Contribution of the SirA regulon to biofilm formation in *Salmonella enterica* serovar Typhimurium

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Orthologues of the *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) BarA/SirA two-component system are important for biofilm formation and virulence in many γ -*Proteobacteria*. In *S. typhimurium*, SirA activates the *csrB* and *csrC* carbon storage regulatory RNAs and the virulence gene regulators *hilA* and *hilC*. The regulatory RNAs antagonize the activity of the CsrA protein, allowing translation of those same virulence genes, and inhibiting the translation of flagellar genes. In this report, it was determined that SirA and the Csr system also control the *fim* operon that encodes type 1 fimbriae. *sirA* orthologues in other bacterial species, and the *fim* operon of *S. typhimurium*, are known to play a role in biofilm formation; therefore, all members of the *S. typhimurium* *sirA* regulon were tested for *in vitro* biofilm production. A *sirA* mutant, a *csrB csrC* double mutant, and a *fimI* mutant, were all defective in biofilm formation. Conversely, inactivation of *flhDC* increased biofilm formation. Therefore, SirA activates *csrB*, *csrC* and the *fim* operon to promote biofilm formation. In turn, *csrB* and *csrC* promote the translation of the *fim* operon, while at the same time inhibiting the translation of flagella, which are inhibitory to biofilm formation.

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INTRODUCTION

There are ~2500 serovars of *Salmonella enterica* with varying host ranges and disease manifestations. *S. enterica* serovar Typhi is the cause of typhoid fever in humans, and serovar Typhimurium (*S. typhimurium*) is one of the most common serovars associated with human gastroenteritis (Zhang *et al.*, 2003). Within animal hosts, *S. typhimurium* actively invades the intestinal epithelial layer preferentially at the Peyer's patches. Bacteria that penetrate the epithelium are engulfed by macrophages. Depending on the *Salmonella* serovar and the animal species, the bacterium can survive and replicate within macrophages, and disseminate to the mesenteric lymph nodes, spleen and liver (Zhang *et al.*, 2003). Typhoidal salmonellae may survive within their asymptomatic human hosts by forming biofilms on the surfaces of gallstones, and this may explain the failure of antibiotic treatments to clear the pathogen from asymptomatic hosts (Prouty *et al.*, 2002). Non-typhoidal salmonella can also colonize gallstones under laboratory conditions (Prouty *et al.*, 2002), and in addition, they can form biofilms on HEp-2 cells that are suspended in a mini-chemostat with tissue culture medium (Boddicker *et al.*, 2002). Biofilm

formation by *gfp*-labelled salmonellae has also been observed on the surfaces of excised chicken intestinal fragments which are grafted onto a continuous-flow tubing apparatus with tissue culture medium and seeded with 10^5 c.f.u. inoculum ml⁻¹ (Boddicker *et al.*, 2002).

In addition to forming biofilms on a variety of biotic surfaces under laboratory conditions, in natural environments, *Salmonella* form biofilms on plant and abiotic surfaces, including plastics, metal and glass (Brandl, 2006; Hood & Zottola, 1997; Momba & Kaleni, 2002). *Salmonella* make up a sizeable proportion of natural mixed-species biofilms isolated from drinking-water pipes (Schmeisser *et al.*, 2003). This ability to survive and proliferate within biofilms, outside of their animal hosts, may explain the persistence of salmonellae as one of the major causes of food- and waterborne bacterial gastroenteritis (Brandl, 2006; Teplitski, 2006).

Establishment of a microbial biofilm is a complex multi-step process, which begins when planktonic bacterial cells make contact with a surface and attach to it. Once attached to a surface (and to each other), bacteria further adapt to the biofilm lifestyle by maturing and forming complex three-dimensional structures (O'Toole *et al.*, 2000; Pasmore & Costerton, 2003; Pratt & Kolter, 1998; Webb *et al.*, 2003). The biofilms are embedded within a polymer matrix of

Abbreviations: FRT, FLP recombinase target; GMSA, gel mobility shift assay; SPI, *Salmonella* pathogenicity island; TTSS, type III secretion system.

bacterial origin, although the composition of the matrix differs depending on the bacterial species (O'Toole *et al.*, 2000; Pasmore & Costerton, 2003; Pratt & Kolter, 1998; Solano *et al.*, 2002). Under certain conditions, bacteria can detach from biofilm communities and resume their planktonic lifestyle (Mireles *et al.*, 2001; Pasmore & Costerton, 2003).

Approximately 1–10 % of bacterial transcripts are differentially regulated between planktonic and sessile bacteria (Beloin & Ghigo, 2005; Schembri *et al.*, 2003). Patterns of gene expression and metabolism also vary during the different steps of biofilm formation (Beloin & Ghigo, 2005; Schembri *et al.*, 2003; Webb *et al.*, 2003). Various environmental cues (e.g. nutrient availability, host metabolites, physical and chemical properties of the colonized surfaces, temperature, oxygen tension, salt concentration and osmolarity) and self-produced signals (e.g. indole, cyclic-diguanylate, acetyl phosphate and *N*-acetylhomoserine lactones) play a role in the establishment, maturation and dispersion of the biofilms formed by Gram-negative bacteria (Hammer & Bassler, 2003; Martino *et al.*, 2003; Prouty *et al.*, 2002; Shirliff *et al.*, 2002; Tischler & Camilli, 2004; Wolfe *et al.*, 2003).

In *S. typhimurium* and closely related bacteria, structural genes required for flagellar motility and pili, and the production of colanic acid, lipopolysaccharide, enterobacterial common antigen lipid II and cellulose, all contribute to the formation of biofilms on different biotic and/or abiotic surfaces (Boddicker *et al.*, 2003; Mireles *et al.*, 2001; Pratt & Silhavy, 1995; Solano *et al.*, 2002; Zogaj *et al.*, 2001). Sensors that perceive osmolarity and envelope stress, and regulatory proteins with functions in motility, carbon storage regulation and polymer matrix synthesis contribute to the regulation of biofilm formation and dispersal (Boddicker *et al.*, 2003; Gerstel *et al.*, 2003; Jackson *et al.*, 2002; Mireles *et al.*, 2001; Romling, 2001; Solano *et al.*, 2002; Zogaj *et al.*, 2001). Orthologues of the *S. typhimurium* two-component BarA/SirA system contribute to biofilm formation in *Escherichia coli* and *Pseudomonas aeruginosa*, although the links of the BarA/SirA regulon to biofilm formation in *Salmonella* have not been investigated (Jackson *et al.*, 2002; Parkins *et al.*, 2001; Suzuki *et al.*, 2002; Teplitski & Ahmer, 2004). BarA/SirA is an important global regulator of virulence, motility and biofilm formation; therefore, better understanding of the BarA/SirA regulatory cascade may lead to the development of treatment/management practices to specifically reduce or eliminate biofilm formation.

