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Type III secretion chaperone-dependent regulation: activation of virulence genes by SicA and InvF in *Salmonella typhimurium*

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Invasion of the intestinal epithelium by *Salmonella* sp. requires a type III secretion system (TTSS) common in many bacterial pathogens. TTSS translocate effector proteins from bacteria into eukaryotic cells. These effectors manipulate cellular functions in order to benefit the pathogen. In the human and animal pathogen *Salmonella typhimurium*, the expression of genes encoding the secreted effector molecules Sip/Ssp ABCD, SigD, SptP and SopE requires both the AraC/XylS-like regulator InvF and the secretion chaperone SicA. In this work, an InvF binding site was identified in the promoter regions of three operons. SicA does not appear to affect InvF stability nor to bind DNA directly. However, SicA could be co-purified with InvF, suggesting that InvF and SicA interact with each other to activate transcription from the effector gene promoters. This is the first demonstration of a contact between a protein cofactor and an AraC/XylS family transcriptional regulator and, moreover, is the first direct evidence of an interaction between a transcriptional regulator and a TTSS chaperone. The regulation of effector genes described here for InvF and SicA may represent a new paradigm for regulation of virulence in a wide variety of pathogens.
Keywords: AraC/binding site/chaperone/regulation

Introduction

Salmonella species are Gram-negative, motile bacteria that cause diseases in humans ranging from a mild gastroenteritis (*S.typhimurium*, *S.enteritidis*) to a systemic disease that can result in death (*S.typhi*, *S.paratyphi*) (for a review see Darwin and Miller, 1999b). In a mouse model of oral infection, *S.typhimurium* can penetrate the intestinal epithelium and reach the deeper tissues of the liver and spleen. The invasion of the intestinal epithelium is an important initial step in pathogenesis by *Salmonella* as well as several other pathogenic Enterobacteriaceae. Much research has involved the use of *in vitro* tissue culture systems in order to identify genes involved in invasion of non-phagocytic cells. So far, the major contributing factors by which *Salmonella* spp. invade epithelial cells are encoded within a pathogenicity island known as *Salmonella* pathogenicity island 1 (SPI1) (Mills *et al*., 1995). The genes within this pathogenicity island are not found in *Escherichia coli* K-12 strains and appear to be specific for invasive *Salmonella* species. SPI1 encodes at least 30 proteins necessary for the production of a type III secretion system (TTSS), which secretes protein effectors out of the bacterium and translocates them into host cells (reviewed in Hueck, 1998). The effector molecules stimulate morphological changes of the eukaryotic cells, resulting in engulfment of the bacteria. Mutations in the TTSS genes result in a significant reduction of bacterial invasion *in vitro* (Galán and Curtiss, 1989; Galán *et al*., 1992; Stone *et al*., 1992; Behlau and Miller, 1993; Groisman and Ochman, 1993; Jones and Falkow, 1994; Kaniga *et al*., 1994) and a 100-fold attenuation in mice that have been infected by an oral route (Penheiter *et al*., 1997). In addition to invasion, the SPI1 TTSS appears to play a role in inducing apoptosis of macrophages (Hersh *et al*., 1999) and stimulating the transmigration of poly-morphonuclear leukocytes (PMN) both *in vitro* and *in vivo* (McCormick *et al*., 1995; Galyov *et al*., 1997; Lee *et al*., 2000). Similar secretion systems are found in other pathogenic bacteria, such as species of *Bordetella*, *Pseudomonas*, *Shigella*, *Yersinia* and pathogenic *E.coli*, and are also required for virulence (reviewed in Hueck, 1998).

A central regulator of SPI1 expression is HilA (Bajaj *et al*., 1995). Currently, it is known that many environmental signals affect the expression of the SPI1 genes via HilA (Bajaj *et al*., 1996). HilA binds to a consensus sequence (HilA box) to activate the expression of the regulatory gene *invF*, the first gene of the large, putative SPI1 *inv-spa-sic-sip/ssp* operon (Lostroh *et al*., 2000). Genes encoding components of the TTSS are included within this and another HilA-dependent operon, *prgHIJKorgAB* (Behlau and Miller, 1993; Jones and Falkow, 1994). InvF, a member of the AraC/XylS family of transcriptional activators (for a review see Gallegos *et al*., 1997), and SicA (*S*almonella invasion chaperone), a type III secretion chaperone specific for Sip/SspB and Sip/SspC (*S*almonella invasion protein/*S*almonella secreted protein) (Tucker and Galán, 2000), are required for the expression of several genes encoding proteins secreted by the TTSS and their cognate chaperones (Darwin and Miller, 1999a, 2000; Eichelberg and Galán, 1999). These include *sigDE* (*S*almonella invasion gene), *sopE* (*S*almonella outer protein), *sicAsip/sspBCDA* and *sicPsptP* (*S*almonella tyrosine phosphatase). Genetic studies have suggested that SicA acts as a cofactor or inducer for InvF-dependent transcription activation of the *sicA* and *sigD* promoters (Darwin and Miller, 2000). However, it was not known whether or how SicA interacted with InvF, RNA polymerase, DNA or RNA. Prior to the suggestion that SicA may serve as a cofactor

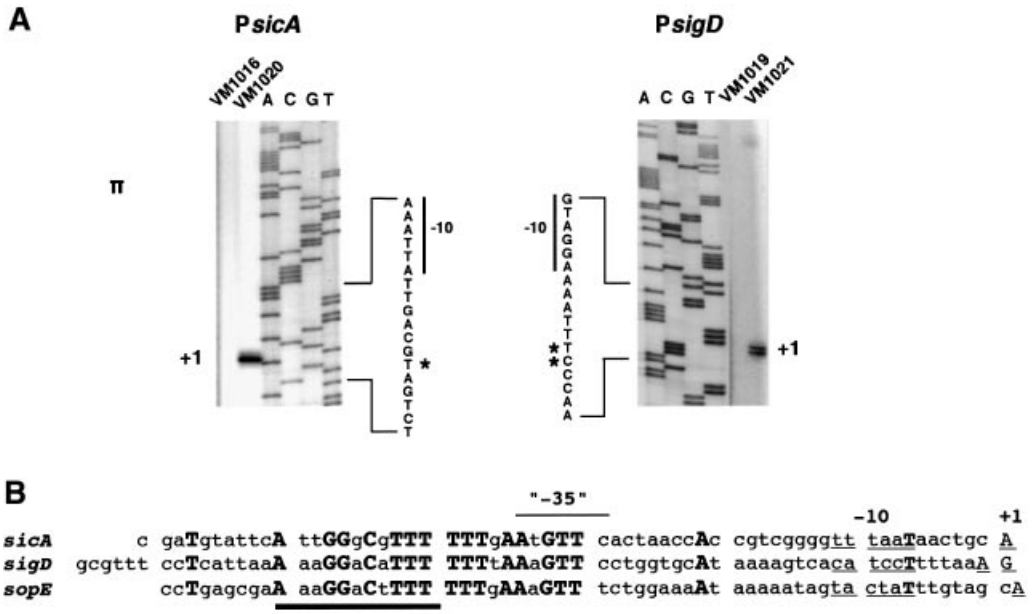


Fig. 1. Primer extension analysis of the *sicA*, *sigD* and *sopE* promoters. (**A**) Primer extension products from the *sicA* and *sigD* promoters. The sequences indicated are of the antisense (bottom) strand. The asterisk indicates the start of transcription sites. See Table V for strain and plasmid descriptions. Shown are results from expression in *E.coli*; similar results were obtained in *Salmonella*. (**B**) Sequence alignment of the *sicA*, *sigD* and *sopE* promoters. Conserved sequences are indicated in bold and the starts of transcription sites (+1) are capitalized and underlined. The –10 hexamers are underlined. The minimal InvF binding domain (based on genetic analyses) is indicated by the heavy line.

Table I. Oligonucleotide pairs used for construction of *lacZYA* reporter fusions in pRW50

5′ primer	3′ primer	Size of fragment	Name of pRW50 derivative
*spaS-Eco*RI-3	*sicA-Bam*HI-1	404	pHD11
*sicA-Eco*RI-12	*sicA-Bam*HI-1	196	pHD83
*sicA-Eco*RI-13	*sicA-Bam*HI-1	173	pHD84
*sicA-Eco*RI-mut1	*sicA-Bam*HI-1	196	pHD98
*sicA-Eco*RI-mut2	*sicA-Bam*HI-1	196	pHD96, pHD97
*sicA-Eco*RI-mut3	*sicA-Bam*HI-1	196	pHD99
*sicA-Eco*RI-12	*sicA*15	65	pHD100
M13reverse primer	*sigP*2	441	pHD86
*sigD-Eco*RI-3	*sigP*2	157	pHD87
*sigD-Eco*RI-5	*sigP*2	129	pHD88
*sopE-Eco*RI-4	*sopE-Bam*HI-4	95	pHD95
*iacP-Eco*RI-1	*sicP-Bam*HI-3	~300	pHD50

for InvF, a protein cofactor or co-activator had not been described for an AraC/XylS-family regulator. In fact, many AraC/XylS-like regulators have been assumed to act alone or to interact with small molecules such as sugars (Gallegos *et al*., 1997).

