R ΔrcsC' wcaH21::MudJ</i>	Low motility	This work
MD1326-J10	<i>igaA3::Cm^R Δ(ompC'-micF) wcaH21::MudJ</i>	Low motility	This work
MD0116	<i>rcsC65::3xFLAG-Km^R</i>	—	This work
MD0233	<i>rcsC65::3xFLAG</i>	—	Dominguez-Bernal <i>et al.</i> (2004)
MD0234	<i>igaA1 rcsC65::3xFLAG</i>	Mucoid, low motility	Dominguez-Bernal <i>et al.</i> (2004)
MD1304-J4	<i>igaA3::Cm^R rcsC' 67::3xFLAG-Km^R</i>	Mucoid, low motility	This work
MD0115	<i>rcsD66::HA-Km^R</i>	—	This work
MD0224	<i>rcsD66::HA</i>	—	Dominguez-Bernal <i>et al.</i> (2004)
MD0225	<i>igaA1 rcsD66::HA</i>	—	Dominguez-Bernal <i>et al.</i> (2004)
MD1330-J10	<i>igaA3::Cm^R Δ(ompC'-micF) rcsD66::HA-Km^R</i>	—	This work

purification kit (Qiagen, catalogue no. 28106) following the instructions of the manufacturer.

β-Galactosidase assays. The levels of β-galactosidase derived from the *wcaH21::lacZ* transcriptional fusion were assayed as described by Miller (1972), using the CHCl₃/SDS permeabilization procedure. Bacteria were grown overnight at 37 °C in ISM-glycerol medium with shaking (180 r.p.m.), reaching a final OD₆₀₀ of ~1.2–1.4. The bacteria were then diluted 1:100 in fresh medium and incubated for a further 6 h, at which time β-galactosidase activity was assessed. Triplicate samples were used for each experiment, which was repeated at least twice.

Protein extracts and Western blot analysis. Total protein extracts were prepared from bacteria grown overnight at 37 °C in ISM-glycerol medium to stationary phase (final OD₆₀₀ ~1.2–1.4). Bacteria contained in 1 ml of culture were collected by centrifugation (20 000 g, 5 min, 4 °C), washed in phosphate-buffered saline (PBS), pH 7.4, and suspended in the appropriate volume of Laemmli sample buffer (1.3 % SDS, 10 %, v/v, glycerol, 50 mM Tris/HCl, 1.8 % β-mercaptoethanol, 0.02 % bromophenol blue, pH 6.8). To estimate relative levels of either RcsC-3xFLAG or RcsD-HA, membrane extracts were prepared as described by Pucciarelli *et al.* (2002). Briefly, 1 ml of culture containing bacteria grown to stationary phase in ISM-glycerol was centrifuged (20 000 g, 5 min, 4 °C), suspended in 0.5 ml cold PBS pH 7.4, and broken by sonication (three pulses of 20 s with resting intervals of 30 s). After a low-speed centrifugation to remove unbroken cells (1500 g, 5 min, 4 °C), the supernatant was centrifuged at high speed to collect envelope material (100 000 g, 60 min, 4 °C). The pellet containing membranes and peptidoglycan was resuspended in PBS pH 7.4 and the appropriate volume of Laemmli buffer. Samples were adjusted to equal number of bacteria for loading in the gels (~5 × 10⁷ bacteria per well). Proteins were resolved by Tris-Tricine-PAGE (Schagger & von Jagow, 1987), using 10 % gels. Conditions of protein transfer and optimal dilutions of primary (anti-IgaA, anti-Flag, anti-HA, anti-flagellin, anti-FtsA, anti-FtsZ) and secondary antibodies have been described elsewhere (Cano *et al.*, 2002; Dominguez-Bernal *et al.*, 2004). Proteins recognized by the antibodies were visualized by

chemoluminescence using the luciferin-luminol reagents. If more sensitivity was required, the ECL Western blotting detection system was used (Amersham Biosciences, catalogue no. RPN2209).