In this study, we determined the contributions of the components of the *S. typhimurium* SirA regulon to biofilm formation. SirA (known as UvrY in *E. coli*, and GacA in *Pseudomonas* spp.) is a response regulator of the FixJ family that is phosphorylated by the tripartite histidine sensor kinase BarA (Heeb & Haas, 2001; Pernestig *et al.*, 2003; Teplitski *et al.*, 2003; Teplitski & Ahmer, 2004; Tomenius *et al.*, 2005). This transphosphorylation has been

demonstrated *in vitro* for both *E. coli* and *S. typhimurium* (Pernestig *et al.*, 2003; Teplitski *et al.*, 2003). In the absence of phosphorylation by BarA, SirA may be phosphorylated from the cellular pool of acetyl phosphate (Lawhon *et al.*, 2002; Tomenius *et al.*, 2005).

Orthologues of the BarA/SirA system contribute to the regulation of flagellar gene expression and/or motility in *Escherichia*, *Salmonella*, *Pseudomonas* and *Vibrio* spp. (Goodier & Ahmer, 2001; Teplitski *et al.*, 2003; Whistler *et al.*, 1998). *S. typhimurium sirA* causes a decrease in expression of *flhDC* via the Csr post-transcriptional regulatory system (Goodier & Ahmer, 2001; Teplitski *et al.*, 2003). The FlhD and FlhC proteins form a heterotetramer required for transcriptional activation of the genes that encode the hook-basal body complexes and the alternative sigma factor FlhA (Chilcott & Hughes, 2000; Kutsukake, 1997). Transcription of the *flhDC* promoter is regulated by Crp, OmpR, H-NS, HdfR, QseBC, FlhA and LrhA, and also by DNA supercoiling, temperature, growth phase and cell cycle (Chilcott & Hughes, 2000; Clarke & Sperandio, 2005; Kutsukake, 1997; Soutourina *et al.*, 1999). Post-transcriptionally, *flhDC* is controlled by the Csr system and ClpXP protease (Tomoyasu *et al.*, 2002; Wei *et al.*, 2001).

The Csr system consists of the RNA-binding protein CsrA and the small regulatory RNAs *csrB* and *csrC*. The transcription of *csrB* and *csrC*, but not that of *csrA*, is under the control of the BarA/SirA orthologues in *E. coli* and *S. typhimurium* (Suzuki *et al.*, 2002; Teplitski *et al.*, 2003, 2006; Weilbacher *et al.*, 2003; Fortune *et al.*, 2006). CsrA is a 61 aa protein that binds the mRNA of target genes, and either promotes or inhibits translation and/or message decay (Romeo, 1998). In *E. coli*, *csrA* promotes motility, biofilm formation and exopolysaccharide synthesis, and inhibits glycogen metabolism and gluconeogenesis (Romeo, 1998; Suzuki *et al.*, 2002). The activity of the CsrA protein is antagonized by small regulatory RNAs: a single *csrB* RNA molecule binds up to 18 CsrA molecules, and the *csrC* RNA can bind up to nine CsrA molecules (Romeo, 1998; Weilbacher *et al.*, 2003). The interactions of CsrA with the *csr* RNAs have not been demonstrated in *S. typhimurium*, but as in *E. coli*, the *S. typhimurium csrA*, *csrB* and *csrC* genes have important roles in controlling motility, carbon storage, virulence and secondary metabolism (Altier *et al.*, 2000a; Fortune *et al.*, 2006; Lawhon *et al.*, 2003).

In addition to controlling the evolutionarily conserved *csr* genes, *Salmonella* SirA also regulates horizontally acquired virulence genes. To effect the expression of virulence genes, phosphorylated SirA directly interacts with promoters of the *Salmonella* pathogenicity island (SPI)-1-encoded virulence regulators *hilA* and *hilC* (Teplitski *et al.*, 2003). HilA is a major regulator of SPI-1 that directly activates the type III secretory apparatus encoded by the *prgH* and *invF* operons (Bajaj *et al.*, 1996; Darwin & Miller, 1999, 2001; Lostroh *et al.*, 2000). Within the InvF-controlled *sic/sip* operon, the *sipBCD* genes encode secreted effectors that form a

translocase complex in the target cell membrane, and are themselves required for the translocation of other effectors into the host cell (Darwin & Miller, 1999, 2001).

In this study, we found that *barA* and *sirA* regulated the *fim* operon that encodes type I fimbriae. Type I fimbriae are known to be important for *Salmonella* biofilm formation (Boddicker *et al.*, 2002). Therefore, we tested *sirA*, *barA* and all components of the SirA regulon for their effects on biofilm formation in *Salmonella*. We determined that *sirA*, *barA*, *csrB*, *csrC* and Type I fimbriae were required for formation of biofilms on plastic surfaces. Conversely, mutations that eliminate flagella caused an increase in biofilm formation. We also found that, although SirA-P directly interacted with the *fimA* promoter, a *csrB csrC* double mutation prevented expression even in the presence of *sirA*. We propose a regulatory model in which BarA/SirA controls the regulon, both by activating the transcription of target genes, and by controlling the Csr post-transcriptional regulatory system that is itself required for the translation of those same target genes.

METHODS

Growth conditions and reporter assays. Unless otherwise stated, bacteria were grown at 37 °C in Luria–Bertani (LB) broth or on LB agar (1.2 % agar, w/v; EM Science). Colonization factor antigen (CFA) medium was prepared with 10 g NZ Amine (Sheffield Products), 1.5 g yeast extract (USB), 50 mg MgSO₄ and 5 mg MnCl₂ per litre of distilled water, pH 7.4 (Suzuki *et al.*, 2002). When necessary, media were supplemented with 200 µg ampicillin ml⁻¹, 50 µg kanamycin ml⁻¹, 20 µg tetracycline ml⁻¹, 30 µg chloramphenicol ml⁻¹, or 40 µg X-Gal ml⁻¹. EGTA (Sigma–Aldrich) was added to a final concentration of 10 mM.

Activity of the β -galactosidase fusions grown in LB broth was assayed using ONPG as a substrate (Miller, 1972). Maximal effects of SirA are observed while cells are actively chemotaxing through motility agar (Goodier & Ahmer, 2001; Teplitski *et al.*, 2003), therefore, the expression of β -galactosidase fusions was also scored qualitatively in LB motility agar (0.3 %, w/v) supplemented with X-Gal.

Expression of luciferase activity by reporter strains growing on agar plates or within motility agar was visualized by a Hamamatsu C2400-32 intensified charge-coupled device (CCD) camera attached to an Argus 20 image processor, or quantified in a Victor-2 multiwell luminometer (PerkinElmer).

Bacterial strains and plasmids. Strains and plasmids used in this study are listed in Table 1. DNA manipulations were performed using standard techniques. Enzymes were purchased from Invitrogen or New England Biolabs, unless otherwise noted. Oligonucleotides were synthesized by IDT Technologies. PCR was performed using *S. typhimurium* 14028 as a template with *Taq* DNA polymerase (New England Biolabs), unless otherwise noted. Preparation of plasmid DNA and isolation of DNA fragments from agarose gels were performed using QIAprep Spin Miniprep and QIAquick Gel Extraction kits, respectively, according to the protocols of the supplier (Qiagen). Electroporation of *S. typhimurium* was achieved using a Gene Pulser II (Bio-Rad). Prior to electroporation into the wild-type *S. typhimurium* 14028 or its derivatives, all plasmids were first propagated in the r⁻m⁺ *S. typhimurium* strain JS198 (Ellermeier *et al.*, 2002).

