INFECTION AND IMMUNITY, Dec. 2000, p. 6790–6797 Vol. 68, No. 12
0019-9567/00/$04.00+0

Regulation of *Salmonella enterica* Serovar Typhimurium Invasion Genes by *csrA*

CRAIG ALTIER,* MITSU SUYEMOTO, AND SARA D. LAWHON

Department of Microbiology, Pathology, and Parasitology, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina 27606

Received 28 July 2000/Accepted 14 September 2000

Penetration of intestinal epithelial cells by *Salmonella enterica* serovar Typhimurium requires the expression of invasion genes, found in *Salmonella* pathogenicity island 1 (SPI1), that encode components of a type III secretion apparatus. These genes are controlled in a complex manner by regulators within SPI1, including HilA and InvF, and those outside SPI1, such as the two-component regulators PhoP/PhoQ and BarA/SirA. We report here that epithelial cell invasion requires the serovar Typhimurium homologue of *Escherichia coli csrA*, which encodes a regulator that alters the stability of specific mRNA targets. A deletion mutant of *csrA* was unable to efficiently invade cultured epithelial cells and showed reduced expression of four tested SPI1 genes, *hilA, invF, sipC*, and *prgH*. Overexpression of *csrA* from an induced *araBAD* promoter also negatively affected the expression of these genes, indicating that CsrA can act as both a positive and a negative regulator of SPI1 genes and suggesting that the bacterium must tightly control the level or activity of CsrA to achieve maximal invasion. We found that CsrA affected *hilA*, a regulator of the other three genes we tested, probably by controlling one or more genetic elements that regulate *hilA*. We also found that both the loss and the overexpression of *csrA* reduced the expression of two regulators of *hilA, hilC* and *hilD*, suggesting that *csrA* exerts its control of *hilA* through one or both of these regulators. We further found, however, that CsrA could affect the expression of both *invF* and *sipC* independent of its effects on *hilA*. One additional striking phenotype of the *csrA* mutant, not observed in a comparable *E. coli* mutant, was its slow growth. Phenotypic revertants that had normal growth rates, while maintaining the *csrA* mutation, were common. These suppressed strains, however, did not recover the ability to invade cultured cells, indicating that the *csrA*-mediated loss of invasion cannot be attributed simply to poor growth and that the growth and invasion deficits of the *csrA* mutant arise from effects of CsrA on different targets.

An early step in the pathogenesis of *Salmonella* infection is bacterial penetration of intestinal epithelium. Many of the genes required for epithelial penetration are found within *Salmonella* pathogenicity island 1 (SPI1), a 40-kb region located at centisome 63 (6, 18, 20, 24, 31, 37, 44). These invasion genes encode the components and substrates of a type III secretion apparatus that exports signaling molecules to the bacterial surface and into adjacent eukaryotic cells. The signaling molecules then induce in these cells cytoskeletal changes that lead to bacterial engulfment (11, 17, 26, 27, 34, 35, 46, 58).

Regulation of SPI1 genes is complex. Oxygen tension, pH, osmolarity, and growth phase have all been shown to alter invasion gene expression (5, 16, 19, 36, 42). Much of the response to these conditions is mediated by HilA, a SPI1 regulator of the OmpR/ToxR family (4, 5). Among the targets of HilA are other invasion genes, including *invF*, which encodes a regulator of the AraC family (32). HilA and InvF have overlapping, but not identical, sets of targets, both inside and outside SPI1 (2, 13, 14, 28). Within SPI1, both regulators control the *sip* operon, but they may do so independently using alternative promoters (13, 14). HilA, in turn, is subject to multiple controls. Two SPI1 regulators control *hilA*: *hilD* and *hilC* (also known as *sirC* and *sprA*). A mutant of the former is deficient in invasion, while the role of the latter has been inferred from its effects by overexpression (15, 30, 50). Regulators outside SPI1 also control invasion genes. A constitutively active mutant of *phoQ* represses *hilA* (4). Since the PhoP/PhoQ two-component regulator is normally activated by low magnesium concentration (21, 23), invasion might be repressed by PhoP/PhoQ in response to the extracellular level of magnesium. The BarA/SirA two-component regulator activates *hilA* expression and can also activate *invF* independently of HilA (3, 30, 47). The environmental signal to which this latter regulator responds is not known.