Type III chaperones typically interact with only one or two cognate effector molecules (Wattiau *et al*., 1996). Several of these chaperones have been suggested to participate in negative feedback regulation of virulence genes in *Yersinia* species (Bergman *et al*., 1991). However, no type III secretion chaperone has been directly associated with transcription regulation. In this work, promoter sequences that require both InvF and SicA for transcription activation were identified. In addition, evidence for a direct interaction between InvF and SicA is presented.

Results

Identification of an InvF/SicA-dependent consensus sequence for the sicA, sigD and sopE promoters

In order to identify an InvF/SicA consensus binding site, the starts of transcription (+1) sites of the *sicA* and *sigD* promoters were mapped by primer extension (see Materials and methods). RNA was purified from wild-type (wt) and Δ*invF Salmonella* strains and *E.coli* containing either a *sicA*- or *sigD-lacZYA* fusion in plasmid pRW50 (1–2 copies per cell) (Figure 1A) (see Table I for primer pairs used to make each pRW50 derivative and Table II for primer sequences). The *sicA-11* primer was used in primer extension analysis of *sicA* and the *sigP2* primer was used in primer extension analysis of *sigD*. *sicA*

Table II. Oligonucleotide sequences used for construction of reporter fusions and primer extension reactions

Primer name	Sequence (5′ to 3′)
M13 Universal[a]	AGCGGATAACAATTTCACACAGGA
*spaS-Eco*RI-3	GGAATTCCGGGTTAAGCAGTTGGTTTTACG AGCGAGGGGCG
*sicA-Eco*RI-12	GGAATTCCGATGTATTCATTGGGCG
*sicA-Eco*RI-13	GGAATTCCTGAATGTTCACTAACCACCG
*sicA-Eco*RI-mut1[b]	GGAATTCCGATGTATTCBTTGGGCG
*sicA-Eco*RI-mut2[c]	GGAATTCCGATGTATTCATTHGGCG
*sicA-Eco*RI-mut3[d]	GGAATTCCGATGTATTCATTGGGDG
*sicA-Bam*HI-1	CGGGATCCCGGCGTGGCGCCTTCACTAACG GCATCC
*sicA*11	ACATTATTTTGATAATCCATTACTTACTTCC TGTTAT
*sicA*15	GCAGTTATTAAACCCCGACGG
*sigD-Eco*RI-3	GGAATTCCGCGTTTCCTCATTAAAAAGGAC
*sigD-Eco*RI-4	GGAATTCCTTAAAGTTCCTGGTGCATAAAAG
*sigP*2	GTGAAGCTGAGTGATAGAAGCTCTGTATTT GC
*sopE-Eco*RI-4	GGAATTCCTGAGCGAAAAGGACTTTTTTTG AA
*sopE-Bam*HI-4	CGGGATCCCGATGAATTAGAAAAATTCGGC TGATTC
*iacP-Eco*RI-1	GGAATTCCCCTTTGCGGATATATGCCTGTTG
*sicP-Bam*HI-3	CGGGATCCCGCGTACCTTCATTATTCGCAGCC
*sptP-Xba*I	GCTCTAGACTGCAGGAATATGCTAAAGTATG
sptP-2	GTCCCTTTAGCGCGATATCGAG

[a]New England Biolabs 24mer reverse sequencing primer (–48) #1233.
[b]B indicates that either a C, G or T nucleotide was incorporated at this position.
[c]H indicates that either an A, C or T nucleotide was incorporated at this position.
[d]D indicates that either an A, C or T nucleotide was incorporated at this position.

and *sigD* transcripts were detectable from wt *Salmonella*, but no transcript was detectable from the Δ*invF* mutant (data not shown). *Escherichia coli* that contained plasmids encoding *invF* and *sicA* produced abundant amounts of the *sicA* and *sigD* transcripts (Figure 1A, strains VM1020 and VM1021, respectively). The starts of transcription were the same in *E.coli* and *Salmonella* (the signal was much weaker in *Salmonella* and, therefore, is not shown in Figure 1A for clarity). The *sigD* promoter appeared to have two possible +1 sites (A or G), while the *sicA* promoter had a single distinct +1 site (A). The start of transcription for *sopE* was also mapped (A) from RNA isolated from wt *Salmonella* (L.Schechter and C.Lee, personal communication) (Figure 1B and data not shown).

All of the promoters have potential σ70-dependent –10 hexamers ranging from 6 to 8 bp upstream of the +1 site. To identify a putative consensus activator binding site in the *sicA*, *sigD* and *sopE* promoters, the DNA sequences beginning with the –10 hexamers were aligned. A conserved region was identified in all three promoters, each with identical spacing from the +1 (Figure 1B). None of the promoters had a conspicuous –35 hexamer located 17 bp upstream of the –10 region. However, this was not surprising because many activator-dependent promoters lack this sequence (Busby and Ebright, 1994). The –35 regions in the *sicA*, *sigD* and *sopE* promoters share conserved nucleotides, suggesting that the putative activator binding site may overlap this region.

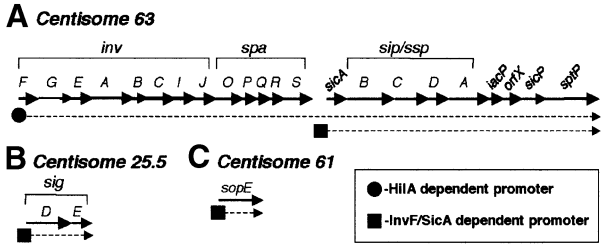


Fig. 2. Transcriptional organization of InvF/SicA regulated genes. Solid arrows indicate genes and dotted lines indicate either known or putative transcripts. The chromosomal location of each locus is indicated above. In the process of characterizing the region between *iacP* and *sicP* for an InvF/SicA-dependent promoter, we sequenced the region and found a small putative open reading frame, which we have designated *orfX*. This figure is not drawn to scale.

sptP, which encodes an effector protein secreted by the SPI1 TTSS (Fu and Galán, 1998b), was previously shown to be InvF and HilA dependent (Eichelberg and Galán, 1999), and is encoded downstream of the InvF/SicA-dependent *sicAsip/sspBCDA* operon. In the absence of InvF, *sptP* expression can be activated by HilA, suggesting that *sptP* can be expressed from a HilA-dependent promoter as well as from the InvF/SicA-dependent *sicA* promoter (Eichelberg and Galán, 1999). We constructed another *sptP-lacZYA* chromosome fusion (by insertion of pHD61) and found it to be, in addition to being InvF dependent, SicA dependent (data not shown). In contrast, a low-copy plasmid reporter, pHD50, containing sequence between *iacP* (encoded immediately downstream of *sip/sspA*) and *sicP* (the gene immediately upstream of *sptP*), failed to show *hilA*-, *invF*- or *sicA*-dependent regulation (data not shown). Moreover, a putative InvF consensus binding site was not found downstream of *sicA* or upstream of *sptP*. Taken together with previous data, it is likely that *sptP* expression is dependent on both a HilA-dependent promoter, i.e. the *invF* promoter (Bajaj *et al*., 1995; Lostroh *et al*., 2000), and the InvF/SicA-dependent *sicA* promoter, but not another promoter downstream of *sicA*. Based on these data, the HilA-dependent transcript would include the *inv-spa-sicA-sip/ssp-iacP-orfX-sicP-sptP* genes, while the InvF/SicA dependent transcript would encode *sicA-sip/ssp-iacP-orfX-sicP-sptP* genes (Figure 2A). Nevertheless, we can not rule out the existence of another promoter upstream of *sptP* that is InvF/SicA independent. In contrast to *sptP* (and *sicAsip/sspBCDA*), *sigDE* and *sopE* appear to be activated by an InvF/SicA-dependent promoter but not a HilA-dependent promoter (Figure 2B and C).

Deletion and point mutation analysis of the conserved sequences

To determine the minimal sequence required for InvF/SicA-dependent activation of the *sicA* and *sigD* promoters, nested deletions of each promoter were constructed from the pRW50 fusions (‘long promoter’ constructs, pHD11 and pHD86) used for primer extension. *Escherichia coli* CC118λ*pir* with plasmids encoding *invF* and *sicA* were transformed with each reporter plasmid. Deletion of sequences (from the 5′ end) of the long promoter constructs up to 3 or 8 bp upstream of the first conserved thymidine (T) (–58) (‘short promoter’ constructs, pHD83

and pHD87, respectively) from either the *sicA* or *sigD* promoter did not reduce β-galactosidase activity in *E.coli* when compared with the long promoter constructs (Figure 3). The slight increase in β-galactosidase activity for the *sigD* short promoter, pHD87, relative to the long *sigD* promoter construct, pHD86, was consistently observed. The significance of this observation is not clear, but raises the possibility that there is a sequence that has a negative impact on transcription upstream of the InvF/SicA activation site. Deletion of 21 or 26 bp from the 5′ end of the *sicA* or *sigD* short promoters, respectively, eliminated all but background levels of *lacZYA* expression (pHD84 and pHD88).