The type III secretion system (TTSS) genes of SPI-1 were deleted using the *sacB*-based allelic exchange method (Edwards *et al.*, 1998). Briefly, the regions flanking SPI-1 were amplified by PCR, cloned into the pCR2.1-TOPO vector (Invitrogen), and subsequently cloned adjacent to each other in plasmid pRE112 to yield pYD25. This creates a deletion of genes between, and including, *avrA* (encoding an effector secreted by a TTSS) and *invH* (encoding an invasion protein). pYD25 was mobilized from *E. coli* BW20767 into *S. typhimurium* 14028 with selection on M9 medium containing chloramphenicol. The resulting transconjugants harboured pYD25 integrated into the chromosome, resulting in the duplication of the regions upstream and downstream of TTSS1. Transconjugants were grown without chloramphenicol selection until the exponential phase, and were plated onto LB agar lacking NaCl and containing 219 mM sucrose, to select for loss of the integrated plasmid by homologous recombination. The clones obtained were screened for the proper chromosomal deletion using PCR with primers hybridizing upstream and downstream of the TTSS1 region.

Wanner mutagenesis (Datsenko & Wanner, 2000) was used to mutate the *csrB* and *csrC* genes. Briefly, primers BA1344 and BA1345 (for *csrB*), and BA1341 and BA1342 (for *csrC*), were used to amplify the kanamycin-resistance gene and the surrounding FLP recombinase target (FRT) sites from pKD4. The primers were synthesized with extensions identical to portions of the *csrB* or *csrC* DNA sequences (Table 2). The PCR products were used to electroporate *S. typhimurium* JS246 carrying pKD46, which encodes an arabinose-inducible phage lambda Red recombinase that performs homologous recombination between the PCR product and the chromosome. This resulted in the *csrB20::kan* and *csrC30::kan* mutations. These mutations were transduced into the wild-type *S. typhimurium* 14028 using phage P22HTint, resulting in TIM104 and TIM97, respectively. The kanamycin-resistance cassettes were deleted, leaving a single FRT site, using FLP-mediated recombination with pCP20, as described by Datsenko & Wanner (2000). This resulted in TIM108 and TIM110, respectively (Table 1). To construct the *csrB csrC* double mutant, the *csrC30::kan* mutation from TIM97 was transduced into TIM108, resulting in TIM111. The kanamycin-resistance gene was removed from TIM111 by FLP-mediated recombination, resulting in TIM118. The mutations were confirmed by PCR amplification and DNA sequencing of the cloned PCR products at the Ohio State University Plant–Microbe Genomics Facility.

Plasmids pMT45 (*fimA-luxCDABE*) and pMT49 (*fimI-luxCDABE*) were constructed by amplifying the predicted promoter regions with primers BA1453 and BA1454, and BA1455 and BA1456, respectively, using Pfu Polymerase (Stratagene). All PCR products were cloned into pCR-Blunt II-TOPO (Invitrogen), removed from this construct with *EcoRI*, and cloned into the *EcoRI* sites of pSB401, replacing the *luxR* fragment of this plasmid (Winson *et al.*, 1998).

Plasmids pMT37 and pMT38, carrying full-length *csrB* and *csrC*, respectively, were constructed as follows. *csrB* and *csrC* were amplified by PCR using Pfu DNA polymerase (Stratagene) and primers BA423 and BA1346, and BA1312 and BA1343, respectively. The PCR products were cloned into pCR-Blunt II-TOPO, removed with *EcoRI*, and cloned into pWSK29 digested with *EcoRI*. The constructs were confirmed by PCR and DNA sequencing.

Biofilm assays. Cultures were inoculated from glycerol stocks (stored at –80 °C) into LB medium with appropriate antibiotics. After overnight incubation at 37 °C (with shaking at 200 r.p.m.), bacterial suspensions were diluted 100-fold into CFA medium without antibiotics (unless required to maintain plasmids). One hundred microlitres of the diluted bacterial suspensions were then inoculated into the wells of polystyrene microtitre plates with lids (Corning). The plates were sealed inside a Ziploc bag with a moist paper towel, and incubated at 37 °C (or 30 °C as indicated in the text). After 24 h