An unusual method of gene regulation is that achieved by the *csrA*/*B* system. Originally identified in *Escherichia coli*, it consists of a protein, CsrA, and an untranslated RNA, CsrB, and controls such diverse properties as carbohydrate biosynthesis, motility, and bacterial surface characteristics (reviewed in reference 49). The mechanism by which *csrA*/*B* functions is known for the control of glycogen biosynthesis. CsrA binds to the *glgCAP* mRNA and enhances its degradation, thus acting as a negative regulator of glycogen production (40). CsrB binds approximately 18 to 20 CsrA molecules, presumably titrating the protein and acting as a positive regulator. Thus, gene regulation is achieved by altering the concentration of free CsrA (38, 57). A similar system also exists in the plant pathogen *Erwinia carotovora*, where the *csrA* and *-B* homologues, *rsmA* and *-B*, control the expression of secreted virulence proteins pectate lyase, polygalacturonase, cellulase, and protease (12, 41, 45), and in *Pseudomonas fluorescens*, where *rsmA* controls extracellular protease and hydrogen cyanide synthesis (8). CsrA homologues have been identified in a number of other bacterial species as well, suggesting that alteration of message stability provides a widely used means of gene regulation (49).

We have previously investigated the effect of *csrB* on invasion gene expression in *Salmonella enterica* serovar Typhimu-

* Corresponding author. Mailing address: College of Veterinary Medicine, North Carolina State University, 4700 Hillsborough St., Raleigh, NC 27606. Phone: (919) 513-6274. Fax: (919) 513-6455. E-mail: craig_altier@ncsu.edu.

TABLE 1. Strains and plasmids used

Strain or plasmid	Genotype	Source or reference
Strains		
ATCC 14028s	Wild type	American Type Culture Collection
CA614	Δ(*csrA*)::Cam	This work
CA678	Δ(*csrA*)::Cam *sup8*; spontaneous suppressor of *csrA* growth defect	This work
EE630	Φ(*invF′-lacZY*$^+$); same as *invF*::*lacZY11-5*	29
EE631	Φ(*sipC′-lacZY*$^+$); same as *sspC*::*lacZY11-6*	29
EE636	Φ(*prgH′-lacZY*$^+$); same as *prgH*::*lacZY-020*	5
EE658	Φ(*hilA′-lacZY*$^+$); same as *hilA*::*lacZY-080*	5
CA701	Φ(*invF′-lacZY*$^+$) in ATCC 14028s	This work
RM5385	Φ(*sipC′-lacZY*$^+$) in ATCC 14028s	3
RM5387	Φ(*prgH′-lacZY*$^+$) in ATCC 14028s	3
RM5938	Φ(*hilA′-lacZY*$^+$) in ATCC 14028s	3
CA702	Δ(*csrA*)::Cam Φ(*invF′-lacZY*$^+$)	This work
CA620	Δ(*csrA*)::Cam Φ(*sipC′-lacZY*$^+$)	This work
CA621	Δ(*csrA*)::Cam Φ(*prgH′-lacZY*$^+$)	This work
CA622	Δ(*csrA*)::Cam Φ(*hilA′-lacZY*$^+$)	This work
CA704	Δ(*csrA*)::Cam *sup8* Φ(*invF′-lacZY*$^+$)	This work
CA684	Δ(*csrA*)::Cam *sup8* Φ(*sipC′-lacZY*$^+$)	This work
CA707	Δ(*csrA*)::Cam *sup8* Φ(*prgH′-lacZY*$^+$)	This work
CA686	Δ(*csrA*)::Cam *sup8* Φ(*hilA′-lacZY*$^+$)	This work
VV114	*hilA339*::Kan; same as *hilA*::Kan-339	29
RM6373	*hilA339*::Kan in ATCC 14028s	3
RM6376	*hilA339*::Kan Φ(*invF′-lacZY*$^+$)	3
CA728	*hilA339*::Kan Δ(*csrA*)::Cam Φ(*invF′-lacZY*$^+$)	This work
RM6377	*hilA339*::Kan Φ(*sipC′-lacZY*$^+$)	3
CA733	*hilA339*::Kan Δ(*csrA*)::Cam Φ(*sipC′-lacZY*$^+$)	This work
RM6378	*hilA339*::Kan Φ(*prgH′-lacZY*$^+$)	3
CA743	*hilA339*::Kan Δ(*csrA*)::Cam Φ(*prgH′-lacZY*$^+$)	This work
SVM725	Δ*invF* Φ(*sipC′-lacZY*$^+$); in-frame deletion of *invF*	13
CA735	Δ*invF* Φ(*sipC′-lacZY*$^+$)	This work
CA736	Δ*invF* Δ(*csrA*)::Cam Φ(*sipC′-lacZY*$^+$)	This work
RM6375	*ara9*; arabinose auxotroph	R. Maurer
RM6380	*ara9* Φ(*invF′*-lacZY$^+$)	R. Maurer
RM6381	*ara9*, Φ(*sipC′-lacZY*$^+$)	R. Maurer
RM6382	*ara9* Φ(*prgH′-lacZY*$^+$)	R. Maurer
RM6383	*ara9* Φ(*hilA′-lacZY*$^+$)	R. Maurer
Plasmids		
pBAD18	Cloning vector with arabinose-inducible *araBAD* promoter; Ap^r	25
pCA114	*csrA* under P_{araBAD} control on pBAD18; Ap^r	This work
pFF584	Cloning vector with pSC101 origin; Str^r Sp^r	3
pCA132	0.7-kb *csrA* fragment on pFF584; Str^r Sp^r	This work
pCA71	*csrB* on pBAD30; Ap^r	3
pVV214	*hilA* expressed from *neo* promoter on pACYC177; Ap^r	4
pLS31	*hilA*::*lacZY* operon fusion with −497 to +420 of *hilA*; Tc^r	50
pLS79	*hilA*::*lacZY* operon fusion with −39 to +420 of *hilA*; Tc^r	50