Tissue culture infection assays. The fibroblast cell line NRK-49F (ATCC CRL1570) was grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 5 % fetal bovine serum. The bacterial infection conditions have been described elsewhere (Cano *et al.*, 2001). The intracellular proliferation rate was calculated as the ratio of viable intracellular bacteria at 24 h versus 2 h post-infection (Cano *et al.*, 2001).

RESULTS

Characterization of *S. Typhimurium* wild-type-derivative clones lacking *IgaA*

igaA is an essential gene in *S. Typhimurium* having a functional RcsCDB system (Cano *et al.*, 2002; Costa *et al.*, 2003). In genetic terms, this essentiality is however not absolute since the requirement of *IgaA* can be spontaneously suppressed, albeit at low frequency. Thus, a few abortive colonies are obtained upon transduction of an *igaA* null allele into wild-type bacteria (Costa *et al.*, 2003). The mutations responsible map in *rcsC*, *rcsD* or *rcsB*, but their exact nature or effect on the function of the RcsCDB system has not been investigated. With that aim, we generated a new collection of *IgaA*-deficient clones upon transduction of the *igaA2::KIXX* allele into the *S. Typhimurium* virulent strain SL1344. This defined null allele was generated by interruption of the open reading frame with a kanamycin-resistance (*KIXX*) cassette (Cano *et al.*, 2002). No truncated part of *IgaA* is produced as revealed by immunoassays with a specific anti-*IgaA* polyclonal antiserum (Cano *et al.*, 2002).

During the construction of this strain collection, we repeatedly observed that a few *igaA2::KIXX* transductants displayed mucoidy on plates, which indicated activity of the RcsCDB system. This suppression mechanism occurred independently of the genetic background since a similar percentage of *igaA2::KIXX* mucoid clones were obtained in other wild-type strains such as LT2 and 14028s (data not shown). The *igaA2::KIXX* derivatives of the wild-type strain were visible only after 48 h of incubation at 37 °C, which contrasted with the 24 h of incubation at which *igaA2::KIXX**rscB* transductants were obtained (Fig. 1a). Strikingly, none of the *igaA2::KIXX* clones obtained in these experiments (in either wild-type or *rscB* backgrounds) displayed growth defects in the subsequent phenotypic tests (see below). As was previously known for the *igaA1* mutant, the IgaA-defective clones did not show signs of defects in envelope stability as assessed by sensitivity to ionic detergents (Supplementary Fig. S1).

The wild-type derivative *igaA2::KIXX* clones displayed two distinct colony phenotypes: non-mucoid (indistinguishable from wild-type) and mucoid. These two classes represented ~90 % and ~10 % of the total number of transductants, respectively. Given our interest in characterizing the mucoid clones, we monitored IgaA levels in a first series of randomly selected transductants consisting of three non-mucoid (J1, J3 and J11) and ten mucoid (J2, J4, J5, J6, J7, J8, J9, J10, J12 and J13) clones. As expected, immunoblot analysis showed that the non-mucoid clones J1, J3 and J11 were deficient in IgaA (Fig. 1b). However, some of the mucoid transductants produced IgaA (Fig. 1b). PCR analysis with *igaA*-specific oligonucleotides showed that these IgaA-producing clones (J2, J5, J6, J8 and J13) were *igaA*⁺/*igaA2::KIXX* merodiploids (data not shown), and these were discarded for further analysis. From the remaining 'mucoid' clones lacking IgaA (J4, J7, J9, J10 and J12), two subgroups were differentiated based on the

