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 10⁵ 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...
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-acylhomoserine 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, Shirtliff 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 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 FlIA (Chilcott & Hughes, 2000; Kutsukake, 1997). Transcription of the flhDC promoter is regulated by Crp, Ompr, H-NS, HdfR, QseBC, FlIA and LlrA, and also by DNA supercoiling, temperature, growth phase and cell cycle (Chilcott & Hughes, 2000; Clarke & Sperrandio, 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 glycolgen 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 sid/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 (Shelﬁeld 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 known to be important for biofilm formation in Salmonella. We found 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.

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 the 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::lucCDABE) and pMT49 (fimI::lucCDABE) were constructed by amplifying the predicted promoter regions with primers BA1453 and BA1454, and BA1455 and BA1456, respectively, using Pfu Polymerase (Strategene). All PCR products were cloned into pCR-Blunt II-TOPO (Invitrogen), removed from this construct with PstI, and ligated into the EcoRI and XhoI sites of pET28a, replacing the lacR 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 (Strategene) and primers BA423 and BA1346, and BA1312 and BA1343, respectively. The PCR products were cloned into pCR-Blunt II-TOPO (Invitrogen), removed from this construct with EcoRI, and cloned into the EcoRI sites of pSV401, replacing the lacR fragment of this plasmid (Winson et al., 1998).