rium (3). Although not required for the invasion of cultured epithelial cells, *csrB* is necessary for maximal expression of SPI1 genes, implying that CsrA inhibits invasion gene expression. Here we examine the effects of *csrA* on invasion. Paradoxically, we find that the loss of *csrA* reduces invasion and invasion gene expression. Further, we show that this positive regulation by CsrA has both HilA-dependent and HilA-independent routes, and we show that CsrA regulates genes above *hilA* in the regulatory pathway. We also find that a *csrA* mutant has a severe growth defect, a phenotype not found in a *csrA* mutant of *E. coli*.

MATERIALS AND METHODS

Bacterial strains and genetic techniques. Strains and plasmids used in this study are shown in Table 1. All strains are derivatives of ATCC 14028s, and strains containing multiple genetic elements were constructed by sequential P22 transductions (53). Transductions involving the *lacZY* operon fusion element, which is carried on a nondisabled transposon, were verified by PCR using one primer in *lacZ* and the other primer in the fused gene. Random *lacZY* fusions were created by transduction of the *hilA*::*lacZY* fusion, marked by tetracycline resistance, into a *hilA339*::Kan strain, maintaining selection for both tetracycline and kanamycin. In this way, new transposon insertions were selected. Four such fusions producing varying shades of blue on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were moved by P22 transduction into a wild-type strain and then used to test the specificity of *csrA* regulation.

Recombinant DNA techniques. Serovar Typhimurium *csrA* was amplified from the chromosome of ATCC 14028s by using the primers 5′-GGAATTCGGTCA GCGCAAAATTG-3′ and 5′-CGGGATCCGCGTCTCACTTTTCGG-3′. These primers were derived from the unfinished *S. enterica* serovar Typhi sequence and were predicted to flank *csrA*, based on homology to *E. coli* (preliminary sequence data were obtained from The Institute for Genomic Research website at http://www.tigr.org). The resulting fragment was cloned into pBluescript II (Stratagene) and sequenced using an ABI automated sequencer.

The Δ(*csrA*)::Cam mutant was created by first PCR amplifying two fragments that flank *csrA* in the serovar Typhimurium chromosome. The primers 5′-GGA ATTCGGTCAGCGCAAAATTG-3′ and 5′-CGGGATCCTTGAAAGATTAA AAGAGTCGG-3′ amplify a 0.25-kb piece immediately upstream from *csrA*, creating *Eco*RI and *Bam*HI ends; primers 5′-GCTCTAGACACTTCACGCTC AATTAGTCTG-3′ and 5′-CGGGATCCGCGTCTCACTTTTCGG-3′ amplify a 0.2-kb piece immediately downstream from *csrA*, creating *Bam*HI and *Xba*I ends. These two fragments were sequentially cloned adjacent to each other in pBluescript II. The resulting plasmid was then cut with *Bam*HI, and a chloram-

phenicol resistance marker (Cam) with *Bam*HI ends was inserted between the fragments. The entire piece, containing the marker and flanking fragments, was next cloned into the allele-exchange vector pKAS32, using *Kpn*I and *Xba*I (52). A spontaneous streptomycin-resistant derivative of ATCC 14028s was transformed with this plasmid, and the *csrA* deletion was inserted into the chromosome as described elsewhere (52), creating a deletion of *csrA* and including 7 bp 5′ and 4 bp 3′ of the open reading frame (ORF). The mutation was moved into the wild-type strain by P22 transduction, and deletion of *csrA* was confirmed by Southern blotting.

