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Regulation of Capsule Synthesis and Cell Motility in *Salmonella enterica* by the Essential Gene *igaA*

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ABSTRACT

Mutants of *Salmonella enterica* carrying the *igaA1* allele, selected as able to overgrow within fibroblast cells in culture, are mucoid and show reduced motility. Mucoidy is caused by derepression of *wca* genes (necessary for capsule synthesis); these genes are regulated by the RcsC/YojN/RcsB phosphorelay system and by the RcsA coregulator. The induction of *wca* expression in an *igaA1* mutant is suppressed by mutations in *rcsA* and *rcsC*. Reduced motility is caused by lowered expression of the flagellar master operon, *flhDC*, and is suppressed by mutations in *rcsB* or *rcsC*, suggesting that mutations in the *igaA* gene reduce motility by activating the RcsB/C system. A null *igaA* allele can be maintained only in an *igaA*$^+$/*igaA* merodiploid, indicating that *igaA* is an essential gene. Lethality is suppressed by mutations in *rcsB*, *rcsC*, and *yojN*, but not in *rcsA*, suggesting that the viability defect of an *igaA* null mutant is mediated by the RcsB/RcsC system, independently of RcsA (and therefore of the *wca* genes). Because all the defects associated with *igaA* mutations are suppressed by mutations that block the RcsB/RcsC system, we propose a functional interaction between the *igaA* gene product and either the Rcs regulatory network or one of its regulated products.

A screen for mutants able to overgrow within a fibroblast cell line identified functions of *Salmonella enterica* involved in growth restraint within nonphagocytic eukaryotic cells (Cano *et al.* 2001). One of such mutants carried a point mutation in a hitherto unknown locus, located near *mcrA* at centisome 75. This novel locus was called *igaA* (*i*ntracellular *g*rowth *a*ttenuator; EMBL accession no. AJ301649). The same study showed that IgaA$^-$ mutants of *S. enterica* are mucoid and avirulent in the murine typhoid model (Cano *et al.* 2001).

Analysis *in silico* of the *igaA* DNA sequence indicated the existence of an open reading frame (ORF) homologous to the *yrfF* ORF of *Escherichia coli*. The *yrfF* and *igaA* ORFs are located at an equivalent position on the chromosomal gene maps of *E. coli* and and *S. enterica* serovar Typhimurium (Blattner *et al.* 1997; McClelland *et al.* 2001). A BLAST databank search (Altschul *et al.* 1997) indicated that the *igaA* and *yrfF* loci are in turn homologous to the *umoB* gene of *Proteus mirabilis* (Dufour *et al.* 1998). The *umoB* gene was identified as a multicopy suppressor of the swarming defect associated with *flgN* mutations and proved to be required for both swarming and motility (Dufour *et al.* 1998). Current evidence suggests that *umoB* encodes a membrane protein that regulates genes involved in flagellar synthesis (Dufour *et al.* 1998).

Below we show that the *igaA* gene of *S. enterica* is essential under laboratory conditions. We also provide evidence that the *igaA* gene product of *S. enterica* is a pleiotropic regulator that exerts positive control on the flagellar master operon *flhDC* and negative control on the colanic acid cluster *wca*. Genetic analysis also unveils a functional relationship between the *igaA* gene product and the two-component regulatory system RcsB-RcsC. Originally described as a regulator of capsule synthesis in *E. coli* (Gottesman *et al.* 1985), the RcsB-RcsC system has been shown to participate in a variety of cellular processes, which include cell division control in *E. coli* (Carballès *et al.* 1999), regulation of Vi antigen synthesis in *Salmonella typhi* (Virlogeux *et al.* 1996), synthesis of flagellin and invasion proteins in *S. typhi* (Arricau *et al.* 1998), expression of the *E. coli tolQRA* operon (Clavel *et al.* 1996), synthesis of the *E. coli* outer membrane protein OsmC (Davalos-Garcia *et al.* 2001), resistance to chlorpromazine-induced stress in *E. coli* (Conter *et al.* 2002), and production of exopolysaccharide by *Erwinia amylovora* (Bereswill and Geider 1997). The RcsB-RcsC system is made of two main components, the sensor protein RcsC and the transcriptional activator RcsB (Brill *et al.* 1988), but includes also a second transcriptional activator, RcsA (Gottesman *et al.* 1985);

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TABLE 1

Strains of *S. enterica* serovar Typhimurium

Strain	Genotype
MST1753[a]	*leuA414 hsdL* (r⁻m⁻)Fels2⁻/F′114(ts) *lac*⁺ *zzf-20*::Tn*10 zzf-3551*::Mu*d*P
SV4215	*igaA1*
SV4254	*igaA1 zhf-6311*::Tn*10d*Tc
SV4321	DUP[*argA9000**Mu*d*P**cysG1573*]
SV4322	DUP[*argA9000**Mu*d*P**cysG1573*] *igaA*2::KIXX
SV4324	DUP4102[*fliC5050**Mu*d*A**flhDC5213*] *zhf-6311*::Tn*10d*Tc
SV4325	DUP4102[*fliC5050**Mu*d*A**flhDC5213*] *igaA1 zhf-6311*::Tn*10d*Tc
SV4343	*igaA1 zhf-6311*::Tn*10d*Tc *rcsA51*::Mu*d*Q
SV4345	*igaA2*::KIXX *rcsC52*::Mu*d*Q
SV4357	*igaA1 zhf-6311*::Tn*10d*Tc *gmm-21*::Mu*d*J
SV4379	*rcsA51*::Mu*d*P
SV4380	*rcsC52*::Mu*d*P
SV4402	*igaA1 zhf-6311*::Tn*10d*Tc *rcsB70*::Tn*10d*Cm
SV4404	*igaA1 zhf-6311*::Tn*10d*Tc *rcsC52*::Mu*d*Q
SV4406	*rcsB70*::Tn*10d*Cm
SV4407	*gmm-21*::Mu*d*J
SV4411	*gmm-21*::Mu*d*J *rcsB70*::Tn*10d*Cm
SV4412	*igaA1 zhf-6311*::Tn*10d*Tc *gmm-21*::Mu*d*J *rcsB70*::Tn*10d*Cm
SV4443	*igaA1 zhf-6311*::Tn*10d*Tc *rcsA51*::Mu*d*Q
SV4450	*igaA1*
SV4577	SL1344/pNG1166
SV4578	*igaA2*::KIXX/pNG1166
SV4614	*zhf-6311*::Tn*10d*Tc *flhDC5213*::Mu*d*A
SV4615	*igaA1 zhf-6311*::Tn*10d*Tc *flhDC5213*::Mu*d*A
SV4616	*igaA1 zhf-6311*::Tn*10d*Tc *flhDC5213*::Mu*d*A *rcsA51*::Mu*d*P
SV4617	*igaA1 zhf-6311*::Tn*10d*Tc *flhDC5213*::Mu*d*A *wcaK21*::Mu*d*P
SV4618	*igaA1 zhf-6311*::Tn*10d*Tc *flhDC5213*::Mu*d*A *manB571*::Mu*d*P
SV4619	*igaA1 zhf-6311*::Tn*10d*Tc *flhDC5213*::Mu*d*A *wcaF22*::Mu*d*P
SV4620	*igaA1 zhf-6311*::Tn*10d*Tc *flhDC5213*::Mu*d*A *gmm*::Mu*d*P
SV4622	*zhf-6311*::Tn*10d*Tc *flhDC5213*::Mu*d*A *rcsB70*::Tn*10d*Cm
SV4623	*igaA1 zhf-6311*::Tn*10d*Tc *flhDC5213*::Mu*d*A *rcsB70*::Tn*10d*Cm
SV4624	*zhf-6311*::Tn*10d*Tc *flhC5213*::Mu*d*A *rcsC52*::Mu*d*P
SV4625	*igaA1 zhf-6311*::Tn*10d*Tc *flhDC5213*::Mu*d*A *rcsC52*::Mu*d*P
SV4629	*igaA2*::KIXX *rcsC52*::Mu*d*P/pNG1166
SV4630	*fliA6002*::Mu*d*A *zhf-6311*::Tn*10d*Tc
SV4631	*fliA6002*::Mu*d*A *igaA1 zhf-6311*::Tn*10d*Tc
TR5878[b]	r(LT2)⁻ m(LT2)⁻ r(S)⁺ *ilv-542 metA22 trpB2* Fels2⁻ *fliA66 thrA120 xyl-404 metE551 hspL56 hspS29*
TT10423[b]	*proAB47*/F′128 *pro*⁺ *lac*⁺ *zzf-1836*::Tn*10d*Tc