To assess the behavior of the promoter fusions under native conditions, wt, *invF* and *sicA S.typhimurium* strains were transformed with the reporter fusions. As in *E.coli*, the expression of the short fusions was virtually identical to that of the long fusions for the *sicA* promoter (long versus short: 132 versus 108 Miller units, respectively) and *sigD* promoter (long versus short: 42 versus 49 Miller units, respectively). The expression of the short promoter constructs was complemented by *invF* and *sicA* in the *invF* and *sicA* mutants, respectively, as previously described for the long promoters (Table III) (Darwin and Miller, 2000). The fusions that were not activated in *E.coli* (pHD84 and pHD88) also exhibited background expression in *S.typhimurium* (5 Miller units for pHD84 and 20 Miller units for pHD88 in either wt or Δ*invF* strains).

Deletion analysis began to define a region required for InvF/SicA-dependent transcription. To determine the importance of the conserved base pairs in this region, three nucleotides, conserved in all three promoters, were selected for site-directed mutagenesis of the *sicA* short promoter. Single base pair substitutions at any of the three positions (–51, –48, –45) significantly reduced activation of the *sicA-lacZYA* fusion in *E.coli* containing *invF* and *sicA* in multicopy (Figure 4). When these reporters, pHD96, pHD97, pHD98 and pHD99, were introduced into wt *S.typhimurium*, *lacZYA* expression was nearly

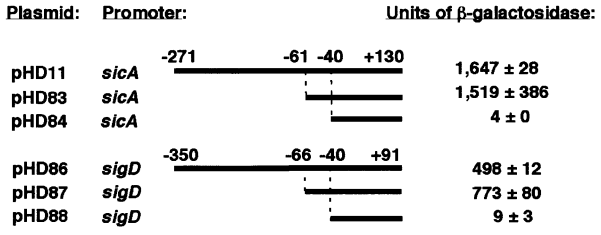


Fig. 3. Deletion analysis of the *sicA* and *sigD* promoters in *E.coli*. Strain CC118λpir containing *invF* (pHD9-1) and *sicA* (pHD30-2) was transformed with reporter plasmids containing either the wt ('long') promoters or nested deletions from the 5′ region of the long promoters. Nucleotide positions relative to +1 are indicated. Units of β-galactosidase activity (Miller units ± SD) are indicated on the right. The reporter vector pRW50 produces 0–1 Miller unit (data not shown). These values are representative of duplicate assays performed on different days.

pHD83 (P*sicA-lacZYA*)

-61 • • • -11
c gaTgtattcA ttGGgCgTTT TTTgAAtGTT cactaaccAc cgtcggggtt

Reporter (mutation):	Units of β-galactosidase: *E. coli*	*Salmonella*
pHD83 (wt)	1,974 ± 159	175 ± 13
pHD98 (-51, A to T)	51 ± 4	21 ± 2
pHD96 (-48, G to T)	166 ± 17	10 ± 0.5
pHD97 (-48, G to C)	104 ± 32	8 ± 0.5
pHD99 (-45, C to G)	52 ± 0	6 ± 0

Fig. 4. Point mutation analysis of the *sicA* promoter in *E.coli* (containing *invF* and *sicA*, on pHD9-1 and pHD30-2, respectively) and wt *S.typhimurium*. Mutations at three conserved nucleotides (indicated with bullets above) were introduced by site-directed mutagenesis. Positions and substitutions of each nucleotide are indicated next to the respective plasmid. Units of β-galactosidase (Miller units ± SD) are indicated on the right. These values are representative of duplicate assays performed on different days.

Table III. Complementation of Δ*invF* and *sicA::aphT* mutations for the expression of *lacZY(A)* fusions to P*sicA,* P*sigD* and P*sopE*

Strain background	Reporter	Units of β-galactosidase activity[a]		
		+ vector	+ *invF*[b]	+ *sicA*[b]
Wild type	Φ(*sicA-lacZYA*) (pHD83)	170 ± 17	2509 ± 56	232 ± 17
Δ*invF*	Φ(*sicA-lacZYA*) (pHD83)	4 ± 0.5	2547 ± 143	6
sicA::*aphT*	Φ(*sicA-lacZYA*) (pHD83)	5	7 ± 1	205 ± 11
Wild type	Φ(*sigD-lacZYA*) (pHD87)	40 ± 7	515 ± 40	43 ± 3
Δ*invF*	Φ(*sigD-lacZYA*) (pHD87)	16 ± 0.5	460 ± 56	15 ± 0.5
sicA::*aphT*	Φ(*sigD-lacZYA*) (pHD87)	16	15	72 ± 0.5
Wild type	Φ(*sopE-lacZYA*) (pHD95)	34 ± 3	1451 ± 28	52 ± 4
Δ*invF*	Φ(*sopE-lacZYA*) (pHD95)	9	1330 ± 68	8
sicA::*aphT*	Φ(*sopE-lacZYA*) (pHD95)	9	10 ± 0.5	53 ± 3
Wild type	Φ(*sopE-lacZY*)/*sopE*⁻ (chromosome)	742 ± 93	1983 ± 394	804 ± 71
Δ*invF*	Φ(*sopE-lacZY*)/*sopE*⁻ (chromosome)	223 ± 42	1628 ± 149	156 ± 6
sicA::*aphT*	Φ(*sopE-lacZY*)/*sopE*⁻ (chromosome)	155 ± 17	164 ± 5	669 ± 241

[a]Units of β-galactosidase (± SD) represent the average of duplicate β-galactosidase assays performed on duplicate cultures and are representative of several assays performed on different days. If the SD was zero, it was not indicated in the table. Cultures were grown without aeration in screw-capped tubes at 37°C for 18 h.

[b]For the pRW50-based reporters, *invF* and *sicA* were provided by pHD10-1 and pHD30-2, respectively, for complementation of Δ*invF* and *sicA*::*aphT*. For the *sopE-lacZY* chromosomal reporter, *invF* and *sicA* were provided by pHD17 and pHD71, respectively, for complementation of Δ*invF* and *sicA*::*aphT*.

abolished when compared with the wt *sicA* short promoter reporter pHD83 (Figure 4). Thus, these base pairs are likely to be an important part of the InvF/SicA consensus binding site.

Expression of sopE requires both InvF and SicA

sopE encodes an invasion protein and is located on a cryptic prophage outside of SPI1 (Hardt *et al*., 1998b). Previous work has shown that *sopE* is InvF regulated (Eichelberg and Galán, 1999). In addition, a mutation in *sicA* has been shown to reduce the expression of *sopE* (Tucker and Galán, 2000). In this study, a putative InvF/SicA-dependent consensus site was identified upstream of *sopE*. In the work by Tucker and Galán (2000), a disruption mutation in *sip/sspC* could suppress the *sicA* defect for *sopE-lacZY* expression. This result suggested that in the absence of SicA and Sip/SspC, InvF could activate transcription of the *sopE* promoter. Because *sicA* and *invF*, but not *invF* alone, are required to activate transcription of the *sicA* and *sigD* promoters in *E.coli* and *Salmonella*, the results of the Tucker and Galán *sopE* study suggested that the *sopE* promoter may be regulated by a different mechanism. We attempted to reproduce their work by combining the same *sicA::aphT* mutation with a complete deletion of *sip/sspC* (Scherer *et al*., 2000) and measuring transcription from both a *sopE-lacZYA* plasmid reporter pHD95 (see Tables I and II for construct description) and a *sigD-lacZYA* chromosomal reporter. In both cases, the deletion in *sip/sspC* did not suppress the *sicA*::*aphT* mutation for activation of the *sopE* (49 ± 13 versus 11 ± 0 for wt versus *sicAΔsspC* strains, respectively) or *sigD* (59 ± 6 versus 3 ± 0.5 Miller units for wt versus *sicAΔsspC* strains, respectively) reporter fusions.

Similarly to the *sicA*- and *sigD-lacZYA* reporters, either the plasmid or chromosomal *sopE* reporter in an *invF* or *sicA* mutant could be fully complemented by *invF* or *sicA*, respectively (Table III). In addition, the *sopE* plasmid reporter could be activated 100-fold in *E.coli* CC118λ*pir* containing *invF* and *sicA* (998 ± 53 versus 10.5 ± 0.5 without *sicA*), as was observed for the *sicA*- and *sigD-lacZYA* plasmid reporters (Darwin and Miller, 2000). Taken together, these results suggest that, like the *sicA* and *sigD* promoters, expression from the *sopE* promoter requires both InvF and SicA. Moreover, these results do not support the model of Tucker and Galán, which suggests that Sip/SspC is involved, directly or indirectly, in transcription of the *sopE* promoter.