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

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or description*</th>
<th>Source, construction or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14028</td>
<td>S. enterica serovar Typhimurium</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>AT343</td>
<td>14028 motA::Tn10</td>
<td>R. Harshay, unpublished data</td>
</tr>
<tr>
<td>AT345</td>
<td>14028 flhA::Tn10</td>
<td>R. Harshay, unpublished data</td>
</tr>
<tr>
<td>AT347</td>
<td>14028 chsZ::Tn10</td>
<td>R. Harshay, unpublished data</td>
</tr>
<tr>
<td>AT351</td>
<td>14028 flhD::Tn10</td>
<td>Teplitski et al. (2003)</td>
</tr>
<tr>
<td>BA715</td>
<td>14028 rpsL</td>
<td>Ahmer et al. (1999)</td>
</tr>
<tr>
<td>BA746</td>
<td>14028 sirA3::cam</td>
<td>Ahmer et al. (1999)</td>
</tr>
<tr>
<td>BA1501</td>
<td>14028 sirr-1501::MudJ</td>
<td>Ahmer et al. (1999)</td>
</tr>
<tr>
<td>BA1526</td>
<td>14028 sopB1526::MudJ</td>
<td>Ahmer et al. (1999)</td>
</tr>
<tr>
<td>BA1550</td>
<td>14028 hilA1550::MudJ</td>
<td>Ahmer et al. (1999)</td>
</tr>
<tr>
<td>BA1557</td>
<td>14028 fimI1557::MudJ</td>
<td>Ahmer et al. (1999)</td>
</tr>
<tr>
<td>BA1577</td>
<td>14028 sipB1577::MudJ</td>
<td>Ahmer et al. (1999)</td>
</tr>
<tr>
<td>BA1572</td>
<td>14028 sirA3::cam sopB1526::MudJ</td>
<td>Ahmer et al. (1999)</td>
</tr>
<tr>
<td>BA1750</td>
<td>14028 sirA3::cam hilA1550::MudJ</td>
<td>Ahmer et al. (1999)</td>
</tr>
<tr>
<td>BA3104</td>
<td>14028 flhD::MudJ flhC::Tn10</td>
<td>Iniguez et al. (2005)</td>
</tr>
<tr>
<td>BW20767</td>
<td>E. coli leu-63::IS10 recA1 creC510 hsdR17 endA1 zbf-5 uiddA(ΔM1ul)::pir+ thi RPs-2-tet::Mu-1kan::Tn7</td>
<td>Metcalf et al. (1996)</td>
</tr>
<tr>
<td>CA513</td>
<td>14028 ΔbarA</td>
<td>Altier et al. (2000b)</td>
</tr>
<tr>
<td>CA678</td>
<td>14028 csrA::cam sup8</td>
<td>Altier et al. (2000a)</td>
</tr>
<tr>
<td>DH5s</td>
<td>E. coli φ80 lacZAM15 deoR endA1 gyrA96 hsdR17 recA1 relA1 supE44 thi-1(lacZYAargF)U169</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>JS198</td>
<td>LT2 met551 met22A ilv452 trpB2 histC527(ram) galE496 xyl-404 rpsL120 flA66 hsd6 hsdSA29 zig8103::pir+ recA1</td>
<td>Ellermeier et al. (2002)</td>
</tr>
<tr>
<td>JS246</td>
<td>14028 zgl8103::res1-tetRA-res1</td>
<td>Merighi et al. (2005)</td>
</tr>
<tr>
<td>KK649</td>
<td>14028 flhF::kan</td>
<td>K. Klose, unpublished data</td>
</tr>
<tr>
<td>MC1000</td>
<td>E. coli F’ araD139 (araA-leu)7697 (lacI-1)74 galE15 galK16 relA1 rpsL150 spoT1 e14+</td>
<td>Pruss et al. (2003)</td>
</tr>
<tr>
<td>MC1000 fhdD::kan</td>
<td>MC1000 fhdD::kan</td>
<td>Pruss et al. (2003)</td>
</tr>
<tr>
<td>RG206</td>
<td>14028 flhD::Tn10 sirA2::kan</td>
<td>Teplitski et al. (2003)</td>
</tr>
<tr>
<td>SM105::pir</td>
<td>E. coli thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu λpir</td>
<td>Miller &amp; Mekalanos (1988)</td>
</tr>
<tr>
<td>TH2775</td>
<td>LT2 fimC5213::MudJ</td>
<td>Karlinsky et al. (2000)</td>
</tr>
<tr>
<td>TIM75</td>
<td>14028 csrC’/csrC-lacZY integrant</td>
<td>Teplitski et al. (2006)</td>
</tr>
<tr>
<td>TIM97</td>
<td>14028 csrC30::kan</td>
<td>This study</td>
</tr>
<tr>
<td>TIM104</td>
<td>14028 csrB20::kan</td>
<td>This study</td>
</tr>
<tr>
<td>TIM108</td>
<td>14028 ΔcsrB20</td>
<td>This study</td>
</tr>
<tr>
<td>TIM110</td>
<td>14028 ΔcsrC30</td>
<td>This study</td>
</tr>
<tr>
<td>TIM118</td>
<td>14028 ΔcsrB20 ΔcsrC30</td>
<td>This study</td>
</tr>
<tr>
<td>TIM138</td>
<td>14028 ΔcsrB20 hilA1550::MudJ</td>
<td>TIM108 x P22/BA1550</td>
</tr>
<tr>
<td>TIM139</td>
<td>14028 ΔcsrC30 hilA1550::MudJ</td>
<td>TIM110 x P22/BA1550</td>
</tr>
<tr>
<td>TIM140</td>
<td>14028 ΔcsrB20 ΔcsrC30 hilA1550::MudJ</td>
<td>TIM118 x P22/BA1550</td>
</tr>
<tr>
<td>TIM144</td>
<td>14028 ΔcsrB20 ΔcsrC30 flhD::Tn10</td>
<td>TIM118 x P22/AT351</td>
</tr>
<tr>
<td>TIM145</td>
<td>14028 flhC5213::MudJ</td>
<td>14028 x P22/TH2775</td>
</tr>
<tr>
<td>TIM146</td>
<td>14028 sirA3::cam flhC5213::MudJ</td>
<td>BA746 x P22/TH2775</td>
</tr>
<tr>
<td>TIM147</td>
<td>14028 ΔcsrB20 ΔcsrC30 flhC5213::MudJ</td>
<td>TIM118 x P22/TH2775</td>
</tr>
<tr>
<td>TIM157</td>
<td>14028 ΔcsrB20 fimI1557::MudJ</td>
<td>TIM118 x P22/BA1557</td>
</tr>
<tr>
<td>TIM158</td>
<td>14028 ΔcsrC30 fimI1557::MudJ</td>
<td>TIM110 x P22/BA1557</td>
</tr>
<tr>
<td>TIM159</td>
<td>14028 ΔcsrB20 ΔcsrC30 fimI1557::MudJ</td>
<td>TIM118 x P22/BA1557</td>
</tr>
<tr>
<td>YD039</td>
<td>14028 Δ araA-invH1</td>
<td>This study</td>
</tr>
<tr>
<td>YK410</td>
<td>E. coli F’ araD139 lacU169 strA thi pyrC46 naiA thyA his</td>
<td>Pruss et al. (2003)</td>
</tr>
<tr>
<td>YK4131</td>
<td>E. coli YK410 flhD (A32D)</td>
<td>Pruss et al. (2003)</td>
</tr>
<tr>
<td>YK4136</td>
<td>E. coli YK410 flhC (N70T)</td>
<td>Pruss et al. (2003)</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBA301</td>
<td>pWSK29 sdiA+ sirA+ (amp’ pSC101)</td>
<td>Ahmer et al. (1998)</td>
</tr>
</tbody>
</table>
Table 1. cont.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or description*</th>
<th>Source, construction or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBA305</td>
<td>pWSK29 sirA&lt;sup&gt;a&lt;/sup&gt;  (amp&lt;sup&gt;b&lt;/sup&gt; pSC101)</td>
<td>Ahmer et al. (1999)</td>
</tr>
<tr>
<td>pBA416</td>
<td>pET24a sirA expression vector (kan&lt;sup&gt;c&lt;/sup&gt; colE1)</td>
<td>Teplitski et al. (2003)</td>
</tr>
<tr>
<td>pFlhDC</td>
<td>pBAD33 flhDC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>R. Harshey, unpublished data</td>
</tr>
<tr>
<td>pKA32</td>
<td>rpsL&lt;sup&gt;a&lt;/sup&gt; suicide vector (amp&lt;sup&gt;b&lt;/sup&gt; oriR6K)</td>
<td>Skorupski &amp; Taylor (1996)</td>
</tr>
<tr>
<td>pKD4</td>
<td>FRT-kan-FRT template (amp&lt;sup&gt;b&lt;/sup&gt; oriR6K)</td>
<td>Datsenko &amp; Wanner (2000)</td>
</tr>
<tr>
<td>pKD46</td>
<td>Lambda Red&lt;sup&gt;b&lt;/sup&gt;  (amp&lt;sup&gt;b&lt;/sup&gt; pSC101 oriTS)</td>
<td>Datsenko &amp; Wanner (2000)</td>
</tr>
<tr>
<td>pMT37</td>
<td>Full-length csrB (amp&lt;sup&gt;b&lt;/sup&gt; pSC101)</td>
<td>This study</td>
</tr>
<tr>
<td>pMT38</td>
<td>Full-length csrC (amp&lt;sup&gt;b&lt;/sup&gt; pSC101)</td>
<td>This study</td>
</tr>
<tr>
<td>pMT45</td>
<td>fimA-luxCDABE fusion in pSB401</td>
<td>This study</td>
</tr>
<tr>
<td>pMT49</td>
<td>fimC-luxCDABE fusion in pSB401</td>
<td>This study</td>
</tr>
<tr>
<td>pRE112</td>
<td>sacB suicide vector (cam&lt;sup&gt;c&lt;/sup&gt;, oriR6K)</td>
<td>Edwards et al. (1998)</td>
</tr>
<tr>
<td>pRG34</td>
<td>flia-luxCDABE fusion in pSB401</td>
<td>Goodier &amp; Ahmer (2001)</td>
</tr>
<tr>
<td>pRG67</td>
<td>pQE30 barA198 expression vector</td>
<td>Teplitski et al. (2003)</td>
</tr>
<tr>
<td>pSB401</td>
<td>luxCDABE transcriptional fusion vector (tet&lt;sup&gt;c&lt;/sup&gt; p15a)</td>
<td>Winson et al. (1998)</td>
</tr>
<tr>
<td>pVik112</td>
<td>lacZYA transcriptional fusion vector (kan&lt;sup&gt;c&lt;/sup&gt; oriR6K)</td>
<td>Kalogeraki &amp; Winans (1997)</td>
</tr>
<tr>
<td>pYD25</td>
<td>pRE112 Δ(avaR-invH)</td>
<td>This study</td>
</tr>
</tbody>
</table>