Fig. 1. Isolation of IgaA-deficient clones of *S. Typhimurium* that retain an operative RcsCDB system. (a) P22 phage-mediated transduction of the *igaA2::KIXX* (Km^R) allele to strains SL1344 (wild-type) and SV4406 (*rscB70::Tn10dCm*). Note that transductants are clearly visible in the wild-type background only at 48 h post-transduction (arrows). (b) Immunodetection of IgaA in total protein extracts of 13 Km^R *igaA2::KIXX* transductants obtained in a wild-type genetic background (series J1 to J13). Except for the clones indicated with an asterisk (J1, J3 and J11), these transductants were mucoid on LB plates. (c) Production of flagellin (FliC, FliB) and cell division proteins (FtsA, FtsZ) by representative clones lacking IgaA. Unlike the non-mucoid strain J1, strains J4 and J10 display mucoidy on LB plates. Strains SV4450 (*igaA1*) and MD0862 (*igaA1 rcsB70::Tn10dCm*) were used as controls (see text). Protein extracts were prepared from bacteria grown to exponential phase ($OD_{600} \sim 0.2$) in minimal ISM-glycerol medium. (d) Morphology of strains with a distinct level of activation of the RcsCDB system. Micrographs were taken from bacteria grown to exponential phase ($OD_{600} \sim 0.2$) in minimal ISM-glycerol medium. Note the coccobacillary shape of bacteria having a highly active RcsCDB system: *igaA1* and J10 [*igaA2::KIXX* $\Delta(ompC'-micF)$]. Scale bars, 2 μm .

mucoidy displayed on LB plates. The first subgroup included J4 and J7, less mucoid than an *igaA1* strain expressing an unstable IgaA protein with an R188H point mutation (Cano *et al.*, 2001). The second subgroup included J9, J10 and J12, which displayed a more pronounced mucoidy. J4 and J10 were selected as representative clones of each of these two phenotypic groups (see below). Besides the production of IgaA, we also monitored the levels of flagellin (FlhC/FlhB), regulated negatively by the RcsCDB system (Arricau *et al.*, 1998), and the cell division proteins FtsA and FtsZ, subjected to positive regulation by this system (Carballes *et al.*, 1999). As controls, strains *igaA1* (RcsCDB system overactivated), *igaA1 rcsB* (no RcsCDB activity), and J1 (non-mucoid strain, expected to have no RcsCDB activity) were used. In these assays, bacteria were grown in the high-osmolarity ISM-glycerol medium. Immunoblot analyses confirmed the inactivation of the RcsCDB system in the J1 strain, which produced large amounts of flagellin and slightly lower levels of FtsA and FtsZ (Fig. 1c). Conversely, the J4 and J10 mucoid strains produced low amounts of flagellin and increased levels of FtsA and FtsZ (Fig. 1c). This assay also revealed that the IgaA1 mutant protein, which has an R188H mutation and is unable to repress the RcsCDB system (Cano *et al.*, 2002), runs anomalously in gels when bacteria are grown in ISM-glycerol medium (Fig. 1c). The nature of these changes is at present unknown. Globally, the monitoring of diverse proteins controlled by the RcsCDB system proved that the system retained activity in the mucoid strains J4 and J10 and that such activity affected other genes besides those involved in capsule synthesis. The J4 and J10 strains also displayed an altered morphology, ranging from rod-shaped bacilli of shorter length (clone J4) to bacteria having a coccobacillary shape (J10) (Fig. 1d). These changes may reflect the alterations observed in the levels of cell-division proteins (Fig. 1c). Changes in cell shape were also evident in the *igaA1* mutant (Fig. 1d). Taken together, these data demonstrated that mutations that abrogate the activity of the RcsCDB system constitute the most common mechanism of suppression of IgaA essentiality (90 % of the cases). On the other hand, the analysis in the J4 and J10 strains revealed for the first time the capacity of *S. Typhimurium* to maintain an RcsCDB response in the absence of IgaA.

The non-mucoid IgaA-deficient strain J1 has a large deletion affecting the entire *rcsD-rcsB-rcsC* locus

We first sought to identify suppressor mutations causing the inactivation of the RcsCDB system in the non-mucoid IgaA-deficient clones. Given the key role of RcsB as response regulator, a search was made for mutations in the *rcsB* locus of two randomly selected non-mucoid strains, J1 and J3 (see Fig. 1b). Oligonucleotides designed to amplify *rcsB* by PCR were used (Fig. 2a). Unexpectedly, whereas an *rcsB* product of identical size was amplified in both wild-type and J3 strains, no such product was obtained in