pCA132 carries a 0.7-kb fragment including *csrA* and ~0.25 kb of upstream DNA that was amplified from the chromosome of ATCC 14028s by using the primers 5′-GGAATTCGGTCAGCGCAAAATTG-3′ and 5′-GCTCTAGACAC TTCACGCTCAATTAGTCTG-3′. It was cloned into pFF584 (3), a *lacI* derivative of pMS421 (22), and the plasmid was maintained by growth in streptomycin and spectinomycin. pCA114 carries *csrA* without upstream sequence and under the control of the *araBAD* promoter on plasmid pBAD18 (25). *csrA* was amplified using the primers 5′-GGAATTCAAGGAGCAAAGAATGCTG-3′ and 5′-GCTCTAGACACTTCACGCTCAATTAGTCTG-3′ and was cloned into pBAD18 cut with *Eco*RI and *Xba*I.

Media and growth conditions. LB broth buffered with 100 mM HEPES to pH 8 was used throughout, and cultures were grown standing at 37°C except where noted. Antibiotics, when included for plasmid maintenance, were added at the following concentrations: ampicillin, 100 μg/ml; streptomycin, 20 μg/ml; spectinomycin, 100 μg/ml; and tetracycline, 25 μg/ml. For all assays that included the Δ(*csrA*)::Cam mutant, cultures to be compared were grown to similar densities (optical densities at 600 nm [OD_{600}] of 0.4 to 0.7), *csrA*$^+$ strains for 12 h, *csrA* strains for 48 h, and suppressed *csrA* strains for 20 h. Glucose or arabinose was added at 0.5% for testing the arabinose-induced expression of *csrA* on pCA114.

Invasion assays. HEp-2 cells were grown in 24-well plates for 3 days in RPMI 1640 with 5% fetal calf serum. Approximately 10^6 bacteria were added to cells, for a multiplicity of infection of about 10 bacteria/cell. Plates were then centrifuged for 10 min at 800 × *g* and incubated for 1 h at 37°C in 95% air–5% CO_2. Medium was removed, the cells were washed three times with phosphate-buffered saline, and the medium was replaced by medium supplemented with gentamicin (20 μg/ml). Cells were incubated for an additional hour, the medium was removed, and monolayers were washed three times with phosphate-buffered saline. The cells were lysed with 1% Triton X-100 for 5 min, and the bacterial titers of the lysates were determined by colony counts. Each bacterial culture was tested in quadruplicate, and results were expressed as percentage of inoculum surviving gentamicin treatment.

β-Galactosidase and growth assays. β-Galactosidase assays were performed as described elsewhere (43). Every strain was assayed at least in triplicate. For growth experiments, strains were grown standing overnight and then diluted 1:150 into fresh medium. Strains were grown in triplicate with aeration at 37°C, and growth was assessed by culture density (OD_{600}).

Northern analysis. For RNA isolation, strains were grown with aeration to an OD_{600} of ≅1. Strains with pCA114 and pBAD18 were grown with ampicillin and 0.5% arabinose. Total bacterial RNA was then isolated using an RNeasy Midi kit (Qiagen) according to the directions of the manufacturer. Equal amounts of RNA were loaded into each well of an agarose-formaldehyde gel (9.5 μg for *hilC*; 2.5 μg for all others), and Northern blotting was performed as described elsewhere (9). Probes were prepared by amplifying fragments internal to ORFs using a PCR digoxigenin probe synthesis kit (Boehringer Mannheim). For *hilC*, the primers 5′-CTTCAACAGCCGAACAAATTTC-3′ and 5′-CTCGCTCAAGGA AATCAAACC-3′ amplify a 510-bp fragment; for *hilD*, the primers 5′-AGCAG GTTACCATCAAAAATCTTTATG-3′ and 5′-TGAGCCGAGCTAAGGATG ATC-3′ amplify a 509-bp fragment. Detection was performed according to the manufacturer's directions, using chemiluminescence and a Lumi-Imager (Boehringer Mannheim).