[a] LT2 derivative, obtained from Stanley R. Maloy.
[b] LT2 derivatives, obtained from John R. Roth (University of Utah, Salt Lake City).

a recently described ancillary protein, YojN (Takeda *et al.* 2001); and additional sensors that transmit external signals to the membrane-bound RcsC sensor (Clarke *et al.* 1997; Kelley and Georgopoulos 1997; Chen *et al.* 2001). We show that mutations affecting any of the *rcsB*, *rcsC*, and *yojN* genes suppress the defects associated with *igaA* mutations (lethality, mucoidy, and reduced motility). Mucoidy is also suppressed by *rcsA* mutations. These observations provide genetic evidence that IgaA might be part of a novel signaling pathway involving the RcsB-RcsC system.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and strain construction: All the *S. enterica* strains listed in Table 1 belong to serovar Typhimurium. Unless indicated otherwise, the strains derive from the mouse-virulent strain SL1344 (Hoiseth and Stocker 1981). Transductional crosses using phage P22 HT 105/1 *int201* (Schmieger 1972; G. Roberts, unpublished results), henceforth called P22 HT, were used for strain construction operations involving chromosomal markers and for transfer of plasmids among Salmonella strains. The transduction protocol was as 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. The alleles *flhDC5213*::Mu*d*A and *fliA6002*::Mu*d*A were originally harbored by strains KK1107 and KK1108, respectively (Kutsukake and Iino 1994). Both strains derive from LT2 and were obtained from Kazuhiro Kutsukake (Okayama University, Okayama, Japan). Mu*d*A is a Mu*d*1 derivative with conditional transposition (Hughes and Roth 1984). The duplication DUP4102[*fliC5050**Mu*d*A**flhDC5213*], originally carried on a LT2-derived strain (TH4313), was a gift from Kelly T. Hughes

(University of Washington, Seattle). Strain MST1753, used for transposon replacement, carries an F-prime containing a Mu*d*P element (Youderian *et al.* 1988); the strain was obtained from Stanley R. Maloy (University of Illinois, Urbana, IL). The allele *rcsB70*::Tn*10d*Cm was obtained from Eduardo Groisman (Washington University School of Medicine, St. Louis). This allele was originally carried by a 14028s derivative (strain EG12711). Allele numbers for *S. enterica* mutants described in this study were obtained from the Salmonella Genetic Stock Center (University of Calgary, Calgary, Alberta, Canada). The *E. coli* recipient of plasmids was strain DH5α (Yanisch-Perron *et al.* 1985). The *E. coli* host for π-dependent suicide plasmids was S17 λ*pir* (Simon *et al.* 1983).

Media and chemicals: The E medium of Vogel and Bonner (1956) was used as the standard minimal medium. The rich medium was Luria broth (LB), prepared according to Maloy (1990). Carbon sources were either 0.2% glucose or 0.2% arabinose. Solid media contained agar at 1.5% final concentration. Auxotrophic requirements and antibiotics were used at the final concentrations described by Maloy (1990). As an exception, viability assays involving plasmid pNG1166 were carried out on plates containing a lower concentration of ampicillin (30 μg/ml), as recommended by Guzman *et al.* (1995). D-Mecillinam, a gift from Juan A. Ayala (Centro de Biología Molecular, CSIC, Cantoblanco, Spain), was used as described below. Green plates were prepared according to Chan *et al.* (1972), except that methyl blue (Sigma, St. Louis) substituted for aniline blue. Motility assays were carried out in LB prepared without yeast extract (Gillen and Hughes 1991). Solid motility medium contained agar at 0.25% final concentration.

Surveys of D-mecillinam resistance: Minimal inhibitory concentrations (MIC) of D-mecillinam were determined in solid media (LB and E) using late exponential cultures of the strains to be tested, prepared in Luria broth. The final concentrations of D-mecillinam ranged from 0.05 μg/ml to 1 μg/ml. Levels of mecillinam resistance were also assessed with filter paper discs soaked in a solution of mecillinam (40 μg/ml). The discs were placed on LB or E plates, previously spread with 10^5 colony-forming units of the strain to be tested. After 18–24 hr incubation at 37°, the diameters of the inhibition halos were measured.

Plasmids: Plasmid pBAD18 (Ap^r) is a member of the pBAD series of vectors, designed for the study of essential genes (Guzman *et al.* 1995). Plasmid pUC4-KIXX (Km^r Ap^r), a product of Pharmacia Biotech Europe (Sant Cugat del Vallès, Spain), carries the Tn*5* kanamycin-resistance gene flanked by restriction sites that facilitate cloning. Plasmid pGEM-3Z (Ap^r) is a cloning vector from Promega (Madison, WI). Plasmid pIZ994, constructed in our laboratory, is a pGEM-3Z derivative that carries the *igaA* locus of *S. enterica*. Plasmid pIZ998 (Ap^r Km^r) is a pIZ994 derivative in which the *igaA* locus has been interrupted with the KIXX cassette from pUC4-KIXX, to generate the *igaA2*::KIXX allele. Plasmid pGP704 (Ap^r) is a pBR322 derivative that carries the origin of replication of R6K and the *mob* region of RP4 (Miller and Mekalanos 1988). Plasmid pIZ1551 (Ap^r) is a pGP704 derivative constructed in our laboratory and carries the *igaA2*::KIXX allele from pIZ998 cloned at the *Eco*RI site of pGP704.