J1 (Fig. 2b). This result suggested that some of the non-mucoid strains could suppress the essentiality of IgaA with deletions affecting the integrity of the *rcsD-rcsB-rcsC* locus. To determine the length of the deletion present in the J1 strain, PCRs were performed with oligonucleotides designed in distinct regions of the *rcsD-rcsB-rcsC* locus (Fig. 2a). No PCR product was obtained with oligonucleotides hybridizing to the coding regions of *rcsD*, *rcsB* or *rcsC* (Fig. 2c). However, primers designed outwards from the *rcsD-rcsB-rcsC* locus (*ada* gene) and the 5' region of *rcsC* resulted in the amplification of a PCR product in the non-mucoid J1 mutant (Fig. 2c). Sequencing of this PCR product revealed that the deletion encompassed a total of 8804 nt, resulting in loss of the entire *ompC-micF-rcsD-rcsB* region, part of the *apbE* gene and most of *rcsC* (Fig. 2d). Based on this finding, the presence of deletions was monitored in another series of IgaA-deficient mutants obtained from independent transduction experiments. From a total of 68 clones tested individually by PCR, ~50 % of them carried deletions in the region of *rcsB*, inferred by either no amplification or amplification of a PCR product of smaller size than that obtained in the wild-type strain (Fig. 2e, and data not shown). The use of a different *igaA3::Cm^R* null allele in the transduction experiments resulted in a similar proportion of deletions (Fig. 2e). Taken together, these data indicated that a common phenomenon occurring in response to the absence of IgaA is the generation of mutations that inactivate the RcsCDB system. A high proportion of these mutations consisted of large deletions affecting the integrity of the *rcsD-rcsB-rcsC* locus.

The IgaA-deficient mucoid strain J4 contains a deletion diminishing the levels of the sensor protein RcsC

The most accepted model indicates that the RcsCDB system must be made non-functional to render dispensable the attenuator IgaA. The isolation of IgaA-deficient clones such as J4 and J10, which produce capsule and repress production of flagella (Fig. 1b), proved however that alternative suppressor mechanisms do exist. To characterize the mutations responsible, we first analysed the integrity of the *rcsD-rcsB-rcsC* locus. In the J4 mutant, a PCR carried out with *rcsC*-specific oligonucleotides uncovered a deletion in this region (Fig. 3a). Consistent with the phenotype of mucoidy displayed by this mutant, no additional deletions were detected in the *rcsD-rcsB* region (data not shown). Sequencing of the *rcsC* region amplified in the J4 strain revealed the loss of 113 nt, encompassing part of the upstream promoter region and the coding sequence of *rcsC* (Fig. 3b). The BPROM program, which predicts σ^{70} promoter sites (<http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb>), was used to analyse *in silico* the *rcsC* sequence present in the J4 mutant. The 113 nt deletion caused the loss of the native -35, -10 and RBS sites of *rcsC* (Fig. 3b). Further analysis revealed potential -35, -10 and RBS sites

Fig. 2. Large deletions in the *rcsD-rcsB-rcsC* locus are a frequent type of mutation suppressing IgaA essentiality. (a) Positions of the oligonucleotide primers used in PCRs designed to assess the integrity of the *rcsD-rcsB-rcsC* locus. (b) PCR carried out with the pair of oligonucleotides *yojN5D-rcsC3D*, which covers the region encompassing the *rcsB* locus. The oligonucleotides *fimY1-fimY2* were used as control. J1 and J3 are non-mucoid clones deficient in IgaA. (c) PCRs carried out in wild-type, J1 and J3 strains with oligonucleotides designed to diverse sites of the *rcsD-rcsB-rcsC* locus and flanking regions. (d) Details of the 8804 nt deletion identified in the J1 strain. (e) PCR products obtained with *rcsB*-specific oligonucleotides in an independent series of *igaA3::Cm^R* transductants.