Table 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or description*	Source, construction or reference
Strains		
14028	<i>S. enterica</i> serovar Typhimurium	American Type Culture Collection
AT343	14028 <i>motA</i> ::Tn10	R. Harshey, unpublished data
AT345	14028 <i>fliA</i> ::Tn10	R. Harshey, unpublished data
AT347	14028 <i>cheZ</i> ::Tn10	R. Harshey, unpublished data
AT351	14028 <i>flhD</i> ::Tn10	Teplitski <i>et al.</i> (2003)
BA715	14028 <i>rpsL</i>	Ahmer <i>et al.</i> (1999)
BA746	14028 <i>sirA3</i> ::cam	Ahmer <i>et al.</i> (1999)
BA1501	14028 <i>svr-1501</i> ::MudJ	Ahmer <i>et al.</i> (1999)
BA1526	14028 <i>sopB1526</i> ::MudJ	Ahmer <i>et al.</i> (1999)
BA1550	14028 <i>hilA1550</i> ::MudJ	Ahmer <i>et al.</i> (1999)
BA1557	14028 <i>fimI1557</i> ::MudJ	Ahmer <i>et al.</i> (1999)
BA1577	14028 <i>sipB1577</i> ::MudJ	Ahmer <i>et al.</i> (1999)
BA1726	14028 <i>sirA3</i> ::cam <i>sopB1526</i> ::MudJ	Ahmer <i>et al.</i> (1999)
BA1750	14028 <i>sirA3</i> ::cam <i>hilA1550</i> ::MudJ	Ahmer <i>et al.</i> (1999)
BA3104	14028 <i>fljB</i> ::MudJ <i>fljC</i> ::Tn10	Iniguez <i>et al.</i> (2005)
BW20767	<i>E. coli</i> <i>leu-63</i> ::IS10 <i>recA1 creC510 hsdR17 endA1 zbf-5 uidA</i> (Δ MluI)::pir ⁺ <i>thi</i> RP4-2-tet::Mu-1kan::Tn7	Metcalf <i>et al.</i> (1996)
CA513	14028 Δ barA	Altier <i>et al.</i> (2000b)
CA678	14028 <i>csrA</i> ::cam sup8	Altier <i>et al.</i> (2000a)
DH5 α	<i>E. coli</i> ϕ 80 <i>lacZ</i> Δ M15 <i>deoR endA1 gyrA96 hsdR17 recA1 relA1 supE44</i> <i>thi-1</i> Δ (<i>lacZYAargF</i>)U169	Life Technologies
JS198	LT2 <i>metE551 metA22 ilv452 trpB2 hisC527</i> (am) <i>galE496 xyl-404 rpsL120</i> <i>flaA66 hsdL6 hsdSA29 zjg8103</i> ::pir ⁺ <i>recA1</i>	Ellermeier <i>et al.</i> (2002)
JS246	14028 <i>zjg8103</i> :: <i>resI-tetRA-resI</i>	Merighi <i>et al.</i> (2005)
KK649	14028 <i>fljF</i> ::kan	K. Klose, unpublished data
MC1000	<i>E. coli</i> F ⁻ <i>araD139 (araA-leu)7697 (lacI-Y)74 galE15 galK16 relA1 rpsL150</i> <i>spoT1 e14</i> ⁻	Pruss <i>et al.</i> (2003)
MC1000 <i>flhD</i> ::kan	MC1000 <i>flhD</i> ::kan	Pruss <i>et al.</i> (2003)
RG206	14028 <i>flhD</i> ::Tn10 <i>sirA2</i> ::kan	Teplitski <i>et al.</i> (2003)
SM10 λ pir	<i>E. coli</i> <i>thi-1 thr leu tonA lacY supE recA</i> ::RP4-2-Tc::Mu λ pir	Miller & Mekalanos (1988)
TH2775	LT2 <i>flhC5213</i> ::MudJ	Karlinsey <i>et al.</i> (2000)
TIM75	14028 <i>csrC</i> ⁺ / <i>csrC-lacZY</i> integrant	Teplitski <i>et al.</i> (2006)
TIM97	14028 <i>csrC30</i> ::kan	This study
TIM104	14028 <i>csrB20</i> ::kan	This study
TIM108	14028 Δ <i>csrB20</i>	This study
TIM110	14028 Δ <i>csrC30</i>	This study
TIM118	14028 Δ <i>csrB20</i> Δ <i>csrC30</i>	This study
TIM138	14028 Δ <i>csrB20</i> <i>hilA1550</i> ::MudJ	TIM108 $\times$ P22/BA1550
TIM139	14028 Δ <i>csrC30</i> <i>hilA1550</i> ::MudJ	TIM110 $\times$ P22/BA1550
TIM140	14028 Δ <i>csrB20</i> Δ <i>csrC30</i> <i>hilA1550</i> ::MudJ	TIM118 $\times$ P22/BA1550
TIM144	14028 Δ <i>csrB20</i> Δ <i>csrC30</i> <i>flhD</i> ::Tn10	TIM118 $\times$ P22/AT351
TIM145	14028 <i>flhC5213</i> ::MudJ	14028 $\times$ P22/TH2775
TIM146	14028 <i>sirA3</i> ::cam <i>flhC5213</i> ::MudJ	BA746 $\times$ P22/TH2775
TIM147	14028 Δ <i>csrB20</i> Δ <i>csrC30</i> <i>flhC5213</i> ::MudJ	TIM118 $\times$ P22/TH2775
TIM157	14028 Δ <i>csrB20</i> <i>fimI1557</i> ::MudJ	TIM108 $\times$ P22/BA1557
TIM158	14028 Δ <i>csrC30</i> <i>fimI1557</i> ::MudJ	TIM110 $\times$ P22/BA1557
TIM159	14028 Δ <i>csrB20</i> Δ <i>csrC30</i> <i>fimI1557</i> ::MudJ	TIM118 $\times$ P22/BA1557
YD039	14028 Δ (<i>avrA-invH</i>)1	This study
YK410	<i>E. coli</i> F ⁻ <i>araD139 lacU169 strA thi pyrC46 nalA thyA his</i>	Pruss <i>et al.</i> (2003)
YK4131	<i>E. coli</i> YK410 <i>flhD</i> (A32D)	Pruss <i>et al.</i> (2003)
YK4136	<i>E. coli</i> YK410 <i>flhC</i> (N70T)	Pruss <i>et al.</i> (2003)
Plasmids		
pBA301	pWSK29 <i>sdia</i> ⁺ <i>sirA</i> ⁺ (amp ^r pSC101)	Ahmer <i>et al.</i> (1998)

Table 1. cont.

Strain or plasmid	Genotype or description*	Source, construction or reference
pBA305	pWSK29 <i>sirA</i> ⁺ (amp ^r pSC101)	Ahmer <i>et al.</i> (1999)
pBA416	pET24a <i>sirA</i> expression vector (kan ^r colE1)	Teplitski <i>et al.</i> (2003)
pFlhDC	pBAD33 <i>flhDC</i> ⁺	R. Harshey, unpublished data
pKAS32	<i>rpsL</i> ⁺ suicide vector (amp ^r oriR6K)	Skorupski & Taylor (1996)
pKD4	FRT-kan-FRT template (amp ^r oriR6K)	Datsenko & Wanner (2000)
pKD46	Lambda Red ⁺ (amp ^r pSC101 oriTS)	Datsenko & Wanner (2000)
pMT37	Full-length <i>csrB</i> (amp ^r pSC101)	This study
pMT38	Full-length <i>csrC</i> (amp ^r pSC101)	This study
pMT45	<i>fimA</i> – <i>luxCDBAE</i> fusion in pSB401	This study
pMT49	<i>fimI</i> – <i>luxCDBAE</i> fusion in pSB401	This study
pRE112	<i>sacB</i> suicide vector (cam ^r , oriR6k)	Edwards <i>et al.</i> (1998)
PRG34	<i>fliA</i> – <i>luxCDABE</i> fusion in pSB401	Goodier & Ahmer (2001)
PRG67	pQE30 <i>barA198</i> expression vector	Teplitski <i>et al.</i> (2003)
pSB401	<i>luxCDABE</i> transcriptional fusion vector (tet ^r p15a)	Winson <i>et al.</i> (1998)
pVIK112	<i>lacZYA</i> transcriptional fusion vector (kan ^r oriR6K)	Kalogeraki & Winans (1997)
pWSK29	Cloning vector (amp ^r pSC101)	Wang & Kushner (1991)
pYD25	pRE112 Δ (<i>avrA</i> – <i>invH</i>)1	This study

*amp^r, ampicillin resistance; cam^r, chloramphenicol resistance; kan^r, kanamycin resistance; tet^r, tetracycline resistance.

incubation, 25 μ l crystal violet solution (1%, w/v, in 95% ethanol) was added to the wells for 15 min. The liquid was then decanted, and all loosely adhering bacteria and dye were gently washed off three times with distilled water. The dye was then solubilized by mixing with 150 μ l 33% acetic acid (v/v), and the A_{570} was measured with a microtitre plate reader (model MR700, Dynatech Laboratories, or Victor 3 multi-mode microtitre plate reader, PerkinElmer). This was a slight modification of the protocol of Suzuki *et al.* (2002). All assays included at least eight replicates. Each strain was assayed on at least three separate occasions with the wild-type *S. typhimurium* 14028 included as a control in all experiments. Typically, flat-bottomed polystyrene microtitre plates were used; however, assays were also carried out in U-bottomed polypropylene and vinyl plates (all from Corning), as indicated in the text.