Construction of plasmid pNG1166: Genomic DNA from strain SL1344 was PCR amplified using primers from both sides of the *igaA* gene: 5′-TCT GTG GTA CCA CGC CTG ACA GAC-3′ and 5′-CAA TAT CTA GAT GCA TGG GGA ACT GC-3′, which introduce *Kpn*I and *Xba*I sites (underlined), respectively. The amplified DNA fragment was digested with *Kpn*I and *Xba*I to permit oriented cloning of the *igaA* gene on pBAD18 (Guzman *et al.* 1995). Ligation mixtures were used to transform *E. coli* DH5α, selecting Ap^r transformants on LB-ampicillin plates. One of the transformants was the source of plasmid pNG1166, which carries the *igaA* gene under the control of the arabinose-dependent P_{BAD} promoter (Guzman *et al.* 1995).

Mutagenesis with Mu*d*J: We employed the *cis*-complementation procedure of Hughes and Roth (1988), in which a defective Mu*d*J element is cotransduced with a Mu*d*1 element that transiently provides transposition functions. Mu*d*1 is the specialized transducing phage Mu*d*1(Ap Lac *cts62*) constructed by Casadaban and Cohen (1979). Mu*d*I1734[KmLac] (Castilho *et al.* 1984) is a transposition-deficient Mu derivative that generates operon fusions upon insertion; the element was renamed Mu*d*J by Hughes and Roth (1988).

Mutagenesis with Tn*10d*Tc: A lysate grown on strain TT10423 was used to transduce the strain to be mutagenized. The latter carried plasmid pNK2280 (Kleckner *et al.* 1991) to permit complementation of the defective Tn*10d*Tc element by ATS transposase. Transducing mixtures were made directly on LB plates containing tetracycline and were incubated 24 hr at 37°. For the preparation of insertion pools, plates containing Tc^r transductants were replica printed to LB-EGTA. Several thousands of colonies were then harvested and used to inoculate a single, mixed culture in LB-EGTA. When saturated, the culture was harvested by centrifugation and washed three to five times with LB. An aliquot was then used for standard lysis with P22 HT.

Replacement of the Mu*d*J element by Mu*d*P: A lysate grown on strain MST1753 was used for transposon replacement by homologous recombination. In each substitution, a Mu*d*J element is replaced by a Mu*d*P element upon recombination between the ends of the elements. P22 HT transductions selecting the incoming marker (Cm^r) were carried out. Cm^r transductants were then scored for loss of the resident marker (Km^r).

β-Galactosidase assays: Levels of β-galactosidase activity were assayed as described by Miller (1972), using the $CHCl_3$-sodium dodecyl sulfate permeabilization procedure.

Bacterial transformation: Transformation of *E. coli* DH5α with plasmids followed the procedure of Inoue *et al.* (1990). Transformation of *S. enterica* was according to Lederberg and Cohen (1974). The Salmonella host for plasmids was strain TR5878. Plasmids transformed into TR5878 were transferred to suitable recipients by transduction with P22 HT.

Matings: Transfer of pIZ1551 from *E. coli* S17 λ*pir* to *S. enterica* was assayed using exponential cultures of both the donor and the recipient. To obtain higher cell concentrations, cultures were harvested by centrifugation and concentrated 10- to 100-fold. Donor and recipient aliquots of 100 μl were mixed on an LB plate and incubated 8 hr at 37° before replica printing to selective plates.

DNA amplification with the polymerase chain reaction: Amplification reactions were carried out in a Perkin Elmer GeneAmp PCR System 2400 (Perkin Elmer Cetus, Foster City, CA). The final volume of all reactions was 50 μl, and the final concentration of $MgCl_2$ was 1 mM. Reagents were used at the following concentrations: dNPTs, 200 μM; primers, 1 μM; and *Taq* polymerase, 1 unit per reaction. The thermal program included the following steps: (i) initial denaturation, 10 min at 94°; (ii) 35 cycles of denaturation (94°, 30 sec), annealing (55°, 30 sec), and extension (72°, 1 min); and (iii) final incubation at 72° for 10 min, to complete extension.

DNA sequencing: Chromosomal DNA was prepared from 1.5-ml overnight cultures in LB. Cells were harvested by centrifugation and resuspended in 1.5 ml of 10 mM Tris-HCl, pH 8.0, 25 mM EDTA, pH 8.0. A volume of 0.55 ml of lysozyme solution (10 mg/ml in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) was added. The mixture was incubated for 20 min at 37°. Proteinase K (100 μg/ml) was then added, and the preparation was incubated for 1 hr at 55°. After three to four extractions with phenol and chloroform-isoamyl alcohol (24:1),

DNA was precipitated with ammonium acetate and absolute ethanol, and finally suspended in 10 mm Tris-HCl, pH 8.0, 1 mm EDTA, pH 8.0. DNA was further sheared with a 23G needle and cleaned in a Sephadex G50 column. The primer used for sequencing of the boundaries of Tn*10* insertions was 5′-CTA ATG ACA AGA TGT GT-3′ (Way and Kleckner 1984). For Mu*d*J insertions, the primer was 5′-CGA ATA ATC CAA TGT CCT CC-3′ (Torreblanca *et al.* 1999), henceforth called MuL. Sequencing was performed as described elsewhere (Cano *et al.* 2001).

Antibodies: Rabbit anti-FtsZ polyclonal antibody was a gift from Miguel Vicente (Centro Nacional de Biotecnología, CSIC, Madrid).

Rabbit anti-IgaA polyclonal antibody was raised against a recombinant N-IgaA-6xHis protein, which was produced and purified as follows: a 707-bp PCR fragment (nucleotide positions 64–771 from the putative translation start of *igaA*; EMBL accession no. AJ272210) was PCR amplified with the primers 5′-TCC GGG CCA TGG CCA GAC GAG GG-3′ and 5′-AAT TTC CTC GAG CGC GCT TTT CGA-3′, which introduce *Nco*I and *Xho*I sites (underlined), respectively. The resulting fragment was digested with *Nco*I and *Xho*I and inserted in-frame upstream from the His tag sequence in the expression vector pET21d+ (Novagen, Madison, WI). The resulting plasmid, pETN1-2, was verified by sequencing the insert from both junctions. This plasmid was then used to transform *E. coli* BL21 (DE3; Novagen). The resulting strain was grown at 37° with shaking until early exponential phase, and expression of IgaA6xHis was induced by addition of 0.1 mm isopropyl thiogalactoside (IPTG). After 3 hr of induction, bacteria were harvested by centrifugation, suspended in column-binding buffer (5 mm imidazole, 500 mm NaCl, and 5 mm HEPES, pH 7.9), and disrupted with a French press. Bacterial debris was removed by centrifugation and protein was purified from the supernatant by cobalt-affinity chromatography according to the manufacturer's instructions. Following elution with 150 mm imidazole, purified IgaA6xHis was concentrated to the desired volume using centriplus YM-10 (Millipore, Bedford, MA). Polyclonal antibodies were obtained by standard immunization of rabbits with the N-IgaA-6xHis protein.