InvF binds to DNA in vitro

The common feature among the AraC/XylS family members is the presence of two helix–turn–helix (HTH) motifs at the C-terminal domain of the protein (Gallegos *et al*., 1997). Although InvF has these two HTH motifs, it has not been shown to bind DNA. A filter binding assay was used to see whether InvF or SicA could bind to the *sicA* promoter (see Materials and methods). A 196 bp end-labeled *sicA* promoter fragment (the same fragment in pHD83) was incubated with InvF-His_6, SicA-His_6, or a mixture of both proteins that was partially purified by nickel–agarose affinity purification. InvF alone could bind to the *sicA* promoter in the absence of SicA (Figure 5). The binding of InvF-His_6 appeared to be specific because it did not bind significantly to the *lac* promoter (Figure 5). The

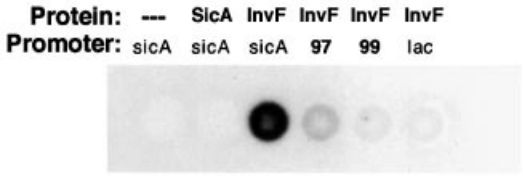


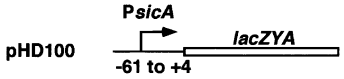
Fig. 5. InvF binds to the *sicA* promoter in a filter binding assay. A total of 15 000 c.p.m. of each ^{32}P-labeled probe were incubated with no protein, SicA-His_6 or InvF-His_6 and filtered through nitrocellulose. The membranes were exposed to film to visualize the bound DNA. The membrane-retained c.p.m. were calculated as described in Materials and methods.

presence of SicA with InvF-His_6 did not appear to increase binding of InvF to the *sicA* promoter (data not shown). In addition, SicA did not bind to DNA in this assay; however, we can not rule out the possibility that SicA plays a role in increasing the binding affinity of InvF to DNA or that SicA itself binds to DNA *in vivo*.

In addition to the wt *sicA* promoter, the mutant *sicA* promoters from pHD97 (–48, G to C) and pHD99 (–45, C to G) (called mut97 and mut99, respectively) were radioactively labeled and tested for binding to InvF-His_6. InvF-His_6 did not bind to the mutant *sicA* promoters as well as it bound to the wt *sicA* promoter (30 and 4% of wt promoter binding for mut97 and mut99 probes, respectively) (Figure 5). In fact, the binding of InvF-His_6 to the mut99 probe was comparable to that of InvF-His_6 to the control *lac* promoter probe (5% of wt promoter binding). Taken together, these results suggest that the base pairs at positions –48 and –45 of the *sicA* promoter are important for InvF binding.

SicA is not an RNA chaperone, nor does InvF require binding sites downstream of the +1 site of the sicA promoter

Recent work by Munson and Scott (2000) has shown that Rns, an AraC-like regulator in enterotoxigenic *E.coli* (ETEC), requires DNA binding sites downstream of +1 for transcription activation. All of the transcriptional fusions to *lacZYA* used in our work include the starts of transcription, untranslated and/or partial coding sequences. It was, therefore, possible that InvF required DNA binding sites downstream of the start site for transcription activation. In other studies, a mRNA signal has been shown to be used for the secretion of two type III secreted proteins (YopE and YopQ) in *Yersinia enterocolitica* (Anderson and Schneewind, 1997, 1999), suggesting that this signal interacts with a protein such as a chaperone or a component of the TTSS. Thus, it was possible that SicA recognized an RNA signal important for translation initiation or a structure that protected the mRNA from degradation. In the absence of the chaperone, *lacZYA* transcripts fused to a potential chaperone-dependent signal might not be translated or might be rapidly degraded by a 5′ exonuclease activity, making β-galactosidase activity undetectable. If the role for SicA were to regulate translation initiation or stabilize an RNA transcript via an RNA sequence, then it is unlikely that SicA is also directly involved in transcription activation. Therefore, in the absence of the RNA signal, SicA would not be needed to initiate translation or stabilize the



Plasmids in *E. coli* + pHD100:	Units of β-galactosidase:
vectors	19 ± 0
p*invF*$^+$/vector	19 ± 0
p*invF*$^+$/p*sicA*$^+$	1,511 ± 104

Fig. 6. The *sicA* promoter does not require sequences downstream of +4 for transcription activation. *Escherichia coli* CC118λ*pir* containing pHD100 along with one of the following plasmid pairs: cloning vectors pWKS130 and pHG329; pHD9-1 (*invF*$^+$) and pHG329; or pHD9-1 (*invF*$^+$)and pHD30-2 (*sicA*$^+$). The sequence relative to the *sicA* start of transcription is indicated in this construct. Units of β-galactosidase (Miller units ± SD) are indicated on the right. These values are representative of duplicate assays performed on different days.

transcript. Furthermore, if SicA were not required for transcription, InvF alone would be sufficient to produce a stable message if the hypothesized SicA–RNA interaction sequence were deleted.

To test these possibilities, a *sicA-lacZYA* fusion in pRW50 that did not include *sicA* sequence downstream of +4 relative to the start of transcription was constructed (pHD100) (Figure 6). *Escherichia coli* CC118λ*pir* containing pHG329 and pWKS130 (cloning vectors), pHG329 and pHD9-1 (*invF*$^+$), or pHD30–2 (*sicA*$^+$) and pHD9-1 (*invF*$^+$) were transformed with this reporter plasmid. *lacZYA* expression was activated only in the presence of both *invF* and *sicA* (Figure 6). This result shows, in contrast to what is observed for Rns in *E.coli*, that there is no requirement for DNA binding sites downstream of +4 for transcription activation. In addition, this result suggests that detection of β-galactosidase does not require an interaction of SicA with an RNA sequence or structure present in the message. Most importantly, these results show that both InvF and SicA are required for transcription activation from the conserved sequence found between –61 and +4 of the *sicA* promoter.

SicA does not affect the steady-state levels of InvF

Although overexpressed and purified InvF-His$_6$ was shown to bind DNA *in vitro*, it was still possible that SicA was required for the stability of InvF *in vivo*. IpgC, a homolog of SicA in *Shigella*, is a chaperone for the secreted invasion proteins IpaB and IpaC (Ménard *et al*., 1994). IpgC prevents the degradation and premature association of IpaB with IpaC in the bacterial cytoplasm. Like IpgC, SicA is a chaperone for Sip/SspB and Sip/SspC, homologs of IpaB and IpaC, respectively (Tucker and Galán, 2000). Because SicA is an intermolecular chaperone, it was possible that SicA was required for the stability or folding of InvF in addition to Sip/SspB and C. To determine whether SicA was required for the stability of InvF, polyclonal rabbit antibodies were raised against the InvF-His$_6$ fusion protein (see Materials and methods). Cell pellets of wt, *invF* and *sicA S.typhimurium* strains were analyzed by western blotting on nitrocellulose membranes using the antibodies raised against InvF-His$_6$. A *sicA* mutant produced wt levels of InvF and multicopy *sicA* did not increase the amount of InvF in the cells (Figure 7).

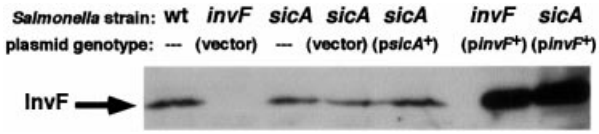


Fig. 7. SicA is not required for the stability of InvF in the cytoplasm. Immunoblot of whole cells of *S.typhimurium* wt, *invF* and *sicA* strains using rabbit polyclonal antibodies to InvF-His$_6$. Strains and plasmids in each strain are indicated above each lane. Equivalent cell numbers as determined by A_{600} were loaded in each lane.

Previous work has shown that multicopy *invF* does not suppress a *sicA* mutation for the activation of *sicA*- and *sigD-lacZYA* expression (Darwin and Miller, 2000). However, it was not known whether InvF was produced or overproduced in a *sicA* mutant containing *invF* on a multicopy plasmid. The western blot in Figure 7 shows that a *sicA* mutant containing multicopy *invF* makes abundant amounts of InvF. Therefore, it does not appear that the reason why a *sicA* mutant can not activate transcription of the *sicA*, *sigD* or *sopE* promoters is due to reduced amounts of InvF. However, this experiment does not rule out the possibility that SicA is involved in changing the conformation of InvF to a state that allows InvF to activate transcription.