Protein purification and gel mobility shift assays (GMSAs).

His₆-tagged full-length SirA protein and truncated BarA protein [lacking the first 198 aa (BarA198)] were purified using nickel affinity chromatography, as described previously (Teplitski *et al.*, 2003). BarA198 was autophosphorylated in the presence of 1.5 mM ATP at room temperature for 15 min, and then used to transphosphorylate SirA for 15 min, as described previously (Teplitski *et al.*, 2003). Predicted promoter regions were amplified with PCR using the primers shown in Table 2, and labelled using [γ -³²P]ATP with T4 polynucleotide kinase, according to the manufacturer's instructions (Amersham Pharmacia). DNA-binding reactions using purified and phosphorylated SirA were carried out in a total volume of 20 μ l containing 5 μ l 4 $\times$ DNA-binding buffer (129 mM Tris/HCl, 90 mM potassium acetate, 24 mM MgSO₄, 81 mM ammonium acetate, 3 mM DTT, 240 mM KCl, 30% glycerol, v/v, 12% PEG 4000) with 5 μ l of a dilution series of His₆-BarA198/SirA-His₆ transphosphorylation reaction mixture, 2 μ l labelled DNA fragment (5 ng; 54 000 c.p.m.), 2 μ l poly(dI-dC) (1 μ g ml⁻¹), and 1 μ l acetylated BSA (1 μ g ml⁻¹). DNA-binding reactions were carried out at room temperature for 25 min, and then samples were subjected to native PAGE with 5% polyacrylamide gels and a buffer containing 90 mM Tris, 90 mM H₃BO₃ and 2 mM EDTA. Radioactive regions of gels were detected with a Storm PhosphorImager and quantified with ImageQuant 5.2 software.

RESULTS AND DISCUSSION

Mutations in the genes of the SirA regulon affect biofilm formation

Throughout the γ -*Proteobacteria*, BarA/SirA orthologues control genes required for motility, virulence and, in some cases, biofilm formation (Heeb & Haas, 2001; Parkins *et al.*, 2001; Teplitski & Ahmer, 2004). However, the role of *barA* and *sirA* of *S. typhimurium* in biofilm formation has not yet been determined. The SirA regulon of *S. typhimurium* has been extensively characterized, and this provides the opportunity to determine which members of the regulon contribute to the biofilm phenotype. The biofilm phenotype of mutant *S. typhimurium* strains was evaluated in polystyrene microtitre plates incubated for 24 h at 37 °C without shaking. The quantity of biofilm was determined using crystal violet staining as described in Methods. The location and pattern of biofilm formation in the wells was also recorded.

The wild-type *S. typhimurium* formed a biofilm on the bottom of the well and at the meniscus. The wild-type also colonized surfaces of the plastic above the meniscus (Fig. 1). A mutation in *sirA* decreased biofilm formation by three- to fivefold (Fig. 2). The *sirA* mutant formed only a thin ring of bacterial cells at the meniscus, and no detectable biofilm on the bottom of the well (Fig. 1). The presence of *sirA* on a low-copy-number plasmid complemented the biofilm defect, while the plasmid vector alone did not (Fig. 3). Deletion of *barA*, the gene encoding the sensor kinase for SirA, also reduced biofilm formation (Fig. 2). Similar to the *sirA* mutant, the *barA* mutant only formed a thin ring of cells at the meniscus (Fig. 1).

Table 2. Primers used in this study

Name	DNA sequence (5′–3′)	Purpose
BA421	GTAATCGTTTGCCCTAACAGGTGTG	For amplifying the region upstream of <i>csrB</i> , binds nt 1738–1762 of GenBank accession number AF076153
BA422	CCTGACGTTTCGCTTCGTTGTACGAC	For amplifying the region upstream of <i>csrB</i> , binds nt 1984–1960 of GenBank accession number AF076153
BA423	CTTGTAAGACAAGGTGAAACAGGCG	For amplifying <i>csrB</i> , binds nt 21 698–21 674 of GenBank accession number AE008835
BA1312	TGCGTCTGTTGATAATTCAGGTTAGT	For amplifying <i>csrC</i> , binds nt 890–915 of GenBank accession number AE008887
BA1314	TCCTGAGTCGTTGATCCTGTAGCGT	For amplifying the region upstream of <i>csrC</i> , binds nt 1225–1200 of GenBank accession number AE008887
BA1341	CGACATGACAGAGTCGTTGTCAGGCTCAAA-GCCAGTTAATATGTAGGCTGGAGCTGCTTCG	For disrupting <i>csrC</i> with Wanner mutagenesis (Datsenko & Wanner, 2000). The first 40 nt correspond to nt 1050–1090 of GenBank accession number AE008887. The last 19 nucleotides correspond to priming site 1 of pKD4 (Datsenko & Wanner, 2000)
BA1342	GCCAAAGGCTTTACTGCGCGGTGCAGAGGT-TGGTTTTTTCAGCATATGAATATCCTCCTTAG	For disrupting <i>csrC</i> with Wanner mutagenesis (Datsenko & Wanner, 2000). The first 40 nt correspond to nt 1542–1502 of GenBank accession number AE008887. The last 21 nucleotides correspond to priming site 2 of pKD4 (Datsenko & Wanner, 2000)
BA1343	AACTCTTCACGCGTTTTACGACGCTG	For amplifying <i>csrC</i> , binds nt 1571–1546 of GenBank accession number AE008887
BA1344	TGATAGAGGATCTCCAGCATCAGGATTTATC-GCGGGCGAATTGTAGGCTGGAGCTGCTTCG	For disrupting <i>csrB</i> with Wanner mutagenesis (Datsenko & Wanner, 2000). The first 41 nt correspond to nt 20 910–20 950 of GenBank accession number AE008835. The last 20 nt correspond to priming site 1 of pKD4 (Datsenko & Wanner, 2000)
BA1345	GATGTGCGCAGGATGCAAACGTTTCAGGATGA-ACAGGCCGTAACATATGAATATCCTCCTTAG	For disrupting <i>csrB</i> with Wanner mutagenesis (Datsenko & Wanner, 2000). The first 42 nt correspond to nt 21 403–21 444 of GenBank accession number AE008835. The last 20 nt correspond to priming site 2 of pKD4 (Datsenko & Wanner, 2000)
BA1346	CGATTTGGTCGCGCACCGTTTGCATG	For amplifying <i>csrB</i> , binds nt 20 800–20 825 of GenBank accession number AE008835
BA1453	AAACGCGACGTCTCCCCCGTCTGTT	For amplifying the region upstream of <i>fimA</i> , binds nt 4649–4673 of GenBank accession number AE008721
BA1454	TACACACACCCGGTTTCGGCATCCTGT	For amplifying the region upstream of <i>fimA</i> , binds nt 5081–5055 of GenBank accession number AE008721
BA1455	TTGAAGGCACCAATACGCTGCGTTTT	For amplifying the region upstream of <i>fimI</i> , binds nt 5545–5570 of GenBank accession number AE008721
BA1456	AATACGCGCAAATTCTGGCTTTCTGT	For amplifying the region upstream of <i>fimI</i> , binds nt 5884–5859 of GenBank accession number AE008721

In *S. typhimurium*, BarA/SirA controls horizontally acquired virulence genes located on SPI-1, SPI-4 and SPI-5 via the *hilA* gene located on SPI-1 (and to a much lesser extent, the *hilC* gene). Mutations in SPI-4 or SPI-5 did not alter biofilm formation. However, a MudJ insertion in *hilA*, a regulator of SPI-1, increased biofilm formation by 1.5–2-fold (Fig. 2). Similarly, deletion of the type III secretion genes within SPI-1, or disruption of *sipB* (part of the type III translocase complex) also increased biofilm formation (Fig. 2). These results suggest that the TTSS encoded by SPI-1 interferes

with biofilm formation, or down-regulates biofilm formation under the conditions tested.