SDS-PAGE and Western blot analysis: Proteins from bacterial extracts were prepared as described by Kaniga *et al.* (1995) and analyzed by SDS-PAGE in Tris/Tricine buffer by using 10% acrylamide gels. Blots were probed with rabbit affinity-purified anti-FtsZ (1:200) or rabbit anti-IgaA (1:5000) polyclonal antibodies. Goat anti-rabbit HRP-conjugated secondary antibody (Bio-Rad Life Science, El Prat de Llobregat, Spain) was used as secondary antibody to detect specific proteins by the enhanced chemiluminescence assay.

Shift to IgaA deprivation conditions: An overnight culture of strain SV4578 [*igaA2*::KIXX/pNG1166 (*igaA*$^+$)] grown in LB medium supplemented with 0.2% arabinose and ampicillin (100 μg/ml) was diluted 1:100 in the same medium. After 2 hr of incubation at 37°, bacteria were washed twice in PBS, suspended in LB medium adjusted to an OD_{600} of 0.05, and transferred to two separate flasks, to which glucose (2%) or arabinose (0.2%) was added. To maintain bacteria in continuous exponential growing conditions, cultures were refreshed every hour. Samples were taken every hour for SDS-PAGE and Western blot analysis.

RESULTS

Suppression of the mucoid phenotype of an *igaA1* mutant by Mu*d*J insertions: The *igaA1* mutation causes mucoidy, which is best observed in colonies grown in rich medium. To identify genes involved in the mucoid phenotype associated with the *igaA1* mutation, an *igaA1* strain was mutagenized with Mu*d*J; nonmucoid colonies were then sought among the the Km^r transductants. The strain used, SV4254, carried a Tn*10* insertion (*zhf-6311*::Tn*10d*Tc) cotransducible with the *igaA1* mutation (Cano *et al.* 2001). Nonmucoid Km^r transductants were subjected to the following tests:

i. For reconstruction analysis the candidates were lysed with P22 HT, and the lysates were used to transduce SV4254, selecting kanamycin resistance. A 100% linkage between the Km^r marker and the nonmucoid phenotype indicated the existence of a suppressor mutation. Only six independent isolates passed this test.
ii. The same lysates were used to transduce the wild-type strain, selecting tetracycline resistance. If true suppression had occurred, the *igaA1* mutation would persist on the chromosome, and its phenotype would reappear in the absence of suppressors. The six candidates passed this test: in all cases, ∼35% of the Tc^r transductants were mucoid, indicating that the *igaA1* mutation had been cotransduced with the insertion *zhf-6311*::Tn*10d*Tc.
iii. Cotransduction between the *zhf-6311*::Tn*10d*Tc insertion and the Mu*d*J element was not detected in any of the suppressor-carrying isolates, indicating that the suppressor mutations were not linked to the *igaA* locus.

To identify the loci where the Mu*d*J element had inserted, DNA sequencing was performed directly on the chromosome, using the MuL primer (Torreblanca *et al.* 1999). The Mu*d*J insertions that suppressed mucoidy in the *igaA1* background were found in the *gmm*, *manB*, *wcaK*, and *wcaF* genes, which are part of the *wca* cluster for colanic acid synthesis (Stevenson *et al.* 1996), and in *rcsA* and *rcsC*, which are part of the Rcs network that controls capsule synthesis (Gottesman and Stout 1991; Gottesman 1995). Interestingly, all the mutations that suppressed the mucoid phenotype also reduced the intracellular proliferation rate in NRK fibroblast cell cultures (data not shown), suggesting that capsule overproduction is necessary for the *igaA1* mutant to overgrow inside eukaryotic cells.

A recent study describes *S. enterica* mutants that display a mucoid phenotype that is suppressed by *rcsC* mutations (Costa and Antón 2001). The map position of the locus affected, *mucM*, is similar to that of *igaA* (Cano *et al.* 2001; Costa and Antón 2001), thus raising the possibility that the *igaA1* allele and *mucM* alleles could all be ascribed to a single class. Because *mucM* mutants were isolated by resistance to D-mecillinam (Costa and Antón 2001), we carried out tests of D-mecillinam resistance in strains SL1344 (*igaA*$^+$) and SV4450 (*igaA1*). Both strains exhibited MIC values around 0.8 μg/ml in LB agar and 0.6 μg/ml in E plates. The diameters of growth inhibition halos were also similar for both

TABLE 2

Effect of *igaA* and *rcsB* mutations on the expression of a *gmm::lac* fusion

Strain	Relevant genotype[a]	β-Galactosidase activity[b]
SV4407	Wild type	6.3 ± 0.7
SV4357	*igaA1*	35.9 ± 1.2
SV4412	*igaA1 rcsB70*::Tn*10d*Cm	7.7 ± 1.2
SV4411	*rcsB70*::Tn*10d*Cm	8.6 ± 0.8

[a] All strains carry, in addition, the *gmm1::lac*(Mu*d*J) fusion.
[b] The data shown are the means and standard deviations of at least three independent experiments.

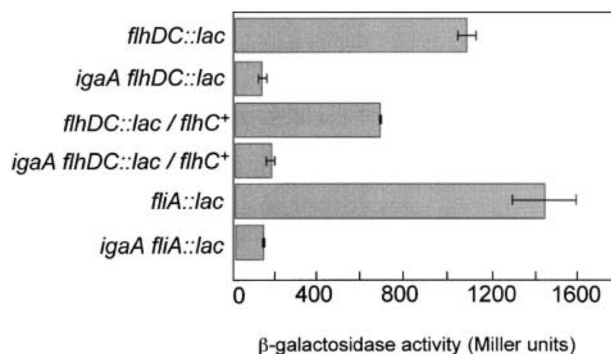

FIGURE 1.—Effect of the *igaA1* mutation on the expression of *flhDC::lac* and *fliA::lac* fusions. The following strains were used, from top to bottom: SV4614, SV4615, SV4324, SV4325, SV4630, and SV4631. The data shown are the means and standard deviations of at least three independent experiments.

strains. This absence of differences suggests that the *igaA1* allele is unrelated to the *mucM* alleles described by Costa and Antón (2001). Further differences between *igaA1* and *mucM* alleles will be described below.