InvF interacts with SicA

Genetic studies have shown that SicA is required for transcription activation in conjunction with InvF (Darwin and Miller, 2000); however, it was not known whether InvF and SicA interacted directly with each other. Several attempts to determine whether InvF and SicA interact with each other *in vivo* were made using two-hybrid systems. An interaction between InvF and SicA was not observed using either a bacterial (Karimova *et al*., 1998) (Table IV) or yeast (Chien *et al*., 1991) two-hybrid system (data not shown). In the bacterial two-hybrid system, two domains (T25 and T18) of the *Bordetella pertussis* adenylate cyclase protein are fused to proteins whose putative interaction is being tested. Although the bacterial two-hybrid system did not show an InvF–SicA (Table IV) or an InvF–InvF interaction (data not shown), it did show that SicA fusion proteins (SicA–T18 and T25–SicA) could interact with each other. This result suggests that SicA dimerizes or forms higher order oligomers. Owing to the nature of this two-hybrid system, it was possible that dimerization of SicA fusion proteins sterically impaired the ability of either SicA–T18 or T25–SicA to interact with a third (T25–InvF or InvF–T18, respectively) fusion protein. A one-hybrid system was also used to test for the presence of an InvF–InvF interaction (Hu *et al*., 1990). Neither full-length InvF nor its N-terminal domain (non-conserved) demonstrated an InvF–InvF interaction (data not shown). However, it is possible that if InvF formed weak dimers, the dissociation constant between the InvF monomers would be too high to detect the interaction.

Because an interaction between InvF and SicA was not detected using a two-hybrid system, this interaction was also tested using a biochemical approach. An *E.coli* strain, BL21(DE3), encoding an inducible T7 polymerase gene was used to express the His-tagged *invF* fusion and *sicA*. Soluble cell lysates were mixed with nickel–agarose and washed extensively (see Materials and methods). When

Table IV. Reconstitution of adenylate cyclase (Cya) activity by a SicA–SicA interaction

Test pair	Genotype of plasmids	Units of β-galactosidase activity[a]
pT18/pHD51	vector/Cya′-SicA	654 ± 20
pHD53/pT25	SicA-′Cya/vector	516 ± 35
pHD53/pHD51	SicA-′Cya/Cya′-SicA	1905 ± 197
pHD52/pHD51	InvF-′Cya/Cya′-SicA	653 ± 16
pHD53/pHD54	SicA-′Cya/Cya′-InvF	648 ± 35
pT18-zip/pT25-zip	leucine zippers (positive control)	7063 ± 138

[a]Units of β-galactosidase (± SD) represent the average of duplicate β-galactosidase assays performed on duplicate cultures and are representative of assays carried out on different days. Cultures were grown in LB supplemented with Ap, Cm, 500 μM IPTG, and aerated on a roller drum at 26°C for 18 h.

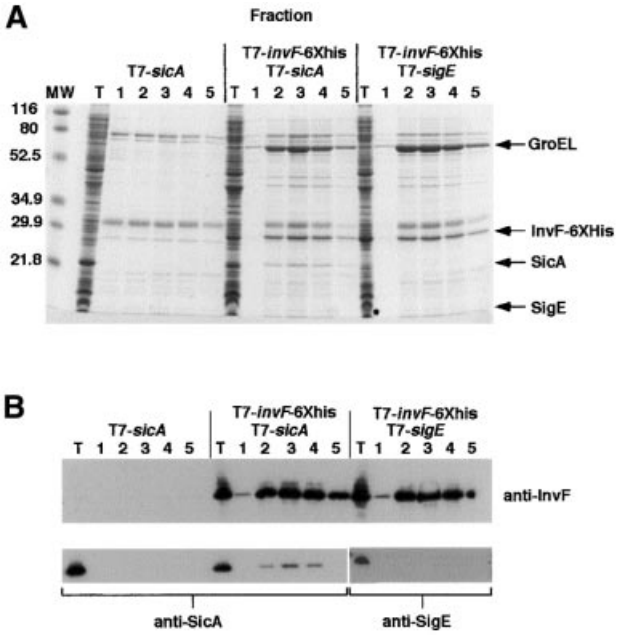


Fig. 8. SicA binds to InvF-His_6. *Escherichia coli* strains were grown and harvested as described in Materials and methods. (**A**) A 12.5% Coomassie Blue-stained gel. InvF-His_6, SicA and SigE are indicated on the right. SigE in the total protein sample (T) is noted with an asterisk. Lanes 1–5 represent consecutive fractions eluted from the nickel–agarose columns. Molecular weight (MW) standards are indicated on the left. (**B**) Immunoblot analysis of the same fractions presented in (A). Top panel: polyclonal antibodies to InvF-His_6 were used. Bottom panel: antibodies to SicA-His_6 or MBP-SigE were used as indicated in the figure.

InvF-His_6 was eluted from the nickel–agarose column, native SicA co-eluted in the same fractions. SicA in the eluted fractions was visible on a Coomassie Blue-stained gel (Figure 8A) and was confirmed to be SicA by western blotting using polyclonal antibodies to SicA (Figure 8B). Two negative controls were performed: BL21(DE3) producing native SicA and containing the His tag fusion vector pET24b(+); and BL21(DE3) making InvF-His_6 and a different *Salmonella*-specific type III secretion chaperone, SigE (Hong and Miller, 1998; Darwin *et al*., 2001). Native SicA did not bind the nickel–agarose column in the absence of InvF-His_6, nor did SigE bind to InvF-His_6 (Figure 8). In a separate study, SicA-His_6 was also shown not to bind SigD or SigE, a TTSS effector and its cognate chaperone, respectively (Darwin *et al*., 2001). These results suggest that SicA can specifically interact with InvF, even under the relatively stringent conditions of a protein purification column.

In addition to SicA, another larger protein consistently eluted from the nickel–agarose columns along with InvF-His_6 (Figure 8A). The sequence of the first 12 amino acids of this protein was determined and it was identical to that of the essential *E.coli* chaperonin GroEL (Hsp60). GroEL binds to newly synthesized polypeptides and is required for proper folding of the protein (Mayhew and Hartl, 1996). Because GroEL is required for folding many cytoplasmic proteins, it is possible that InvF, especially when overexpressed, interacts with GroEL. Because GroEL has not been suggested to bind to DNA, it is not clear whether GroEL serves a direct role in transcription regulation or if it is simply stabilizing or folding overexpressed InvF-His_6 into its proper conformation. Whether or not GroEL binds to InvF under normal conditions, i.e. when not overexpressed, this experiment suggests for the first time that the type III secretion chaperone SicA can interact directly with the transcriptional activator InvF.

Discussion

Many environmental signals are known to stimulate or repress expression of the *Salmonella* invasion regulon (for a review see Lucas and Lee, 2000). These signals are transduced by an unknown mechanism to HilA, which then directly activates *invF* (Lostroh *et al*., 2000). In this study, we examined the role of InvF and SicA in the regulation of a specific subset of invasion genes encoding secreted effector proteins and their cognate chaperones. A consensus sequence, the InvF binding site, was identified in three chromosomally unlinked invasion loci. The *sicA* promoter controls the expression of the *sicAsip/sspBCDA* and, most likely, the *sicPsptP* genes. Sip/SspB and C have been shown to be required for the translocation of several other proteins, including SigD, SopE, and SptP (Wood *et al*., 1996; Collazo and Galán, 1997; Galyov *et al*., 1997; Fu and Galán, 1998b). SipB has been assigned several functions from effector translocator to stimulator of bacterial induced apoptosis (Kaniga *et al*., 1995b; Collazo and Galán, 1996; Hersh *et al*., 1999). SipC has been shown to insert into epithelial cell plasma membranes (Scherer *et al*., 2000) and is also important for effector translocation into eukaryotic cells (Collazo and Galán, 1997). SipA has been shown to stimulate the transmigration of PMN across polarized monolayers in an *in vitro* system (Lee *et al*., 2000), suggesting a role for SipA in causing disease. SigD was identified as a protein required for the efficient invasion of *S.typhimurium* into epithelial cells *in vitro* (Hong and Miller, 1998) and has been shown to contribute to diarrhea in a calf model of infection by *Salmonella dublin* (Galyov *et al*., 1997). SopE was also found to stimulate membrane ruffling of cultured epithelial cells by *S.typhimurium* (Hardt *et al*., 1998a) and *S.dublin* (Wood *et al*., 1996) by stimulating rho GTPases. SptP appears to be an antagonist to SopE (Fu and Galán,

1999), raising the intriguing question: why are two proteins that have opposing effects on the eukaryotic cell co-regulated? It is not known how much SopE is made compared with SptP or how efficiently each is translocated into host cells; therefore, it is possible that a certain amount of SptP must accumulate before antagonizing the effects of SopE on membrane ruffling in the eukaryotic host cell.