and a distinct start codon for *rcsC* originated as a result of the deletion (Fig. 3b). The deletion of the J4 mutant could lead to the production of a 'variant' RcsC protein with an altered amino acid sequence at the N-terminus, but complete identity with wild-type RcsC beyond amino acid residue 18 (Fig. 3c). Of interest, the first transmembrane domain (TM1) of RcsC is predicted to span from residues 20 to 41, a region followed by the periplasmic sensory domain (Majdalani & Gottesman, 2005). Therefore, the J4 strain could produce a mutated RcsC having an unaltered sensory capacity compared to wild-type RcsC (Fig. 3c). We also examined whether the deletion in the 5' regulatory region of *rcsC* could ultimately result in altered protein

levels of RcsC. To that end, we performed 3'-FLAG chromosomal tagging of *rcsC* (see Methods). Immunoblot analyses showed that the J4 mutant contained much less RcsC protein than wild-type or *igaA1* strains (Fig. 3d). These data revealed that the J4 strain suppressed the lack of IgaA by a drastic reduction in the number of RcsC molecules, an alteration that otherwise did not abrogate responsiveness in the RcsCDB system. Two further analyses were subsequently made to prove that this mutation was the only basis of the suppression of IgaA essentiality in the J4 strain. First, we brought back an *igaA⁺* allele into the original J4 (*igaA2::KlXX rcsC'* 67) strain by using as donor the SV4255 (*igaA⁺ zhf-6311::Tn10dTet*) strain (Cano *et*

Fig. 3. Decrease in the relative levels of the sensor RcsC makes the essential protein IgaA dispensable. (a) Identification by PCR of a deletion in the *rcsC* gene of the J4 mucoid strain. (b) Details of the 113 nt deletion affecting the native regulatory region of *rcsC*. Probable regulatory sites driving transcription of *rcsC* in the J4 strain are indicated. (c) N-terminal sequence of the RcsC mutated protein deduced from the sequence obtained in the J4 strain. Note that the deletion does not affect transmembrane domain 1 (TM1), extending from residues 20 to 41 of RcsC and preceding the sensor periplasmic domain (Majdalani & Gottesman, 2005). (d) Levels of RcsC in wild-type (MD0233), *igaA1* (MD0234) and J4 (MD1304-J4) isogenic strains carrying in the chromosome a 3xFLAG tag at the 3'-end of *rcsC*. The band marked as loading control corresponds to a protein non-specifically recognized by the anti-FLAG antibody. Molecular mass markers (in kDa) are indicated on the right.

al., 2001). As expected, the Tet^R/Km^S (*igaA*⁺) colonies were all non-mucoid. The presence of the *rcsC'* 67 allele in these clones was confirmed by PCR. Two of these Tet^R/Km^S colonies were further used as recipients of the *igaA2::KXX* null allele. The resulting Km^R transductants had gained the mucoidy phenotype. Importantly, the frequency of *igaA2::KXX* transductants obtained in the *igaA*⁺ *zhf-6311::Tn10dTet rcsC'* 67 background was similar to that obtained when using an *rcsB* mutant as recipient (data not shown). Second, we transduced the *rcsC'* 67 allele harbouring the 113 nt deletion to a wild-type genetic background. That was possible by first transducing the *rcsC'* 67::3xFLAG-Km^R tag. As mentioned in Methods, tagging of the RcsC protein at its C-terminus does not affect function. The new strain generated, with genotype *igaA*⁺ *rcsC'* 67::3xFLAG-Km^R (the deletion was confirmed by PCR), showed a high frequency of transduction of the *igaA3::Cm*^R allele similar to that of an *rcsB* strain (data not shown). These controls discarded the presence of additional mutations required for the suppression observed

in the J4 mucoid mutant. They also allowed us to conclude that the 113 nt deletion in the 5'-region of *rcsC* is responsible for the decrease in the levels of RcsC and sufficient to suppress *IgaA* essentiality.

The *IgaA*-deficient mucoid clone J10 has a deletion affecting the levels of the phosphotransmitter protein RcsD

To identify the suppressor mutation present in the mucoid strain J10, PCR assays covering the *rcsD-rcsB-rcsC* region were performed (Fig. 4a). A deletion of 1551 nt extending from the upstream regulatory region of *rcsD* to a site located within the coding region of *ompC* was identified (Fig. 4b). Sequencing of the PCR product obtained in this mutant and containing the flanking regions of the deletion revealed that the coding region of *rcsD* remained intact. Nonetheless, the putative -35 site in the promoter region could be removed as a result of the deletion (Fig. 4b). To determine whether this deletion affected the relative levels