The *igaA1* mutation increases *wca* expression: In the *gmm*::Mu*d*J insertion described above as a suppressor of the mucoid phenotype of the *igaA1* mutant (henceforth called *gmm-21*::Mu*d*J), the Mu*d*J element had inserted in the appropriate orientation to generate a *lac* fusion: The insertion was Lac^- in an $IgaA^+$ background and Lac^+ in the presence of the *igaA1* mutation. Analysis of β-galactosidase activity confirmed that the *igaA1* mutation caused an increase in the expression of the *gmm-21::lac* fusion (Table 2). For these experiments, cultures were prepared in LB, where the mucoidy caused by the *igaA* mutation is higher. In the wild type, expression of *gmm* was low, as previously described for *E. coli wca* genes (Gottesman 1995). An additional observation was that the presence of an *rcsB* mutation suppressed derepression of *gmm* in the *igaA1* background (Table 2). This result suggests that derepression of colanic acid synthesis by the *igaA1* mutation is exerted via the Rcs regulatory system. This view is supported by the observation, described above, that *rcsA* and *rcsC* mutations suppress the mucoid phenotype of an *igaA1* mutant.

The *igaA1* mutation causes a defect in motility: The DNA sequence relatedness between the *igaA* ORF and the *umoB* gene of *P. mirabilis* (Dufour *et al.* 1998) raised the possibility that *igaA1* mutants might be impaired in motility. Hence, we compared the motility of isogenic $IgaA^+$ and $IgaA^-$ strains. Cultures were prepared in motility medium and incubated at 37° with shaking. At the stage of midexponential growth, a sterile toothpick was soaked in the culture and used to inoculate a motility agar plate. The diameters of the bacterial growth halos were calculated every hour. Halo formation by the *igaA1* mutant proceeded at a speed about half that of the wild type: 3.2 ± 0.2 mm/hr *vs.* 7.5 ± 0.9 mm/hr, respectively. The doubling time of the *igaA1* mutant in liquid motility medium was identical to that of the wild type (data not shown). Therefore, delayed halo formation associated with the *igaA1* mutation was interpreted as a motility defect.

The *igaA* gene regulates the expression of the flagellar master operon *flhDC*: The motility defect of $UmoB^-$ mutants of *P. mirabilis* is known to be caused by their inability to activate the *flhDC* operon (Dufour *et al.* 1998). In enteric bacteria, as in *P. mirabilis*, the *flhDC* operon encodes transcription factors necessary to trigger the genetic cascade that controls flagellar synthesis (Gygi *et al.* 1995; Macnab 1996; Furness *et al.* 1997; Claret and Hughes 2000). Hence, a defect in *flhDC* expression causes reduced motility (Macnab 1996). The homology between *igaA* and *umoB* led us to investigate whether the *igaA1* mutation affected the expression of flagellar genes, using transcriptional *lac* fusions in *flhDC* and *fliA*. Evidence that *flhDC* undergoes autogenous positive regulation (Kutsukake 1997) led us also to compare *flhDC* expression in $FlhDC^+$ and $FlhDC^-$ backgrounds. The latter experiments were performed in a merodiploid strain (SV4325), which permits analysis of the *flhDC5213::lac* fusion in the presence of a wild-type *flhDC* operon. The results shown in Figure 1 can be summarized as follows:

i. Expression of *flhDC* decreased sixfold in an *igaA1* background. The difference was only threefold when *flhDC* expression was measured in a $FlhDC^-/FlhDC^+$ merodiploid.
ii. As expected, the *igaA1* mutation also reduced expression of an *fliA::lac* fusion, used as representative of class II genes, which require FlhDC activation (Macnab 1996).

The correlation between reduced motility and lowered *flhDC* expression in an *igaA1* background is in agreement with the report that other mutations that lower *flhDC* expression (*e.g.*, *crp*, *cya*, and *hns*) also decrease motility (Kutsukake 1997).

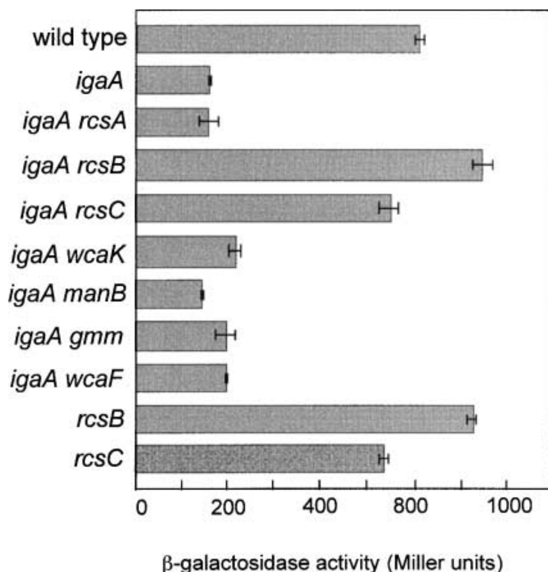

Figure 2.—Effects of *rcs* and *wca* mutations on *flhDC* operon expression in an *igaA1* background. The strains used were, from top to bottom, SV4614, SV4615, SV4616, SV4623, SV4625, SV4617, SV4618, SV4620, SV4619, SV4622, and SV4624. The data shown are the means and standard deviations of at least three independent experiments.

Mu*d*J insertions in *rcsB* or *rcsC*, but not in *rcsA*, suppress the defect in *flhDC* expression caused by the mutation *igaA1*: To investigate whether reduced expression of the *flhDC* operon in an *igaA1* background was suppressed by suppressors of mucoidy, we constructed a set of strains that carried an *flhDC::lac* fusion and the *igaA1* mutation in combination with *wca* or *rcs* mutations. Aside from the *rcsA* and the *rcsC* alleles described above, a collection *rcsB* allele (*rcsB70*::Tn*10d*Cm) was also used. As shown in Figure 2, *rcsB* and *rcsC* mutations suppressed the *flhDC* expression defect in an *igaA1* background. None of the *wca* insertions exerted any effect on *flhDC* expression. An *rcsA* mutation failed also to restore *flhDC* expression in an *igaA1* mutant, suggesting that IgaA might regulate *flhDC* via RcsB/RcsC and that RcsA is not required. It must be noted that other examples of loci regulated by RcsB/RcsC, but not by RcsA, are found in the literature (Gottesman 1995; Clavel *et al.* 1996; Virlogeux *et al.* 1996; Davalos-Garcia *et al.* 2001).