One common denominator of the InvF/SicA-regulated operons is that they are all required for invasion. Many other proteins, including AvrA (Hardt and Galán, 1997), SopD (Jones *et al*., 1998), SopE2 (Bakshi *et al*., 2000), SlrP (Miao and Miller, 2000) and SspH1 (Miao *et al*., 1999), have been shown to be secreted by the SPI1 TTSS. The genes encoding these proteins (with the exception of *sopE2*) have not been implicated in invasion nor do any of these genes possess an InvF binding site. Therefore, it is not surprising that the expression of *avrA* (Eichelberg and Galán, 1999) and *sopD* (our unpublished results) is not InvF/SicA dependent. Reporter fusions to the other genes encoding secreted effectors that do not have the InvF binding site will have to be constructed in order to determine whether they are also InvF/SicA independent. At this point, it is clear that not all genes encoding proteins secreted by the type III system are regulated by InvF and SicA. Interestingly, *slrP* (Miao and Miller, 2000), *sspH1* (Miao *et al*., 1999), *sopD* (our unpublished results) and *avrA* (Eichelberg and Galán, 1999) are not part of the *hilA* regulon either.

InvF is a member of the growing AraC/XylS family of transcriptional regulators (Gallegos *et al*., 1997). The hallmark of this family of regulators is the presence of HTH motifs at their C-terminal domains. AraC contains an N-terminal domain that allows it to form dimers; the AraC dimer can bind to different regulatory sequences depending on its conformation (Soisson *et al*., 1997). Unlike AraC, InvF does not appear to bind multiple DNA sites. The InvF binding site lacks direct and inverted repeats, and does not require any distal binding sites to stimulate transcription. In several aspects, the InvF binding site resembles those sites that are bound by monomer AraC/XylS family members, such as SoxS or MarA (Gallegos *et al*., 1997; Rhee *et al*., 1998). SoxS and MarA binding sites generally span no more than 20 bp and are believed to be bound by monomeric proteins. Because bacterial one- and two-hybrid systems using the entire InvF protein or just the N-terminal domain (lacking the putative DNA binding domain) have not shown that InvF dimerizes, it seems likely that InvF, like SoxS or MarA, binds to DNA as a monomer. Nevertheless, these negative results do not yet rule out the possibility that InvF multimerizes.

The crystal structure of MarA bound to DNA revealed that two HTH motifs of MarA interact with two tandem major grooves on one face of a DNA molecule (Rhee *et al*., 1998). Based on this model, one could propose potential major groove interactions of the InvF binding site of the *sicA* promoter by a monomer of InvF: three conserved nucleotides (–48, –47 and –45), two of which have been shown to be important for activation by InvF, could be part of one major groove making contacts with the C-terminal HTH (relative to the other HTH in InvF). The following conserved region consists of six T nucleotides (–44 to –39), perhaps providing flexibility of the binding site. This sequence is followed by five more conserved nucleotides (–37, –36, –34, –33, –32) near or in the –35 region of these promoters. This set of conserved base pairs starts ~10 bp from the first conserved region and is partially (–37, –36,–34) included in the major groove adjacent to the first major groove. This may suggest that these base pairs interact with the N-terminal HTH motif of InvF.

Although it is possible that InvF alone can bind DNA, it is not sufficient for activation of the *sicA*, *sigD* and *sopE* promoters. In addition to InvF, the type III secretion chaperone SicA is required for transcription activation of invasion effector genes. Several type III secretion chaperones have been implicated to participate in negative feedback regulation of virulence genes in *Yersinia* sp. (Bergman *et al*., 1991). However, none has been shown to directly regulate transcription. Moreover, none of these chaperones has been suggested to, directly or indirectly, activate transcription. Previous work has provided genetic evidence that *sicA* is required for the activation of two operons encoding secreted proteins (*sicAsip*/*sspBCDA* and *sigDE*) (Darwin and Miller, 2000). In this work, a third invasion gene promoter (*sopE*) was shown to require both proteins in *S.typhimurium* and an *E.coli* K-12 strain. All three of these genetically unlinked promoters have a highly conserved sequence upstream of the start of transcription, which appears to be sufficient for transcription activation.

Our work also demonstrates that SicA interacts with InvF, the first evidence of an interaction between a member of the AraC/XylS family of regulators and a type III secretion chaperone. Unlike some chaperones, such as those involved in heat shock (Mayhew and Hartl, 1996), type III secretion chaperones are usually associated with only one or two cognate effector molecules. These chaperones have been shown to protect effector molecules from degradation as well as prevent the inappropriate association of effectors prior to secretion out of the bacterium (Ménard *et al*., 1994; Fu and Galán, 1998a; Tucker and Galán, 2000; Darwin *et al*., 2001). Interestingly, SicA is not required to protect InvF from degradation and is not required for InvF binding to DNA *in vitro*, nor does SicA appear to bind DNA directly. Therefore, it appears that SicA either changes the conformation or binding specificity of InvF, allowing it to activate transcription, or SicA is itself directly interacting with RNA polymerase in order to stimulate transcription. Further characterization of the InvF–SicA interaction will require the purification of significant amounts of active InvF. This work is ongoing but, as is the case for many AraC-like regulators, this is proving difficult.

From the results of this work, we present a model for how InvF and SicA may activate transcription (Figure 9). In the absence of SicA, InvF is predicted to bind to DNA, but can not activate transcription. Once SicA is produced, it binds to InvF, stimulating transcription, perhaps by changing the conformation of InvF and/or DNA, or interacting with RNA polymerase. It is also possible that if SicA changes the conformation of InvF, this change results in InvF interacting with σ70 or other domains of RNA polymerase (e.g. the α-C-terminal domain or region 4 of σ70) (Busby and Ebright, 1994). Another scenario may be that interactions of SicA with InvF increase the

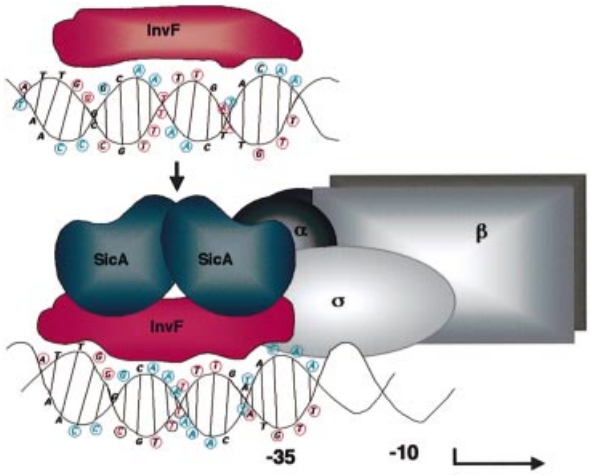


Fig. 9. Hypothetical model for activation of transcription of the *sicA*, *sigD* and *sopE* promoters. InvF binds to DNA in the absence of SicA, but can not activate transcription. Conserved base pairs are circled and colored in magenta for one strand and in cyan for the other. Based on the MarA model, it is likely that two HTH motifs of InvF (in red) interact with two consecutive major grooves on one face of the DNA molecule. Once SicA (in blue) is made, it interacts with InvF, but it is not known whether SicA binds as a dimer or monomer. The binding of SicA to InvF may create a bend in the DNA in a region where there are six conserved A–T base pairs susceptible to this conformation. This interaction results in the activation of transcription by an unknown mechanism, but may involve contacts between InvF, SicA or both proteins with subunits of RNA polymerase (in gray). This figure is not drawn to scale.

binding affinity of InvF to DNA, although we did not observe this *in vitro*.

InvF represents a unique member of the AraC/XylS family of regulators because it interacts with another protein required for transcription activation. Nearly all, if not all, of the Gram-negative bacteria that possess TTSSs have AraC/XylS homologs involved in expression of the TTSS genes. In SPI1 alone, there are three known AraC/XylS homologs with different functions (Rakeman *et al*., 1999; Schechter *et al*., 1999). Most of these regulators have not been extensively characterized; therefore, it is possible that these homologs also require type III chaperones for transcription regulation.

SicA is an interesting type III chaperone in that it interacts with two secreted proteins (Tucker and Galán, 2000) and a transcriptional activator. An intriguing possibility is that SicA contacts RNA polymerase in order to activate transcription. It is also compelling to speculate that SicA may participate in the targeting of proteins destined for type III secretion by somehow 'docking' the transcription machinery to or near the secretion apparatus. Because transcription and translation are typically coupled in prokaryotes, this would also imply that the translation machinery could be brought to the secretion apparatus. This model would suggest that, in addition to interacting with effectors of invasion and apoptosis (Sip/SspB and Sip/SspC) and a regulator (InvF), SicA might also interact with a component of the secretion machinery. It has been proposed that *Y.enterocolitica yop* (*Yersinia* outer protein) mRNA signals recognize components of the TTSS, coupling translation of *yop* genes and secretion of Yops (Anderson and Schneewind, 1997, 1999). Perhaps SicA acts by targeting the transcription and translation machinery near or at the TTSS rather than coupling translation and secretion via a mRNA signal.