Fig. 4. The IgaA-deficient mucoid strain J10 has diminished levels of RcsD. (a) PCR indicating the deletion in the region of *rcsD* in the J10 mutant. (b) Sequencing of the region flanking the 1551 nt deletion reveals the loss of the native -35 site of *rcsD* as predicted by the BPROM program. (c) Relative levels of RcsD in wild-type (MD0224), *igaA1* (MD0225) and J10 (MD1330-J10) isogenic strains carrying in the chromosome an HA tag at the 3'-end of *rcsD*. Loading controls correspond to two proteins non-specifically recognized by the anti-HA antibody. Molecular mass markers (in kDa) are indicated on the right.

of the RcsD protein, we tagged the 3'-end of *rcsD* with HA-Km^R (see Methods). Immunoblot analyses showed that the levels of RcsD were significantly diminished in the J10 mutant compared to the wild-type or *igaA1* strains (Fig. 4c). Since the *rcsD66::HA-Km^R* allele affects the functionality of the RcsCDB system, we could not employ a similar strategy to that used in the mucoid J14 strain to assess whether secondary suppressor mutations were present in strain J10. Nonetheless, we brought back the *igaA*⁺ allele into the J10 strain with the aforementioned closely linked *zhf-6311::Tn10dTet* marker. Two randomly selected *igaA*⁺ transductants (both Tet^R and non-mucoid) were used to verify by PCR the maintenance of the $\Delta(ompC'-micF)$ deletion present in the original J10 strain. These two clones were used as recipients of an *igaA2::KIXX* allele. The frequency of transductants, all gaining the mucoidy phenotype, was comparable to that

obtained when using an *rcsB* strain as recipient. These observations suggested that the deletion $\Delta(ompC'-micF)$ affecting the levels of the RcsD protein could be responsible for the suppression of IgaA essentiality observed in the J10 strain.

Decreased levels of RcsC or RcsD affect the response to stimuli

The stoichiometry of the three main components of the RcsCDB system is unknown. It could be therefore postulated that, in the absence of IgaA, a reduced level of either RcsC or RcsD (J4 and J10 strains, respectively) may be sufficient to ensure proper responsiveness of the RcsCDB system. To get insights into the functional status of the RcsCDB system, we first transduced the *igaA1* allele (R188H point mutation) into the IgaA-deficient strains J4

and J10 (Fig. 1c). Unlike the *igaA1* mutant, neither the *igaA1 rcsC'67* strain (J4 derivative) nor the *igaA1 Δ(ompC'-micF)* strain (J10 derivative) was mucoid on plates (data not shown). This result suggested that the status of an RcsCDB system operating with low levels of RcsC or RcsD may substantially differ from that of wild-type bacteria. To verify this assumption, the expression of a *lacZ* transcriptional fusion to a gene required for colanic acid capsule synthesis, *wcaH* (formerly *gmm*), was monitored in wild-type, *igaA1*, J1, J4 and J10 strains. The β -galactosidase activity measured was significantly lower (≤ 8 -fold) in strains J4 and J10 than in the *igaA1* strain (Fig. 5a). Likewise, a reproducible two- to threefold lower expression of the *wcaH::lacZ* fusion was noticed when comparing J4 to J10 (Fig. 5a), which differ in having reduced levels of either RcsC or RcsD, respectively. These data indicated that the RcsCDB system is activated to a different extent upon exposure of three 'mucoid' mutants (*igaA1*, J4 and J10) to the same stimulus. Remarkably,

the gradation observed in *wcaH::lacZ* expression (*igaA1*>J10>J4) matched the behaviour of these strains inside fibroblasts (Fig. 5b). In this eukaryotic cell type, we have previously shown that the activation of the RcsCDB system correlates with an increased intracellular growth rate (Cano *et al.*, 2001). Taken together, these data unequivocally demonstrated that a reduction in RcsC or RcsD translates into a 'debilitated' RcsCDB response. This alteration may explain why the deletions present in J4 and J10 were positively selected as mutations suppressing the requirement of IgaA.