The *igaA1* mutation is recessive: Strain 4215 carries a G → A transition in the putative *igaA* ORF (Cano *et al.* 2001). To examine whether this mutation was dominant or recessive, the *igaA1* mutation was introduced into a merodiploid strain carrying a chromosomal duplication of the *argA-cysG* region, which encompasses centisomes 64–75 (Camacho and Casadesús 2001). For this purpose, strain SV4321 was transduced with a P22 HT lysate grown on SV4254. Tc^r Cm^r transductants were selected. Because the *zhf-6311*::Tn*10d*Tc insertion is 35% linked to the *igaA1* mutation (Cano *et al.* 2001), dominance can be expected to result in ∼35% of mucoid transductants. A screen of >2000 independent transductants did not reveal any mucoid colony, suggesting that the *igaA1* mutation was recessive.

Segregation analysis (Anderson and Roth 1977) confirmed the recessivity of the *igaA1* mutation. Twenty-four Tc^r Cm^r transductants were grown in LB without antibiotics. Cultures were diluted and spread on LB agar. Single colonies were then replica printed to LB agar with tetracycline. Eight of the 24 transductants segregated mucoid colonies. All the mucoid colonies were Cm^s, thereby confirming that the nonmucoid merodiploids carried the *igaA1* mutation. The *igaA1* mutation is therefore recessive and, in principle, may cause loss of function.

The *igaA* gene of *S. enterica* is essential: The *igaA1* mutation is a base substitution that putatively changes a histidine moiety to arginine (Cano *et al.* 2001); thus, *a priori*, the *igaA1* allele could be null or leaky. To compare the properties of *igaA1* with those of a definite null allele, the *igaA* gene was knocked out *in vitro*. The wild-type *igaA* allele carried on plasmid pIZ998 was interrupted at its single *Bam*HI site with the KIXX (Km^r) cassette from pUC4-KIXX. Attempts to replace the chromosomal *igaA* gene with this construct failed (data not shown), thereby raising the possibility that *igaA* was an essential gene. To test this hypothesis, a suicide plasmid containing the *igaA2*::KIXX mutation (pIZ1551) was constructed. Plasmid pIZ1551 is a pGP704 derivative (Miller and Mekalanos 1988) and was stably maintained in *E. coli* S17 λ*pir* (Simon *et al.* 1983). Plasmid pIZ1551 was then transferred by conjugation to strain SV4321, which carries a Mu*d*P-held duplication of the *cysG-argA* chromosomal region (Camacho and Casadesús 2001). Transconjugants were selected on LB plates supplemented with kanamycin (to select plasmid transfer) and chloramphenicol (to counterselect the donor). Because the plasmid cannot replicate in a strain devoid of π-protein, Km^r transconjugants can be formed only by either plasmid integration in the chromosome or homologous recombination between one resident *igaA* gene and the incoming *igaA2*::KIXX allele. The latter event yields Km^r Ap^s transconjugants, while plasmid integration generates Km^r Ap^r transconjugants. An ampicillin-sensitive $igaA^+$/*igaA2*::KIXX merodiploid (SV4322) was obtained by this procedure. To confirm the presence of both alleles, DNA from SV4322 was PCR amplified using *igaA*-flanking primers. Two amplification fragments of 4.2 and 5.8 kb were obtained (data not shown). The size of the *igaA* locus is 4.2 kb and that of the KIXX cassette is 1.6 kb: hence the merodiploid contained both an intact *igaA* locus and a knockout allele.

When the duplication carried by strain SV4322 was allowed to segregate in chloramphenicol-free medium, all Cm^s colonies were kanamycin sensitive. The absence of haploid segregants carrying the *igaA2*::KIXX allele supported the view that a null *igaA* allele might be lethal. This view received further support from experiments in

which a P22 HT lysate grown on strain SV4322 (*igaA*$^+$/*igaA2*::KIXX) was used to transduce SL1344 (*igaA*$^+$, haploid) and SV4321 (*igaA*$^+$/*igaA*$^+$, merodiploid). Kanamycin-resistant transductants appeared at frequencies 1000-fold higher in SV4321 than in SL1344: around 10^{-6} and 10^{-9} per plaque-forming unit (PFU), respectively. Altogether, these observations suggest that the *igaA* locus of *S. enterica* is essential, at least under the conditions assayed. A corollary is that the original *igaA1* mutation may be leaky. Interestingly, the essential condition of *igaA* in *S. enterica* is not shared by the related gene *umoB* of *P. mirabilis*, where insertion mutations have been described (Dufour *et al.* 1998).

Because *igaA* is the promoter-proximal gene within a putative operon that may contain four genes (*yrfF*, *yrfG*, *yrfH*, and *yrfI*), we investigated whether the lethality of the *igaA2*::KIXX mutation was caused by a polar effect on downstream genes. For this purpose, a KIXX cassette was introduced in the second ORF of the putative operon, *yrfG* (data not shown). The *yrfG*::KIXX mutant was viable, as indicated by the observation that strains SL1344 (*igaA*$^+$, haploid) and SV4321 (*igaA*$^+$/*igaA*$^+$, merodiploid) could be transduced at similar frequencies ($\sim10^{-6}$/PFU) with a P22 lysate carrying the *yrfG*::KIXX construct. We thus concluded that the lethality of the *igaA2*::KIXX mutation was solely due to *igaA* inactivation. Furthermore, the *yrfG*::KIXX mutant was nonmucoid and unable to derepress the *flhDC5213::lac* fusion (data not shown), indicating that mucoidy and *flhDC* derepression were also *igaA*-associated traits.

Finally, we tested whether the lethality caused by a null *igaA* mutation could be complemented by a wild-type *igaA* allele carried on a plasmid. For this purpose, plasmid pNG1166 was transduced to SL1344. The resulting strain was then transduced with a P22 HT lysate grown on strain SV4322 (*igaA*$^+$/*igaA2*::KIXX), selecting kanamycin resistance on LB plates containing 0.2% arabinose. Km^r transductants appeared at frequencies $\sim10^{-6}$/PFU, suggesting that the *igaA2*::KIXX mutation did not impair viability in the presence of the *igaA*$^+$ allele carried on pNG1166. To confirm that the *igaA2*::KIXX construct had recombined with the recipient chromosome (and not with plasmid pNG1166), a putatively complemented isolate was lysed with P22 HT. The lysate was used to transduce SL1344, selecting Ap^r. Transductants were obtained at a frequency of 10^{-5}/PFU, and all (100/100) were Km^s. If the same lysate was used to select kanamycin resistance, transductants were extremely rare ($\sim10^{-9}$/PFU) and were Ap^s. Transduction of kanamycin resistance to the merodiploid strain SV4321 was, however, successful. These experiments confirmed that the donor carried both an intact plasmid pNG1166 and the chromosomal *igaA2*::KIXX construct. This strain was propagated as SV4578.