*a<sup>b</sup>, ampicillin resistance; cam<sup>c</sup>, chloramphenicol resistance; kan<sup>c</sup>, kanamycin resistance; tet<sup>c</sup>, tetracycline resistance.

incubation, 25 µl 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 µl 33 % acetic acid (v/v), and the A<sub>570</sub> 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 <i>S. typhimurium</i> 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). His<sub>6</sub>-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 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<sup>32</sup>P]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 µl containing 5 µl 4 x DNA-binding buffer (129 mM Tris/HC1, 90 mM potassium acetate, 24 mM MgSO<sub>4</sub>, 81 mM ammonium acetate, 3 mM DTT, 240 mM KCl, 30 % glycerol, v/v, 12 % PEG 4000) with 5 µl of a dilution series of His<sub>6</sub>-BarA198/SirA-His<sub>6</sub> phosphorylation reaction mixture, 2 µl labelled DNA fragment (5 ng; 54 000 c.p.m.), 2 µl poly(dI-dC) (1 µg ml<sup>−1</sup>), and 1 µl acetylated BSA (1 µg ml<sup>−1</sup>). 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 H<sub>3</sub>BO<sub>3</sub> 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 <i>γ-Proteobacteria</i>, 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 <i>S. typhimurium</i> in biofilm formation has not yet been determined. The SirA regulon of <i>S. typhimurium</i> 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 <i>S. typhimurium</i> 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 <i>S. typhimurium</i> 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 <i>sirA</i> decreased biofilm formation by three- to fivefold (Fig. 2). The <i>sirA</i> 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 <i>sirA</i> on a low-copy-number plasmid complemented the biofilm defect, while the plasmid vector alone did not (Fig. 3). Deletion of <i>barA</i>, the gene encoding the sensor kinase for SirA, also reduced biofilm formation (Fig. 2). Similar to the <i>sirA</i> mutant, the <i>barA</i> mutant only formed a thin ring of cells at the meniscus (Fig. 1).
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.