It is important to note that, unlike a *Yersinia* secretion mutant, a *S.typhimurium* secretion mutant can still make effector proteins that remain within the bacterium (Kaniga *et al*., 1995a). Therefore, it appears that the SPI1 TTSS and the *Yersinia* virulence plasmid TTSS have, so far, different ways of regulating expression and translation of their effector genes. Nevertheless, it seems likely that multiple mechanisms of transcription, translation and secretion regulation occur in any single bacterial species. Future work will determine how other *S.typhimurium* effector genes that are not InvF or SicA dependent are regulated and how all secreted proteins, InvF/SicA dependent or not, are targeted for secretion.

Materials and methods

Bacterial strains and plasmids

See Table V for bacterial strains and plasmids used in this study. PCRs were carried out using the high-fidelity polymerase Pfu (Stratagen). All plasmids with PCR amplified genes were sequenced. Electroporation of plasmids into bacteria was carried out as previously described (Sambrook *et al*., 1989). Plasmids that were purified from *E.coli* were passaged through a restriction-minus (*hsd*) *S.typhimurium* LT2 strain (LB5000) (Sanderson and Stocker, 1987) prior to electroporation into other *S.typhimurium* strains. For transductions, P22 HT *int* lysates were harvested and used as described previously (Maloy *et al*., 1996).

To construct the *sicA*::*aphT*Δ*sspC* double mutant, the *sicA*::*aphT* mutation was transduced from the SL1344 strain background into strain CAS108 (14028s *phoN* Δ*sspC*) (Scherer *et al*., 2000). Because *sicA* and *sspC* are linked, PCR was used to check that the Δ*sspC* deletion was not crossed out of the chromosome during the transduction. Primers to *sspB* and *sspD* (which flank *sspC*) were used to amplify DNA between *sspB* and *sspD* in the wt and Δ*sspC* strains, and six transductants. Kan^r transductants missing *sspC* were then transformed with the plasmid reporter pHD95 (*sopE*). To make the SL1344 *sicA*::*aphT* Δ*sspC sigD-lacZYA*/*sigDE*$^+$ strain, a P22 HT lysate was made from strain 14028s *sicA*::*aphT* Δ*sspC* and Kan^r (from *sicA*::*aphT*) was transduced into SL1344 *sigD-lacZYA*/*sigDE*$^+$. As above, because *sicA* and *sspC* are linked, Kan^r transductants were checked for the *sspC* deletion by PCR.

Growth conditions

Salmonella typhimurium and *E.coli* strains were grown in Luria–Bertani (LB) Miller Broth (Difco) at 37°C with aeration on a roller drum or without aeration in standing cultures, depending on the assay. Evans Blue uranine (EBU) agar was made as described previously (Maloy *et al*., 1996). Antibiotics were used at the following final concentrations: ampicillin (Ap), 100–200 μg/ml; chloramphenicol (Cm), 25 μg/ml; kanamycin (Kn), 100 μg/ml; tetracycline (Tc), 15 μg/ml. For the detection of β-galactosidase activity, solid medium was supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) at 40 μg/ml. For induction of the T7 polymerase gene in BL21(DE3), isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 100 μM.

Purification of RNA and primer extension

RNA was purified from *Salmonella* (strains SL1344 pHH10, SL1344 pHD30-2, SVM579 pHH10 and SVM579 pHD30-2) or *E.coli* (strains VM1016, VM1020, VM1019 and VM1021) using Trizol reagent (Lifetechnologies). [^{32}P]ATP labeling of the *sicA* and *sigD* primers and primer extension were performed as described previously (Ausubel *et al*., 1992). For the *sicA* promoter, oligonucleotide *sicA*-11 was used (Table II). For the *sigD* promoter, primer *sigP*2 was used (Table II). Sequencing reactions (see below) were performed with the same primers used for primer extension.

Table V. Bacterial strains and plasmids used in this work

Strain	Genotype	Source/reference
S.typhimurium		
SL1344 derivatives		
SL1344	wt	Hoiseth and Stocker (1981)
SVM473	Cm^r; Φ(*sigD-lacZYA*)/*sigDE*$^+$	Darwin and Miller (1999a)
SVM579	Δ*invF* (in-frame deletion of 465 bp)	Darwin and Miller (1999a)
SVM609	Cm^r; Δ*invF*, *sigD-lacZYA*/*sigDE*$^+$	Darwin and Miller (1999a)
SVM687	Km^r; *sicA*::*aphT*	Darwin and Miller (2000)
GG5	Amp^r; *sopE-lacZY/sopE*$^-$	gift from Catherine Lee
SVM935	*sicA*::*aphT* Δ*sspC sigD-lacZYA*/*sigDE*$^+$	this work
14028s derivatives		
14028s	wt	ATCC[a]
CAS108	Δ*sspC phoN*::Tn*10*dCm	Scherer *et al*. (2000)
SVM933	CAS108 with *sicA*::*aphT*	this work
LT2 strain		
LB5000	LT2, *flaA66, metA22, trp-2, rpsL, xyl-401, ilv-452, leu, hsd, mod*$^+$	Sanderson and Stocker (1987)
E.coli strains		
DH5α	F$^-$ p80Δ*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoP recA1 endA1 hsdR17* ($r_k^-m_k^-$)	Gibco-BRL
DHP1	F$^-$ *cya glnV44* (AS) *recA1 endA1 gyrA96* (Nal^r) *thi1 hsdR17 spoT1 rfbD1*	Karimova *et al*. (1998)
BL21(DE3)	F$^-$ *ompT gal* [*dcm*] [*lon*] *hsdS$_B$* ($r_B^-m_B^-$) λ prophage carrying T7 polymerase	Studier *et al*. (1990)
CC118λ*pir*	*araD139* Δ(*ara,leu)7697* Δ*lacX74 phoA20 galE galK thi rpsE rpoB argEam recA1* λ*pir*	Herrero *et al*. (1990)
VM1016	Tet^r, Km^r, Ap^r; CC118λ*pir* pRW50, pHD9-1, pHG329	this work
VM1020	Tet^r, Km^r, Ap^r; CC118λ*pir* pHD11, pHD9-1, pHD30-2	this work
VM1019	Tet^r, Km^r, Ap^r; CC118λ*pir* pRW50, pHD9-1, pHD30-2	this work
VM1021	Tet^r, Km^r, Ap^r; CC118λ*pir* pHD34, pHD9-1, pHD30-2	this work
Plasmids		
pAJD107	Ap^r; medium copy-number cloning vector	A.J.Darwin (Darwin and Miller, 2001)
pHG329	Ap^r; medium copy-number cloning vector	Stewart *et al*. (1986)
pWKS130	Kn^r; low copy-number cloning vector	Wang and Kushner (1991)
pWSK29/pWKS30	Ap^r; low copy-number cloning vector	Wang and Kushner (1991)
pFUSE	Cm^r;MobRP4 *ori*R6K, polylinker upstream of promoterless *lacZYA*	Bäumler *et al*. (1996)
pHD9-1	Kn^r; 1.7 kb *Pst*I fragment containing *invF* in pWKS130	Darwin and Miller (1999a)
pHD10-1	Ap^r; 1.7 kb *Pst*I fragment containing *invF* in pHG329	Darwin and Miller (1999a)
pHD17	Cm^r; pACYC184 with a 1.7 kb *Hin*dIII fragment containing *invF* from pHD10-1	Darwin and Miller 1999a)
pHD30-2	Ap^r; 0.9 kb *sicA* fragment in pHG329	Darwin and Miller (2000)
pHD50	Tc^r; pRW50 with ~300 bp fragment containing the *iacP* to *sicP* intergenic region	this work
pHD57	Ap^r; pWKS30 with a 0.9 kb *Eco*RI–*Hin*dIII fragment from pHD30-2 containing *sicA*	this work
pHD61	Cm^r; pFUSE containing an ~550 bp fragment fusing the first 535 bp of *sptP* to *lacZYA*	this work
pHD71	Cm^r; 0.9 kb *Sal*I–*Xba*I fragment containing *sicA* in pACYC184	this work
pRW50	Tc^r; low copy transcriptional reporter fusion vector	Lodge *et al*. (1992)
pHD11	Tc^r; Φ(*sicA-lacZYA*) in pRW50 (–271 to +130)	Darwin and Miller (1999a)
pHD83	Tc^r; Φ(*sicA-lacZYA*) in pRW50 (–61 to +130)	this work
pHD84	Tc^r; Φ(*sicA-lacZYA*) in pRW50 (–35 to +130)	this work
pHD98	Tc^r; same as pHD83 but with an A to T mutation at –51	this work
pHD96	Tc^r; same as pHD83 but with a G to T mutation at –48	this work
pHD97	Tc^r; same as pHD83 but with a G to C mutation at –48	this work
pHD99	Tc^r; same as pHD83 but with a C to G mutation at –45	this work
pHD86	Tc^r; Φ(*sigD-lacZYA*) in pRW50 (–350 to +91)	this work
pHD87	Tc^r; Φ(*sigD-lacZYA*) in pRW50 (–66 to +91)	this work
pHD88	Tc^r; Φ(*sigD-lacZYA*) in pRW50 (–37 to +91)	this work
pHH10	Ap^r; 4.1-kb *Eco*RI *sigDE* fragment cloned into pHG329	Hong and Miller (1998)
pAJD*sicA*61	Ap^r; pAJD107 with a 61 bp PCR *sicA* fragment from –61 to +4	this work
pHD100	Tc^r; *Eco*RI–*Hin*dIII fragment from pAJD*sicA*61 containing the *sicA* promoter from –61 to +4 cloned into pRW50	this work
pT25	Cm^r; encodes the N-terminal domain of *B.pertussis* adenylate cyclase	Karimova *et al*. (1998)
pHD51	Cm^r; pT25 with a *Pst*I–*Kpn*I *sicA* PCR fragment	this work
pHD54	Cmr; pT25 with a *Pst*I–*Kpn*I *invF* PCR fragment	this work
pT18	Ap^r; encodes the C-terminal domain of *B.pertussis* adenylate cyclase adenylate cyclase	Karimova *et al*. (1998)
pHD52	Ap^r; pT18 with a *Kpn*I–*Xho*I *invF* PCR fragment	this work
pHD53	Ap^r; pT18 with a *Kpn*I–*Xho*I *sicA* PCR fragment	this work
pHD101	Ap^r; pWSK29 with a 1.27 kb *Kpn*I fragment containing *sigE* downstream of a T7 promoter	this work