Occurrence of complementation was confirmed in plate tests, shown in Figure 3. Strain SV4578 (*igaA2*::KIXX/pNG1166) was able to grow on arabinose-containing plates, but not on glucose. In contrast, IgaA$^+$ and IgaA$^-$ RcsC$^-$ strains (SV4577 and SV4629) grew well on both media. These experiments confirm that lack of *igaA* expression alone prevents growth of *S. enterica* in standard laboratory conditions.

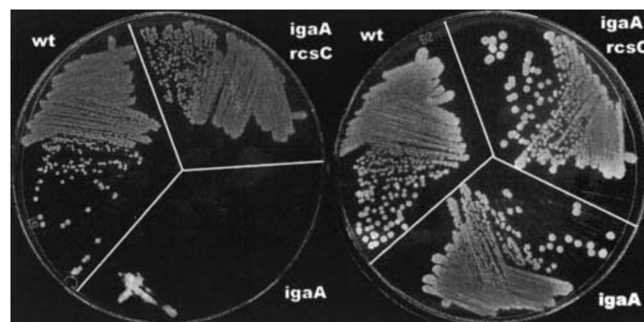

Figure 3.—Growth of IgaA$^+$, IgaA$^-$, and IgaA$^-$ RcsC$^-$ strains on LB-glucose-ampicillin (left) and LB-arabinose-ampicillin (right). The strains used were SV4577 (*igaA*$^+$/pNG1166), SV4629 (*igaA2*::KIXX *rcsC52*::Mu*d*P/pNG1166), and SV4578 (*igaA2*::KIXX/pNG1166). In the latter, expression of the plasmid-borne *igaA* gene is conditional, driven by the arabinose-dependent P_{BAD} promoter. The assays were carried out as described by Guzman *et al.* (1995), and the concentration of ampicillin was 30 mg/liter.

Mu*d*J insertions in *rcsB* or *rcsC*, but not in *rcsA*, suppress the lethality caused by a null *igaA* allele: We have described above that *rcsA* and *rcsC* mutations suppress mucoidy and reduced motility caused by the viable allele *igaA1*. To investigate whether mutations affecting the Rcs regulatory system also suppressed the lethality caused by the null allele *igaA2*::KIXX, transductional crosses were performed. The recipients were SV4379 (RcsA$^-$), SV4380 (RcsB$^-$), and SV4406 (RcsC$^-$). The donor was an *igaA*$^+$/*igaA2*::KIXX merodiploid (SV4322). Kanamycin-resistant transductants were selected upon P22 HT transduction. Their frequencies (per PFU) were $\sim10^{-6}$ in the RcsB$^-$ and RcsC$^-$ recipients and $<10^{-9}$ in the RcsA$^-$ recipient. Hence, lack of RcsB or RcsC functions does suppress the lethality of an *igaA* null mutation, but lack of RcsA does not. This suppressor pattern is identical to that found for *flhDC* expression in an *igaA1* background (see above).

Search for Tn*10* insertions that suppress the lethality caused by a null *igaA* allele: Mutagenesis with Tn*10d*Tc was carried out in strain SL1344; five pools of Tn*10d*Tc insertions, each containing some 10,000 independent isolates, were then prepared. The pools were grown in LB + EGTA until midexponential phase and used as recipients in P22 HT-mediated transductional crosses. The donor was the merodiploid strain SV4322 (*igaA*$^+$/*igaA2*::KIXX). Km^r transductants were selected on LB-kanamycin plates. Each cross yielded 5–30 Km^r transductants. Segregation analysis after nonselective growth (in LB without kanamycin) allowed us to discard *igaA*$^+$/*igaA2*::KIXX merodiploids that segregated Km^s colonies. Stable Km^r isolates carried putative Tn*10d*Tc inser-

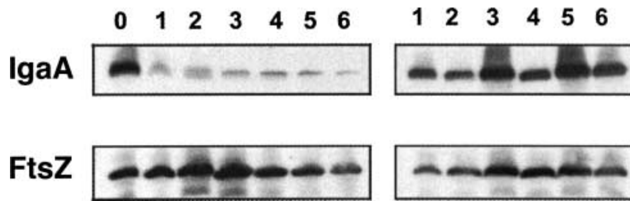

FIGURE 4.—Levels of IgaA and FtsZ in protein extracts from strain SV4578 (*igaA2*::KIXX/pNG1166) upon transfer to glucose medium (left) or to fresh arabinose medium (right). Samples were taken every hour for SDS-PAGE and Western blot analysis. The time of sampling (in hours) is indicated on the top of each sample; zero designates the time of transfer to fresh media.

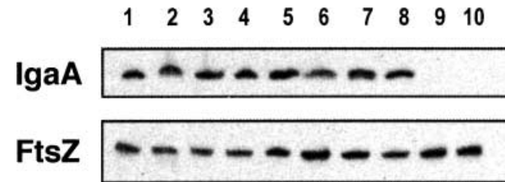

FIGURE 5.—Levels of IgaA and FtsZ in protein extracts from SL1344 (wildtype, lane 1), SV4215 (*igaA1*, lane 2), SV4379 (*rcsA*, lane 3), SV4406 (*rcsB*, lane 4), SV4380 (*rcsC*, lane 5), SV4343 (*igaA1 rcsA*, lane 6), SV4402 (*igaA1 rcsB*, lane 7), SV4404 (*igaA1 rcsC*, lane 8), SV4345 (*igaA2*::KIXX *rcsC*, lane 9), and SV4443 (*igaA2*::KIXX *rcsB*, lane 10). The extracts were prepared from bacteria growing exponentially in LB.

tions that suppressed the lethality associated with the *igaA2*::KIXX mutation. These isolates were then subjected to reconstruction analysis:

i. Fifty independent candidates were lysed with P22 HT, and the resulting lysates were used to transduce SL1344, selecting tetracycline resistance.
ii. Tc^r transductants obtained in these crosses were transduced with an SV4322 lysate, selecting kanamycin resistance. As a control, the merodiploid strain SV4321 ($igaA^+/igaA^+$) was also transduced with the same lysate. Whenever Km^r transductants were obtained at similar frequencies in SV4231 and in the putative suppressor-carrying isolates, evidence existed that the *igaA2*::KIXX mutation was viable in the genetic background of the latter. In such cases, the frequency of transductants was $\sim 10^{-6}$/PFU. Otherwise, transduction occurred at frequencies $\sim 10^{-9}$/PFU; these rare transductants were presumed to carry suppressor mutations of spontaneous origin and were discarded.