Table 2. Primers used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>DNA sequence (5′–3′)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA421</td>
<td>GTAATCGTTTGCCCTAACAGGTGTG</td>
<td>For amplifying the region upstream of <em>csrB</em>, binds nt 1738–1762 of GenBank accession number AF076153</td>
</tr>
<tr>
<td>BA422</td>
<td>CCTGACGTTCCTTCGTTGTAACGAC</td>
<td>For amplifying the region upstream of <em>csrB</em>, binds nt 1984–1960 of GenBank accession number AF076153</td>
</tr>
<tr>
<td>BA423</td>
<td>CTTGAAAGAAGGTGAAACAGGCC</td>
<td>For amplifying <em>csrB</em>, binds nt 21 698–21 674 of GenBank accession number AE008835</td>
</tr>
<tr>
<td>BA1312</td>
<td>TGCCTCAGTTGATAATTCAAGTTAGT</td>
<td>For amplifying <em>csrC</em>, binds nt 890–915 of GenBank accession number AE008887</td>
</tr>
<tr>
<td>BA1314</td>
<td>TCCTGAGTCTCGTTAGCTTAGTACG</td>
<td>For amplifying the region upstream of <em>csrC</em>, binds nt 1225–1200 of GenBank accession number AE008887</td>
</tr>
<tr>
<td>BA1341</td>
<td>CGCATGACAAGTGTTGCGAGGTCTGAGAGTGGTCG</td>
<td>For disrupting <em>csrC</em> with Wanner mutagenesis (Datsenko &amp; Wanner, 2000). The first 40 nt correspond to nt 1005–1009 of GenBank accession number AE008887. The last 19 nucleotides correspond to priming site 1 of pKD4 (Datsenko &amp; Wanner, 2000)</td>
</tr>
<tr>
<td>BA1342</td>
<td>GCCAAAGCTTTAAGGGCGGCCAGGTTGTGTTTTCAGCATATGAAATATCCTCGGTAGT</td>
<td>For disrupting <em>csrC</em> with Wanner mutagenesis (Datsenko &amp; 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 &amp; Wanner, 2000)</td>
</tr>
<tr>
<td>BA1343</td>
<td>AACTCTTTACGGCTTAGCAGGT</td>
<td>For amplifying <em>csrC</em>, binds nt 1571–1546 of GenBank accession number AE008887</td>
</tr>
<tr>
<td>BA1344</td>
<td>TGATAGAGGATCTCCAGCATCAGGTTATCCCTCG</td>
<td>For disrupting <em>csrB</em> with Wanner mutagenesis (Datsenko &amp; Wanner, 2000). The first 41 nt correspond to nt 20910–20950 of GenBank accession number AE008835. The last 20 nt correspond to priming site 1 of pKD4 (Datsenko &amp; Wanner, 2000)</td>
</tr>
<tr>
<td>BA1345</td>
<td>GATGTGGCTAGGATGCAAACGTTCAGAGGCAATATGAAATATCCTCGGTAGT</td>
<td>For disrupting <em>csrB</em> with Wanner mutagenesis (Datsenko &amp; Wanner, 2000). The first 42 nt correspond to nt 21 403–21 434 of GenBank accession number AE008835. The last 20 nt correspond to priming site 2 of pKD4 (Datsenko &amp; Wanner, 2000)</td>
</tr>
<tr>
<td>BA1346</td>
<td>CGATTGGTCCGGCCAAGGT</td>
<td>For amplifying <em>csrB</em>, binds nt 20 800–20 825 of GenBank accession number AE008835</td>
</tr>
<tr>
<td>BA1453</td>
<td>AAACCGACGTCTCCCTCCCTGTT</td>
<td>For amplifying the region upstream of <em>fimA</em>, binds nt 4649–4673 of GenBank accession number AE008721</td>
</tr>
<tr>
<td>BA1454</td>
<td>TACACACACCCGGTTCCGCACTCGTG</td>
<td>For amplifying the region upstream of <em>fimA</em>, binds nt 5081–5055 of GenBank accession number AE008721</td>
</tr>
<tr>
<td>BA1455</td>
<td>TTGAGGCACCAATAGCTGGGTTTTT</td>
<td>For amplifying the region upstream of <em>fimI</em>, binds nt 5545–5570 of GenBank accession number AE008721</td>
</tr>
<tr>
<td>BA1456</td>
<td>AATACGCCAAAATCTGGCTTCTTG</td>
<td>For amplifying the region upstream of <em>fimI</em>, binds nt 5884–5859 of GenBank accession number AE008721</td>
</tr>
</tbody>
</table>
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. 1. Patterns of biofilm formation by the mutant indicated below each well. The cultures were grown in 100 μl CFA medium for 24 h at 37 °C. The individual polystyrene wells with adhering biofilms were stained with crystal violet, and photographed, as described in Methods.