Deletion of either *csrB* or *csrC*, which encode small regulatory RNAs with redundant functions, slightly reduced biofilm formation (Fig. 2). A double *csrB csrC* mutant was strongly reduced in its ability to form a biofilm (Fig. 2). Neither *csrB* nor *csrC* expressed on a low-copy-number plasmid increased biofilm formation in the *sirA* mutant (data not shown). Similarly, expression of *sirA* from a

Fig. 1. Patterns of biofilm formation by the mutant indicated below each well. The cultures were grown in 100 μ l CFA medium for 24 h at 37 $^{\circ}$ C. The individual polystyrene wells with adhering biofilms were stained with crystal violet, and photographed, as described in Methods.

low-copy-number plasmid in the *csrB csrC* double mutant did not restore biofilm formation (Fig. 2), although this same plasmid complemented a *sirA* mutation (Fig. 3). These results demonstrate that both *sirA* and the two regulatory

RNAs, *csrB* and *csrC*, are required for biofilm formation. One model to explain these findings is that the genes required for biofilm formation require SirA for activation and the regulatory RNAs for translation. The CsrA protein

Fig. 2. Biofilm formation by wild-type and isogenic mutants of *S. typhimurium* 14028. Biofilms were allowed to form during growth in 100 μ l CFA medium in 96-well microtitre plates at 37 $^{\circ}$ C for 24 h. After 24 h, the biofilm formation was quantified with crystal violet, as described in Methods. Eight replicates were assayed for each strain, and mean values are plotted. Error bars indicate SE. All assays were repeated at least three times.

Fig. 3. Complementation of *sirA* and *flhD* biofilm phenotypes. Biofilm formation was assayed as described in the legend of Fig. 2. The wild-type was strain 14028, the *sirA* mutant was strain BA746, and the *flhD* mutant was strain AT351. The *sirA* gene was expressed from pBA305 (pWSK29 is the vector control). The *flhDC* genes were expressed from p*FlhDC* under the control of an arabinose-inducible promoter. Arabinose was added at 0.2%, w/v, to the wells indicated. Biofilm formation was measured and presented as in Fig. 2.

may prevent translation and/or cause degradation of the genes required for biofilm formation, and the *csrB* and *csrC* RNAs are required to antagonize CsrA. Therefore, while SirA is required to activate a particular gene, SirA also needs to activate *csrB* and *csrC* to allow translation of that gene.

Based on this model, the phenotype of the *csrA* mutant is expected to be opposite to that of the *sirA* or *csrB* *csrC* mutants, i.e. a *csrA* mutant should form more biofilm (similar to the *flhDC* mutants). While *E. coli* *csrA* mutants grow well, *csrA* mutants of *S. typhimurium* grow poorly and rapidly accumulate suppressor mutations (Altier *et al.*, 2000a). Therefore, to examine the biofilm phenotype of a *csrA* mutant of *S. typhimurium*, we used a *csrA* mutant strain that contains a second-site suppressor that partially restores growth, CA678 (Altier *et al.*, 2000a). This mutant was not strongly affected in relation to total biofilm formation under the conditions tested, although the attachment pattern was different from that of the wild-type strain (Figs 1 and 2). This result is not entirely consistent with the predicted increase in biofilm formation, and the phenotype observed in *E. coli* (Weilbacher *et al.*, 2003). In an attempt to mimic the *csrA* mutation without causing growth defects or the accumulation of second-site mutations, the *csrB* or *csrC* genes were expressed from plasmids in *Salmonella*. We hypothesized that the overexpression of *csrB* or *csrC* would titrate the CsrA protein and reduce its function (therefore functionally mimicking the *csrA* mutation). However, the *S. typhimurium* strains carrying either the *csrB* or the *csrC* genes on high-copy-number plasmids grew much more

slowly than did the wild-type, in a similar manner to *csrA* mutants (data not shown). These observations are consistent with previous reports that *csrA* mutants have serious growth deficiencies in *S. typhimurium* (Altier *et al.*, 2000a; Lawhon *et al.*, 2003).

Flagellar regulators contribute to *S. typhimurium* biofilm formation

The flagellar regulon is regulated by the BarA/SirA and Csr systems. The *flhDC* regulator is at the top of the flagellar regulatory cascade. Mutations in *flhD* and *flhC* caused an increase in biofilm formation of approximately fivefold (Fig. 2). This phenotype is different from that observed with *E. coli*, where mutations in *flhDC* cause a decrease in biofilm formation (Pratt & Kolter, 1998). Expression of *flhDC* from a plasmid with an arabinose-inducible promoter complemented the *S. typhimurium* *flhD*::Tn10 mutant, reducing biofilm formation to the wild-type level (Fig. 3). Therefore, the effect observed in *S. typhimurium* was indeed due to *flhDC* and not a secondary mutation in the strain. To test the hypothesis that there are indeed differences between the organisms rather than a difference in laboratory procedures, we compared the biofilm formation of *S. typhimurium* and *E. coli* *flhD* and *flhC* mutants. In our assays, three different *E. coli* *flhD* and/or *flhC* mutants (YK4131, YK4136 and MC1000 *flhD*::*kan*) demonstrated a decrease in biofilm formation compared to that of the parent strains MC1000 or YK410, when grown in LB or CFA medium at 30 or 37 °C, consistent with earlier reports (data not shown). Therefore, the effect of *flhDC* was indeed different in *E. coli* and *S. typhimurium*. Double *S. typhimurium* mutants lacking *sirA* and *flhD*, or *sirA* and *flhC*, had the *sirA* phenotype of reduced biofilm production (Fig. 2).

The attachment patterns of the *Salmonella* *flhD* and *flhC* mutants were different from those of the wild-type (Fig. 1). Unlike the wild-type, *flhD* and *flhC* mutants did not form a ring of tightly adhering bacteria at the meniscus, and no bacterial attachment was visible above the meniscus. The *flhD* and *flhC* mutants adhered most strongly to the sides of the polystyrene wells, and to the bottom of the wells. During prolonged incubation (5 days), wild-type bacteria formed a thick, loosely adhering biofilm on the bottom of the well, while the *flhD* and *flhC* mutants formed only a thin film on the bottom of the wells, with most cells adhering to the walls (data not shown).