DISCUSSION

In this work, we extended previous genetic studies inferring a functional connection between the essential protein IgaA and the RcsCDB system of *S. enterica* serovar Typhimurium. Such a functional link was first revealed by the observation that mutations inactivating the RcsCDB system render IgaA dispensable (Cano *et al.*, 2002; Costa *et al.*, 2003). Transduction of an *igaA::Km* null allele into wild-type bacteria was later shown to select for abortive colonies harbouring mutations mapping in *rcsD*, *rcsB* and *rcsC* (Costa *et al.*, 2003). However, neither the underlying suppression mechanism nor the exact nature of the mutations was determined. In this study, we revisited these observations with the aim of demonstrating whether IgaA is a repressor absolutely required to maintain the RcsCDB system operative. Although we found conditions in which the suppression of IgaA essentiality was possible without complete abrogation of the RcsCDB system (mucoid strains J4 and J10), defects in the integrity and responsiveness of the RcsCDB system were observed in all the cases analysed. IgaA therefore behaves as a protein with a dedicated negative regulatory role in the RcsCDB system. A similar action as a dedicated repressor of a TCS was previously shown for CpxP (DiGiuseppe & Silhavy, 2003). This periplasmic protein downregulates the activity of the CpxA-CpxR TCS, implicated in diverse responses to envelope stress related to misfolding of periplasmic proteins. Unlike CpxP with its cognate TCS, IgaA is however not regulated itself by the RcsCDB system (Dominguez-Bernal *et al.*, 2004).

A frequent mechanism of suppression found in this study was the loss of integrity of the *rcsD-rcsB-rcsC* locus due to deletions of variable length. Among the ~90 % of IgaA-deficient transductants displaying a wild-type (non-mucoid) colony phenotype, about half harboured deletions of considerable extent (visualized by PCR). It cannot be discounted that small deletions, undetectable by the standard PCR technique, may also be present in the non-mucoid clones representing the remaining 50 % of the transductants. At present, it is unclear why large deletions constitute the most common mechanism suppressing IgaA essentiality. Large deletions of more than 10 nt have been estimated to occur spontaneously at frequencies similar to single-base frame shifts but at significantly lower rates

Fig. 5. The RcsCDB system is not equally activated in the J4 and J10 mucoid mutants lacking IgaA. (a) Expression of *wcaH* (formerly *gmm*), regulated positively by the RcsCDB system, in the mucoid strains J4 and J10 compared to wild-type and *igaA1* strains. The relative expression level was assessed with the *wcaH21::lacZ* transcriptional fusion. Values of β -galactosidase activity correspond to the mean and standard deviation from a total of three independent experiments. Mean values are shown at the top of each bar. (b) Intracellular proliferation rate (ratio of viable intracellular bacteria at 24 h versus 2 h) of the mutants inside the fibroblast cell line NRK-49F. The values correspond to the mean and standard deviation of a total of three independent experiments.

(~10-fold) than base substitutions (Kanie *et al.*, 2007). Loss of RecA has been shown to increase selectively spontaneous mutations comprising large deletions and frame-shifts versus base substitutions (Kanie *et al.*, 2007). The appearance of deletions has also been related to the presence of direct or inverted repeats (Bzymek & Lovett, 2001; Collins *et al.*, 1982). We analysed the sequence obtained at the end-points of deletions present in three non-mucoid clones (J1, J17, 18) and two mucoid clones (J4 and J10). No clear evidence for direct or inverted repetitions was found (Supplementary Fig. S2). Based on these observations, we favour the hypothesis of a 'transient' genome instability provoked by the stress accompanying the loss of IgaA and the concomitant overactivation of the RcsCDB system. This hypothesis is currently being tested by analysing the capacity of suppression of IgaA essentiality in mutants with defects in repair-recombination proteins.