These experiments provided us with six independent suppressor-carrying isolates in which suppression of lethality appeared to be associated with a Tn*10d*Tc insertion. One boundary of each Tn*10d*Tc insertion was then sequenced using a Tn*10*-derived primer (WAY and KLECKNER 1984). Two of the insertions mapped in *rscC*, thereby confirming that lack of RcsC suppresses the lethality of an *igaA* null mutation. The four remaining Tn*10d*Tc insertions mapped in *yojN*, a locus located between *ompC* and *rcsB* on the Salmonella chromosome (MCCLELLAND *et al.* 2001). Mutations in *yojN* also suppressed the mucoidy associated with *igaA* mutations and the low expression of *flhDC* (data not shown). Suppression by *yojN* mutations strengthens the evidence that IgaA interacts with the RcsB-RcsC regulatory system, since YojN has been shown to participate in the phosphorelay signaling pathway RcsC → RcsB (TAKEDA *et al.* 2001).

The lethality caused by a null *igaA* allele does not involve changes in the level of the cell division protein FtsZ: Because IgaA appears to interact with the RcsB-RcsC regulatory system and the latter has been shown to regulate positively the cell division genes *ftsA* and *ftsZ* (CARBALLÈS *et al.* 1999), a conceivable explanation for the lethality associated with null *igaA* alleles might be cell division arrest, as a consequence of overproduction of these cell division proteins. In *E. coli*, the key protein that regulates the frequency and the timing of cell division is FtsZ (WARD and LUTKENHAUS 1985). On these grounds, we investigated whether a decrease in IgaA synthesis altered the level of FtsZ. For this purpose, we carried out shift-down experiments in which strain SV4578 (*igaA2*::KIXX/pNG1166) was transferred from arabinose to glucose medium. The amounts of IgaA and FtsZ in cell extracts obtained at different times after arabinose depletion were analyzed by Western blotting using anti-IgaA and anti-FtsZ polyclonal antibodies. The results were clear-cut: While a swift decrease in IgaA was observed, the level of FtsZ remained unchanged (Figure 4). These data suggest that the viability defect of *igaA* mutants is not caused by FtsZ overproduction and reveal a further difference between *igaA* and *mucM* alleles, since the latter increase transcription from the *ftsA1* promoter at the *ftsQAZ* operon (COSTA and ANTÓN 2001). Further evidence that the proposed functional interaction between IgaA and the RcsB-RcsC system does not involve changes in the level of FtsZ was provided by the following observations: (i) The levels of FtsZ remained largely unchanged in strains carrying *igaA* mutations, alone or combined with *rcsA*, *rcsB*, and *rcsC* alleles (Figure 5) and (ii) strains carrying the *igaA1* mutation do not form minicells (not shown).

DISCUSSION

Mutants of *S. enterica* carrying the *igaA1* allele show a mucoid phenotype caused by derepression of *wca* genes. The induction of *wca* gene expression in an *igaA1* mutant is suppressed by mutations in *rcsA* and *rcsC*. The *igaA1* mutation also causes lowered expression of the flagellar master operon, *flhDC*, which results in lowered motility. The latter defect is suppressed by mutations in *rcsB* or *rcsC*. Altogether, these observations support a model in which loss of IgaA causes activation of the

RcsB/C system, leading both to derepression of *wca* genes and to repression of the *flhDC* operon. However, a noteworthy observation is that, in an *igaA1* background, activation of colanic acid synthesis requires RcsA while repression of flagellar formation is RcsA independent.

The opposite effects of the *igaA* gene product on colanic acid synthesis and flagellar formation are reminiscent of the inverse relationship between capsule synthesis and flagellar biogenesis described in several bacterial species: (i) Biofilm-forming *E. coli* repress flagellar genes and induce capsular gene expression (Prigent-Combaret *et al.* 1999); (ii) production of alginate and synthesis of flagella by *Pseudomonas aeruginosa* show inverse and mutually exclusive regulation (Garrett *et al.* 1999); and (iii) absence of flagella is a signal to induce capsular polysaccharide synthesis in *Vibrio cholerae* (Watnick *et al.* 2001). One may thus hypothesize that formation of flagella and capsule synthesis could be also mutually exclusive in Salmonella and that the *igaA* gene product might coordinate them, perhaps in response to environmental signals. This coordination would be exerted via the RcsB/C regulatory system.

The idea that IgaA might be a sensor protein is speculative at this stage. However, analysis *in silico* of the predicted IgaA amino acid sequence unveils the presence of putative phosphorelay motifs similar to those found in two-component regulatory systems (data not shown). Several potential transmembrane domains (Hofmann and Stoffel 1993) are also detected. Immunodetection upon subcellular fractionation has confirmed that the IgaA protein is membrane bound (data not shown). With these features in mind, it is tempting to speculate that IgaA might be part of a novel pathway of signal transduction through the RcsB-RcsC system. A membrane protein, DjlA, that interacts with RcsB/C and participates in *wca* activation has been described (Clarke *et al.* 1997; Kelley and Georgopoulos 1997), and additional transmitters appear to exist (Chen *et al.* 2001). IgaA may thus be a tentative addition to the list of sensors converging in the Rcs system. An alternative possibility is that the functional interaction between IgaA and the RcsB/C system might be indirect. In *E. coli*, mutants affected in the *mdoH* gene (Ebel *et al.* 1997) show several defects similar to those of *igaA1* mutants of *S. enterica* (*e.g.*, mucoidy suppressed by mutations in *rcsA*, *rcsB*, or *rcsC*, and impaired motility). The *mdoH* gene product is involved in the synthesis of membrane-derived oligosaccharides (MDOs; Kennedy 1996). A model proposes that periplasmic levels of MDOs act to signal RcsC to activate capsule synthesis (Ebel *et al.* 1997). Mutations in genes for lipopolysaccharide synthesis also activate RcsB/C (Parker *et al.* 1992). In an analogous fashion, the *igaA* gene product might affect the RcsB-RcsC system through specific components of the cell envelope.

Null *igaA* mutations are lethal and can be maintained only in a merodiploid carrying a wild-type *igaA* allele, either chromosomal or plasmid borne. However, null IgaA^{-} mutants are viable in the presence of *rcsB*, *rcsC*, or *yojN* mutations, indicating again the existence of a functional relationship between IgaA and the RcsB-RcsC system. A tentative explanation is that, in the absence of IgaA, activation of the RcsB-RcsC system might lead to derepression of a gene or operon whose overexpression is lethal. An obvious candidate was the *ftsQZA* operon, which is known to be positively regulated by the RcsB/C system (Carballès *et al.* 1999; Costa and Antón 2001). However, we provide evidence that the viability defect of null *igaA* mutants does not involve FtsZ-mediated cell division arrest.

The viability of the *igaA1* allele admits a simple (albeit at this stage unproved) explanation. The mutation causes a single amino acid substitution and is recessive. Hence, the *igaA1* allele may be leaky, and residual function may permit cell survival. However, the view that only point *igaA* mutations may be tolerated is countered by observations made in *E. coli*, where a partial deletion in the *igaA* homolog, *yrfF*, appears to be viable and causes mucoidy (Meberg *et al.* 2001).

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