To further explore the basis for the *flhDC* phenotype, we tested whether the presence of flagella, motility, chemotaxis or motility-independent regulatory functions of *flhD* control biofilm formation. All non-motile strains used in this study (*csrA*, *flhD*, *flhC*, *fliF*, *fliA*, *cheZ*, *motA* and a non-flagellated *fljB* *fliC* double mutant) differed from the wild-type in their ability to form biofilms. Of these, CsrA is known to directly bind and regulate the *flhDC* mRNA in *E. coli* (Romeo, 1998; Wei *et al.*, 2001). The remaining mutations (*flhD*, *flhC*, *fliF*, *fliA*, *cheZ* and *motA*) belong to the *flhDC* regulon (Chilcott & Hughes, 2000). A mutation in

fliA, which encodes an alternative sigma factor, was similar to a mutation in *flhD* in that biofilm formation was increased (Fig. 2). A 'bald' mutant lacking *fliF* (*fliF* mutants have a motor apparatus but no flagella on the surface) also formed more biofilm than did the wild-type (Fig. 2). A *fljB fljC* double mutant, which lacks flagellin but has the flagellar secretory machinery, also demonstrated increased biofilm formation (Fig. 2). The *fliF* mutant and *fljB fljC* double mutant both attached to the wells similarly to the *flhD* and *fliA* mutants, by adhering to the sides of the wells, without forming a tight ring at the meniscus or colonizing the surfaces above the meniscus (Fig. 1). In contrast, a *motA* mutant with paralysed flagella, and a *cheZ* mutant that is flagellated but non-chemotactic, formed less biofilm than did the wild-type (Fig. 2). These mutants formed only a thin ring at the meniscus, and did not colonize the surfaces above or below the meniscus (Fig. 1). Therefore, it appears that the presence of the flagellum on the surface of the cell, functional or not, is inhibitory to biofilm formation. Loss of the flagellum causes an increase in biofilm formation. The flagellum may act as a repellent, or interfere with the ability of an adhesin to attach. Non-functional flagella on the surface of the cell (*motA*), or a lack of chemotaxis (*cheZ*), are more inhibitory to biofilm formation than are functional (wild-type) flagella.

A novel small regulatory RNA, *csrC*, contributes to the regulation of *Salmonella* motility genes

Recently, *csrC*, a novel small regulatory RNA, and a member of the Csr post-transcriptional regulatory system, has been identified and shown to regulate *Salmonella* virulence genes located within SPI-1 (Fortune *et al.*, 2006; Teplitski *et al.*, 2006). In *S. typhimurium*, the regulatory effect of *sirA* on flagellar genes is indirect and mediated through the activation of the *csrB* RNA which then antagonizes the activity of the CsrA protein (Lawhon *et al.*, 2003; Teplitski *et al.*, 2003). We hypothesized that, in the proposed regulatory model, *csrC* functions similarly to *csrB*, and contributes to biofilm formation by repressing *flhDC*. Indeed, when the mutations were combined, the *csrB csrC* double mutant phenotype of reduced biofilm formation was obtained, rather than the *flhDC* phenotype of increased biofilm formation (Fig. 2). This is consistent with the above results in which *sirA flhD* or *sirA flhC* double mutants had the *sirA* phenotype rather than the *flhDC* phenotype (Fig. 2).

To determine how *csrC* contributes to the *sirA*-dependent regulation of flagellar genes and biofilm formation, the expression of a representative flagellar fusion *fliA-luxCDABE* was tested in the wild-type, *sirA*, *csrB*, *csrC* and *csrB csrC* double mutant backgrounds. Expression of the fusion was quantified during growth of the bacteria on the surface of LB agar because *sirA* is known to have only marginal regulatory effects on flagellar genes in liquid culture (Goodier & Ahmer, 2001). As shown in Fig. 4, when grown on the surface of LB agar, the expression of the *fliA-luxCDABE* fusion was strongly increased by mutation

of *sirA*, *csrB* and/or *csrC*. This is consistent with *csrB* and *csrC* being in the same pathway as *sirA*. The *sirA* gene expressed from a low-copy-number plasmid, pBA305, could not restore function to the *csrB csrC* double mutant. Neither *csrB* nor *csrC* expressed from low-copy-number plasmids pMT37 or pMT38, respectively, could restore function to a *sirA* mutant (data not shown). These results are consistent with the proposed model in which SirA activates the same genes that are post-transcriptionally regulated by *csrB* and *csrC*.

The type I fimbrial operon is regulated by *sirA* and *csr*, and contributes to biofilm formation

Previously, random MudJ fusions (which create *lacZY* transcriptional fusions) were screened for regulation by *sirA* (Ahmer *et al.*, 1999). Some of the fusions identified were responsive to *sirA* overexpression, but not to *sirA* expressed from its natural position in the chromosome (Ahmer *et al.*, 1999). These fusions were called 'class 2' and were not studied further. We have since determined that *sirA*-dependent regulatory effects are strongest during growth in motility agar (Goodier & Ahmer, 2001), and we therefore re-evaluated the regulation of some of the class 2 fusions using this growth condition. One of the fusions, *svr-1557::MudJ*, was indeed found to be regulated by *sirA* during growth in motility agar containing the colorimetric β -galactosidase substrate X-Gal (data not shown). DNA

Fig. 4. Mutations in *sirA*, *csrB*, *csrC* or *csrB csrC* caused an increase in *fliA-luxCDABE* expression during growth on an LB agar plate at 37 °C. All strains carried the *fliA-luxCDABE* fusion plasmid pRG34. The wild-type was strain 14028, the *sirA* mutant was strain BA746, the *csrB* mutant was strain TIM108, the *csrC* mutant was strain TIM110, and the *csrB csrC* double mutant was strain TIM118. Relative luminescence is indicated by the pseudocoloured image overlaid on the black and white image of the colonies (red indicating more luminescence than blue). The difference in luminescence between the wild-type and that of the various mutants was >100-fold.

sequencing of the fusion junction revealed that the MudJ is inserted at nucleotide 1879 of accession number L19338 (strain LB5010), within the *fimI* gene. The *fimI* gene is the second gene of the *fim* operon that encodes mannose-sensitive type I fimbriae (Valenski *et al.*, 2003). The MudJ is in the proper orientation for the *lacZ* fusion to be regulated by the *fim* promoter. Therefore, we renamed *svr-1557::MudJ* to *fimI1557::MudJ*. Type I fimbriae are known to contribute to biofilm formation in *E. coli* and *S. typhimurium* (Boddicker *et al.*, 2003; Pratt & Kolter, 1998; Schembri *et al.*, 2003). Consistent with these previous reports, we found that the *fimI1557::MudJ* mutant was indeed mildly deficient in biofilm formation (Fig. 2). *Salmonella* type 1 fimbriae, encoded by the *fim* genes, are surface appendages that specifically bind mannoseylated glycoproteins; therefore, the role of these fimbriae in biofilm formation on abiotic surfaces was not entirely expected. The regulation of fimbrial genes by a *sirA* orthologue has not been previously demonstrated in any γ -proteobacterium.