[a]American Type Culture Collection.

Purification of His-tagged proteins and production of polyclonal antibodies

Oligonucleotide primers (Lifetechnologies) were used to amplify *invF* and *sicA* by PCR using Pfu polymerase (Stratagene). PCR products were digested with *Eco*RI and *Xho*I, and cloned into pET24+ (for *sicA*) or pET24b(+) (for *invF*). The resulting plasmids, pHD40-1 and pHD48 for *sicA* and *invF*, respectively, were sequenced to ensure that the fusions were in-frame and that the *sicA* and *invF* coding regions were unaltered. For making polyclonal antibodies, SicA-His_6 was purified under native conditions and InvF-His_6 was purified under denaturing conditions as described in the QIAexpressionist manual (Qiagen). The His-tagged proteins were separated from contaminating proteins by running the most concentrated fractions on a 3 mm 12.5% SDS–PAGE gel (Ausubel *et al*., 1992). The gels were stained in a solution of 0.05% Coomassie Blue in water to visualize the proteins and the His-tagged fusion proteins were sliced from the gels using a clean razor blade (Harlow and Lane, 1988). The gel slices were lyophilized and used for immunization of Elite New Zealand white rabbits by Covance (Denver, PA).

Co-purification of SicA with InvF-His_6

invF was cloned into plasmid pET24b(+) (Novagen), resulting in plasmid pHD48 as described above. This plasmid with pHD57 (T7-*sicA*) was able to activate transcription of the *sicA* promoter in the *lac*-minus *E.coli* strain ER2566, which encodes an inducible T7 polymerase gene (data not shown) (Chong *et al*., 1997). For purification of soluble InvF-His_6, BL21(DE3) containing pHD48 was transformed with plasmid pHD57 (T7-*sicA*) or pHD101 (T7-*sigE*). In addition, BL21(DE3) with pET24b(+) was transformed with pHD57 as an additional negative control. Although the majority of InvF-His_6 was insoluble, a portion of the fusion protein was soluble when purified as follows: 25 ml overnight cultures were subcultured into 1 l of LB broth with Kn and Ap and grown for 2 h at 37°C with aeration. At 2 h, the cultures were moved to a 12°C water bath and incubated for 20 min with shaking before adding 1 ml of 100 mM IPTG (final concentration 100 μM). The cultures were incubated with shaking at 12°C overnight (~20 h). Cells were harvested by centrifugation (10 min, 6000 *g*) and pellets were stored at –20°C. Pellets were frozen and thawed twice before resuspension in 10 ml of lysis buffer supplemented with a Complete Mini protease inhibitor tablet (Roche/Boehringer Manheim). Cell lysates and fractions were prepared under native conditions as described in the QIAexpressionist manual with one exception: the column washes were increased from two 4-ml washes to three 10-ml washes.

Proteins were analyzed by either Coomassie Blue staining (Ausubel *et al*., 1992) or immunoblotting using the ECL Western Blotting Detection System (Amersham Pharmacia Biotech) as described previously.

Radioactive DNA probes and filter binding assays

Promoter fragments were isolated by PCR amplification using Pfu (Stratagene). For the *sicA* promoter, primers *sicA-Eco*RI-12 and *sicA-Bam*HI-1 (Table II) were used. For the mutant *sicA* promoters, the same sequence as the *sicA-Eco*RI-12 primer was used, but with the appropriate base changes found in pHD97 and pHD99. To amplify the *lac* promoter from chromosomal DNA purified from *E.coli* S17-1λ*pir*, primers *lacP*1-*Eco*RI (5′-GGAATTCGGCGCCCAATACGCAAACCGCC-3′) and *lacP*2 (5′-CGAGCTCGTCCACACAACATACGAGCCGGAAGC-3′) were used. Each promoter had an *Eco*RI site engineered at the 5′ end. PCR products were purified using QIAEX II (Qiagen) and digested overnight with *Eco*RI. The digested fragments were purified again with QIAEX II, eluted in 50 μl of water, and 10 μl of each fragment were labeled with [^{32}P]dATP and [^{32}P]dTTP (Amersham Pharmacia Biotech) using Klenow (New England Biolabs) (Ausubel *et al*., 1992). Each probe was purified using DyeEx spin columns (Qiagen). The final amount (μg/ml) of DNA was determined by ethidium bromide dot quantitation (Ausubel *et al*., 1992). The counts per minute (c.p.m.) of each probe were measured by diluting in TE (10 mM Tris pH 8, 1 mM EDTA) and measuring the c.p.m. in a scintillation counter. For final use, each probe was diluted in TE to 15 000 c.p.m./μl, where 15 000 c.p.m. represented 0.2–0.6 ng/μl DNA.

Filter binding assays were performed based on methods described previously (McEntee *et al*., 1980; Ausubel *et al*., 1992). Briefly, to prepare nitrocellulose filters for the assay, 0.45 μm pure nitrocellulose membranes (Schleicher and Schuell BA85) were soaked for 20 min in 0.5 M KOH, rinsed extensively for 10 min in deionized water, and soaked in 0.1 M Tris–HCl pH 7.4 for 45 min. Native InvF-His_6 used in these assays was purified as described above and SicA-His_6 was purified under native conditions as described in the QIAexpressionist manual. All proteins used were dialyzed in 50 mM sodium phosphate, monobasic, 10 mM Tris–HCl and 100 mM NaCl. The pH was adjusted to 6.5. The proteins were diluted in 50% glycerol in 0.5× dialysis buffer to a final concentration of 1 μg/μl. Binding reactions (50 μl) were carried out by incubating protein (5 μg, a non-saturating amount), 20 mM Tris–HCl pH 8, 2 mM dithiothreitol, 10 mM $MgCl_2$, 50 μg/ml bovine serum albumin, 100 μM EDTA, 10 μM $MnCl_2$, 200 mM NaCl and 3 μg of poly(dI–dC). After 20 min at room temperature, 15 000 c.p.m. of labeled DNA (0.2–0.6 ng/μl) were added, gently tapped to mix, and incubated at room temperature for 30 min. The prepared nitrocellulose membrane and two sheets of Whatman 3 mm paper soaked in 0.1 M Tris–HCl pH 7.4 were mounted on a Millipore Milliblot system and the samples were slowly vacuumed through the membrane. Each well was washed twice with 200 μl of 1× binding buffer. Filters were wrapped in plastic wrap and exposed to Hyperfilm (Amersham) overnight at –80°C or room temperature. After exposure to film, each spot on the nitrocellulose membrane was excised and placed in a scintillation vial with scintillation fluid (Fisher Scintiverse) and the c.p.m. on each membrane piece were determined.

Enzyme assays

β-galactosidase assays were performed and values calculated as described previously (Miller, 1972).

Sequence analysis

Nucleotide (except for the primer extension experiments) and protein sequencing (N-terminal) were performed by the Washington University Protein and Nucleic Acid Chemistry Laboratory (St Louis, MO). For the primer extension experiments, sequencing was performed using the dideoxy chain termination method (Sanger sequencing) with Sequenase (USB). Sequence analyses (homologies, mapping, etc.) were performed using the Wisconsin Sequence Analysis Package by the Genetics Computer Group, Inc. (GCG).

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