The regulation of the RcsCDB system has been proposed to rely on the rate of the RcsC→RcsD→RcsB phosphorelay or on alternative pathways ending in RcsB phosphorylation (Huang *et al.*, 2006; Majdalani & Gottesman, 2005). The data collected for the mucoid strains J4 and J10 show for the first time that the RcsCDB response also relies on the relative levels of RcsC and RcsD. This scenario contrasts with that reported in more simple TCSs, such as EnvZ-OmpR of *E. coli*. In this system, different expression levels of either the sensor EnvZ or the regulatory protein OmpR do not alter the relative expression of genes under control of these regulators (Batchelor & Goulian, 2003). The data collected in our study suggest that, under steady-state conditions, the RcsCDB system of *S. Typhimurium* might have an excess of sensor and phosphotransmitter proteins, which would ensure a rapid response upon encountering stimuli. This configuration would be essentially regulated at the level of phosphate transfer, a concept in agreement with the constant protein level of RcsC, RcsD and RcsB observed in wild-type and *igaA1* strains (Dominguez-Bernal *et al.*, 2004). Such a model would also explain why 'dedicated' repressors might be required to fine-tune the output of the response in both basal and inducing conditions. Determination of the relative levels of these proteins in diverse growth conditions will certainly provide valuable information to assess the validity of this model.

Another interesting aspect found in our study was the apparent bias in the type of deletions identified. Our PCR assays were designed in a way in which we first determined the integrity of the *rcsB* locus. From this location, in the case of no amplification of the expected PCR product, we used new primers hybridizing to upstream and downstream locations. Surprisingly, a procedure that started from *rcsB* revealed that most of the deletions extended far beyond this locus. The rationale for the necessity of deleting larger regions than the *rcsB* gene for suppressing IgaA essentiality is at present unclear. In fact, the PCR assays showed that of a total of 50 deletions identified in

non-mucoid suppressor clones carrying *igaA2::KIXX* or *igaA3::Cm^R* null alleles, only five appeared to affect exclusively the *rcsB* locus (see mutant J18 as representative example in Fig. 2e). The remaining 45 deletions therefore affected in addition the open reading frames of either *rcsC* or *rcsD* (see three examples in Fig. 2e). These calculations of the frequency of deletions should however be taken with caution as they are probably underestimates. Thus, not all the 'non-mucoid' clones giving a wild-type PCR product with primers covering *rcsB* (yojN5D/*rcsC3D*) were examined for additional deletions in either *rcsC* or *rcsD*. Such a type of analysis, when performed in the J4 and J10 mucoid mutants, identified deletions located rather far from *rcsB*. The high frequency of deletions covering more than the *rcsB* gene is intriguing considering that the loss of the response regulator should make the RcsC→RcsD cycle futile and IgaA dispensable. Interestingly, the types of suppressors described in our study agree with those reported by Costa *et al.* (2003), who estimated that the suppressor mutations mapped mostly in *rcsC* and *rcsD* (46 and 33 %, respectively, of the suppressors analysed) with few of them in *rcsB* (5 % of suppressors). The procedure used in that study was based on complementing plasmids expressing RcsC, RcsD or RcsB. Taking our findings into account, the remaining 10 % of suppressors non-mapped by Costa *et al.* (2003) could correspond to deletions covering more than one gene in the *rcsD-rcsB-rcsD* locus. In addition, we previously characterized six *Tn10dTc* insertions suppressing IgaA essentiality, with four of them mapping in *rcsD* and two in *rcsC* (Cano *et al.*, 2002). These differences are more pronounced than would be expected from the relative size of the respective genes, *rcsC* and *rcsD* being fourfold larger than *rcsB*. This consideration discards the possibility that the uneven proportion of deletions in these genes is only due to this 'size' factor. Nonetheless, since defined *rcsB* null mutants suppress IgaA essentiality, it remains to be defined why a lower proportion of spontaneous mutations map in *rcsB* relative to *rcsC* and *rcsD*. Future work is required to discern whether these differences reflect the necessity of losing either RcsC or RcsD to render IgaA dispensable. Given that a phosphorelay is proposed to occur between RcsC and RcsD upon receipt of the external signal (Chen *et al.*, 2001), a tempting speculation is to assume that IgaA could somehow modulate such RcsC→RcsD phosphotransfer. This working hypothesis is an excellent platform to unravel the as yet elusive mechanism of action of this important regulator of the RcsCDB response.

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