Statistical analysis. For β-galactosidase and invasion results, a one-way analysis of variance was used to determine whether the mean of at least one strain differed from that of any of the others. Then multiple comparison tests (least squares differences *t* test at a *P* of ≤0.05) were used to determine which means differed (SAS System for Windows 7.0).

Nucleotide sequence accession number. The nucleotide sequence of serovar Typhimurium *csrA* has been deposited with GenBank under accession number AF203976.

RESULTS

Growth defect of the *csrA* mutant. In previous work, we showed that loss of the regulatory RNA CsrB decreased the expression of a number of SPI1 genes, while multicopy expression of *E. coli csrA* in serovar Typhimurium had more pronounced repressive effects on these same genes. This suggested that CsrA acts as a negative regulator of invasion and that CsrB antagonizes that effect (3). To directly study the role of *csrA* in the control of invasion genes, we first cloned serovar Typhimurium *csrA* from the chromosome of the wild-type strain, ATCC 14028s. The gene and adjacent sequence were amplified using primers predicted to flank *csrA*, based on homology to *E. coli*. Sequence analysis showed the predicted amino acid sequence of serovar Typhimurium CsrA to be identical to that of *E. coli* (GenBank accession numbers AF203976 and L07596, respectively).

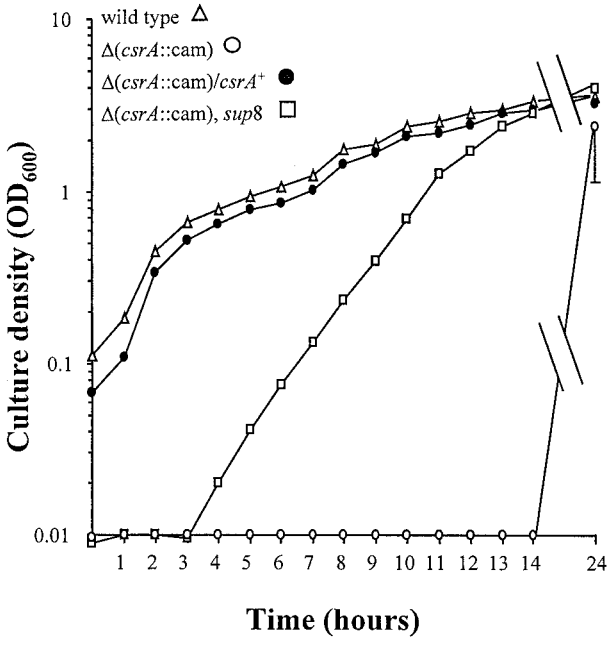

FIG. 1. Effect of *csrA* on bacterial growth. Strains were grown with aeration in LB broth buffered with HEPES to pH 8. The *csrA*$^+$ plasmid used was pCA132. Values represent mean and standard error for strains tested in triplicate.

We next created a precise chromosomal deletion-substitution mutant in which the entire *csrA* ORF was replaced by a chloramphenicol resistance marker. The inserted marker was found to be linked to *recA* by P22 transduction, placing *csrA* near centisome 63, analogous to its position in *E. coli* and outside SPI1. Surprisingly, the most distinctive phenotype of the Δ(*csrA*)::Cam mutant was a severe growth defect, a characteristic not observed in a *csrA* mutant of *E. coli* (48). Poor growth, which was apparent both on solid and in liquid media, is illustrated in Fig. 1 for LB broth. This defect was fully complemented by plasmid pCA132, which carries *csrA* under the control of its native promoter on a low-copy-number vector (approximately five copies per bacterium). Although growth of the Δ(*csrA*)::Cam mutant was initially very poor, some cultures achieved densities comparable to those of the wild type after overnight aerated growth. This change in growth rate almost certainly represents the accumulation of suppressor mutations (discussed further below) rather than an authentic *csrA* phenotype. There was great variation among replicate cultures (note the large error bar at 24 h in Fig. 1), and a large proportion of clonal isolates obtained from the later time points of these experiments continued to grow faster when subcultured (data not shown). We further noted that suppressors were uncommon in cultures grown without aeration and to low density (OD_{600} of 0.4 to 0.7), presumably because the disparity between the growth rates of the *csrA* mutant and its suppressed derivatives was not as great. Under these conditions, suppressors accounted for less than 0.2% of the bacterial population. Therefore, to ensure that suppressors did not obscure the effects of *csrA* loss, we grew bacteria for assays described in this work as standing cultures with optical densities of less than 0.7.