To further explore the role of SirA in regulating *fim* genes, we first studied the region upstream of *fimI*, the gene disrupted by the MudJ insertion. To test whether there is a functional promoter upstream of *fimI*, the region upstream of *fimI* was cloned upstream of a promoterless *luxCDABE* cassette. The resulting construct pMT49 was not regulated by *sirA* or *csrB* *csrC* (Fig. 5). Next, we tested the promoter region of the *fimA* gene, which lies immediately upstream of *fimI*. The promoter for *fimA* and its start site have been identified (Yeh *et al.*, 2002). The expression of the *fimA-luxCDABE* fusion was indeed dependent upon *sirA* and *csrB* *csrC* (Fig. 5). In the *sirA* background, luminescence was about 50-fold lower than that in the wild-type, and in the *csrB* *csrC* background the luminescence was ~ 10 -fold lower than that in the wild-type strain. Therefore, the *sirA*- and *csrB* *csrC*-dependent expression of the *fimI1557::MudJ* fusion originated from the *fimA* promoter.

To test whether SirA directly activates the *fim* operon, we carried out GMSAs using the same promoter fragments that were used for the construction of the *lux* reporters. As shown in Fig. 6, phosphorylated SirA specifically interacted with the *fimA* but not the *fimI* region. The binding of SirA-P to the *fimA* promoter was specific because it was not affected by the presence of non-competitive DNA oligomers, but was diminished when a specific unlabelled promoter fragment was added to the reactions (Fig. 6).

Conclusions

The ability of *Salmonella* to form biofilms on a variety of biotic and abiotic surfaces contributes to the remarkable persistence of this pathogen in a variety of environments and under different environmental conditions (Brandl 2006; Prouty *et al.*, 2002; Momba & Kaleni, 2002; Hood & Zottola, 1997). To learn about genes and signals involved in biofilm formation, several laboratory protocols have been developed: they range from fairly straightforward microtitre plate assays

Fig. 5. SirA and *csrB* *csrC* regulate the *fimA* promoter. The putative promoter regions for *fimA* and *fimI* were cloned in front of a promoterless *luxCDABE* operon in pSB401, and tested for regulation by *sirA* and *csrB* *csrC* *in vivo*. The wild-type was strain 14028 (◆), the *sirA* mutant was strain BA746 (△), and the *csrB* *csrC* double mutant was strain TIM118 (◇). The *fimA-luxCDABE* fusion was pMT45, and the *fimI-luxCDABE* fusion was pMT49. Bacteria were cultured overnight with shaking at 37 °C, subcultured at 1 : 100 into LB + tetracycline, cultured at 37 °C with shaking for 1 h, and subcultured again at 1 : 50. Twenty microlitres of this culture were then stabbed into freshly poured LB motility agar + tetracycline in black microtitre plates with flat clear bottoms, and read immediately (time 0). The plates were incubated in a moist chamber (a sealed plastic bag containing a wet paper towel) at 37 °C, and luminescence readings were taken at different time points with a Victor 3 luminometer. Growth rates of the strains were roughly equal, as determined by *A*₄₅₀. Each point is the mean of triplicate cultures. Error bars represent SD. CPS, light counts per second: the unit of measurement of the luminometer.

(used in this study) to sophisticated organ grafts and tissue culture chemostats. In most cases, the goal of these *in vitro* studies is to identify biofilm-related regulatory pathways and structural mechanisms, which can be targeted either to prevent biofilm formation or to disperse existing structures.

Fig. 6. SirA alters the gel mobility of the *fimA* promoter. The regions upstream of *fimA* and *fimI* were tested for their ability to bind purified SirA in EMSA. The DNA fragments tested are indicated by the black bars above the gene map (nucleotide coordinates are given in Methods and Table 2). SirA was phosphorylated *in vitro* by BarA198 and ATP, as described in Methods. An asterisk indicates that a 30- to 50-fold excess of unlabelled promoter DNA fragment was added to the reaction as a specific competitor. All reactions contained non-specific competitor DNA and protein, as described in Methods.

In this study, we report that mutations in *sirA* or *csrB* and *csrC* regulated the expression of flagellar genes and type I fimbrial genes, which contribute to biofilm formation. The effects of single mutations in either *csrB* or *csrC* were not as severe as the effects obtained from a double mutation lacking both genes, consistent with a recent study of the effects of *csrB* and *csrC* on SPI-1 virulence gene regulation (Fortune *et al.*, 2006). Phenotypes of a double *csrB csrC* mutant approximated the phenotypes of a *sirA* mutant in all cases, consistent with the effect of the Csr RNAs on virulence gene expression (Fortune *et al.*, 2006). However, this may seem unexpected, as SirA can directly bind the *hilA*, *hilC* and *fimA* promoters, so SirA would be expected to activate these genes independently of *csrB* and *csrC*. Given that both *sirA* and the *csrB/C* genes are required, we hypothesize that *hilA*, *hilC* and *fim* gene expression requires SirA for transcription initiation, and requires the *csrB* and *csrC* RNAs for stabilization and/or translation of the messages (via inhibition of CsrA activity). This is consistent with a model in which SirA/BarA regulates the *csr* system, and also regulates at least a subset of the genes that are post-transcriptionally controlled by *csr* (Fig. 7). Furthermore, we have identified the *fim* operon as a target locus of the SirA

Fig. 7. Current model of the SirA regulon of *S. typhimurium*. SirA has regulatory effects on all genes shown in the central column of the figure (Ahmer *et al.*, 1999; Goodier & Ahmer, 2001; Teplitski *et al.*, 2003; Teplitski & Ahmer, 2004). Direct activation of *csrB*, *fimA*, *hilA* and *hilC* is supported by *in vitro* EMSA (Teplitski *et al.*, 2003; this study). In *E. coli*, direct activation of *csrB* by UvrY (SirA) is supported by S-30 in *in vitro* transcription/translation assays (Suzuki *et al.*, 2002). The *csrC* gene is regulated by SirA, but it is not yet known if this regulation is direct (Fortune *et al.*, 2006). In *E. coli*, the *csrB* and *csrC* RNAs inhibit the activity of the CsrA protein (Liu & Romeo, 1997; Weilbacher *et al.*, 2003). CsrA is an RNA-binding protein that directly promotes the expression of *flhDC* (Wei *et al.*, 2001). In *E. coli*, CsrA inhibits the expression of glycogen metabolism and other genes (Romeo, 1998), while in *Salmonella* it appears to inhibit type III secretion genes and type I fimbrial genes (Altier *et al.*, 2000a, b; Lawhon *et al.*, 2003; Teplitski *et al.*, 2003; this study).

and *csr* systems that contributes to biofilm formation. SirA and the *csr* system promote biofilm formation by increasing the expression of type I fimbriae and decreasing the expression of flagella.

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