Fig. 2. Biofilm formation by wild-type and isogenic mutants of S. typhimurium 14028. Biofilms were allowed to form during growth in 100 μl CFA medium in 96-well microtitre plates at 37 °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.
accumulation of second-site mutations, the

csrB
genes were expressed from plasmids in

S. typhimurium
strains carrying either the

csrA
functionally mimicking the

hypothesized that the overexpression of

csr genes on high-copy-number plasmids grew much more
grow well,
csrA
flhDC
(similar to the

et al.
rapidly accumulate suppressor mutations (Altier

csrA
in

E. coli
increase in biofilm formation, and the phenotype observed
different from that of the wild-type strain (Figs 1 and 2).

With the conditions tested, although the attachment pattern was
strongly affected in relation to total biofilm formation under

growth, CA678 (Altier

et al.
that contains a second-site suppressor that partially restores
expression of the

sirA
Based on this model, the phenotype of the

csrB
csrC
and

csrB csrC
mutants). While

S. typhimurium
mutant should form more biofilm

mutants were different from those of the wild-type (Fig. 1).
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

fliB fliD
c 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 regulen (Chilcott & Hughes, 2000). A mutation in

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
(Weibacher

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 titer the CsrA protein and reduce its function (therefore
functionally mimicking the

csrA mutation). However, the

S. typhimurium
strains carrying either the

csrB or

csrC genes on high-copy-number plasmids grew much more
flfA, 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 flfB (flfB mutants have a motor apparatus but no flagella on the surface) also formed more biofilm than did the wild-type (Fig. 2). A fljB flfC double mutant, which lacks flagellin but has the flagellar secretory machinery, also demonstrated increased biofilm formation (Fig. 2). The flfB mutant and flfB flfC double mutant both attached to the wells similarly to the flhD and flfA 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 flaA–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 flaA–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 flaA–luxCDABE expression during growth on an LB agar plate at 37°C. All strains carried the flaA–luxCDABE fusion plasmid pRG94. 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 fiml gene. The fiml 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 fiml1557::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 fiml1557::MudJ mutant was indeed mildly deficient in biofilm formation (Fig. 2). Salmonella type I fimbriae, encoded by the fim genes, are surface appendages that specifically bind mannosylated 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 fiml, the gene disrupted by the MudJ insertion. To test whether there is a functional promoter upstream of fiml, the region upstream of fiml 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 fiml. 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 fiml1557::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 fiml 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 (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.
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 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.

ACKNOWLEDGEMENTS

We are grateful to Craig Altier (North Carolina State University), Jim Slauh (University of Illinois), Kelly Hughes (University of Utah), Karl Klose (University of Texas Health Science Center), Birgit Prues (North Dakota State University), Adam Toguchi (University of Texas at Austin) and Rasika Harshey (University of Texas at Austin) for sharing strains and plasmids; and to Yakhya Dieye (Ohio State University) for constructing the SPI-1 deletion mutant. This publication was supported by Public Health Service Grant 5 R01 AI050002-04 from the National Institute for Allergy and Infectious Diseases (B. M. M. A.) and by the Institute of Food and Agricultural Sciences at the University of Florida (M. T.). Support for A. A.-A. is provided, in part, by the National Institute for Allergy and Infectious Diseases (B. M. M. A.) and by the Institute of Food and Agricultural Sciences at the University of Florida Scholars Program.

REFERENCES