TABLE 2. Invasion and invasion gene expression

Strain/plasmid	% Invasion[b]	β-Galactosidase[a] (Miller units)			
		Φ(*sipC'-lacZY*$^+$)	Φ(*invF'-lacZY*$^+$)	Φ(*prgH'-lacZY*$^+$)	Φ(*hilA'-lacZY*$^+$)
Wild type	3.0 ± 0.67	2,700 ± 110	1,400 ± 60	890 ± 24	280 ± 14
Δ(*csrA*)::Cam	0.003 ± 0.001	200 ± 9	25 ± 1	72 ± 3	29 ± 0
Δ(*csrA*)::Cam/*csrA*$^{+c}$	3.3 ± 0.28	4,200 ± 160	2,000 ± 60	1,200 ± 18	460 ± 12
Δ(*csrA*)::Cam *sup8*	0.019 ± 0.006	73 ± 6	15 ± 2	34 ± 2	21 ± 1
Δ(*csrA*)::Cam *sup8*/*csrA*$^{+c}$	7.8 ± 1.1	3,800 ± 370	1,500 ± 3	920 ± 10	440 ± 24

[a] Mean and standard error for strains tested at least in triplicate.
[b] Mean and standard error for strains tested at least in quadruplicate.
[c] The plasmid used was pCA132.

***csrA* affects invasion.** We next tested the effect of *csrA* on epithelial cell invasion. Since *csrB* functions as a positive regulator of invasion gene expression, and multicopy expression of *E. coli csrA* in serovar Typhimurium reduces both invasion and expression of SPI1 genes (3), the loss of *csrA* might be expected to increase invasion. This, however, was not the case; invasion of cultured HEp-2 cells by the mutant was 1,000-fold lower than the wild-type level (Table 2). Invasion was complemented to the level of the wild type by *csrA* expressed from pCA132.

To examine the cause of this invasion defect, we tested the expression of four SPI1 genes, *hilA, invF, sipC*, and *prgH*, using chromosomal *lacZY* fusions (5, 29). *hilA* encodes a regulator of the other three genes (4), and InvF is a regulator of *sipC* (13, 14). The product of *sipC* is secreted (29, 33), while the *prgH* product is required for the secretion of Sip proteins (35). In all cases, expression of these fusions was significantly decreased in the *csrA* mutant (Table 2). The reduction ranged from 10-fold (for *hilA*) to 56-fold (for *invF*). This effect also proved to be specific for invasion genes; expression of several random fusions was not altered by the loss of *csrA* (not shown). Expression of *csrA* from pCA132 completely complemented the expression of the SPI1 genes in the *csrA* mutant.

Extragenic suppression of the growth defect. Expression of invasion genes has been shown to depend on bacterial growth phase (16, 42). Although all assays described here used cultures grown to similar densities, we considered the possibility that loss of invasion gene expression was due to the poor growth of the Δ(*csrA*)::Cam strain. To study this, we isolated suppressor mutants having improved growth. These suppressors commonly appeared during aerated growth in broth or upon plating bacteria at high density on solid media. One such suppressor, represented by *sup8*, appears to enter log phase later than the wild type when grown in aerated cultures but then achieves similar doubling times (Fig. 1). When tested for its ability to invade HEp-2 cells, however, the suppressed strain invaded as poorly as did the unsuppressed Δ(*csrA*)::Cam mutant (Table 2). Similarly, *sup8* did not restore expression of any of the four SPI1 genes tested. To ensure that the suppressor mutation itself had no effect on invasion, we complemented the *sup8* strain with wild-type *csrA* on pCA132. This plasmid fully restored both invasion and invasion gene expression, indicating that the suppressor did not confer an invasion defect (Table 2). Taken together, these results show (i) that the loss of invasion gene expression in the Δ(*csrA*)::Cam mutant cannot be attributed to its poor growth and (ii) that the *csrA*-mediated effects on growth and invasion are separable and thus at some level require effects on different target genes.

Overexpression of *csrA*. To test the effect of *csrA* overexpression on SPI1 genes, we expressed *csrA* from pCA114, which carries *csrA* under the control of the arabinose-inducible *araBAD* promoter, in an *ara*-mutant strain. Consistent with our previous results using *E. coli csrA*, overexpression of serovar Typhimurium *csrA* using 0.5% arabinose caused a significant 2- to 12-fold decrease in expression of invasion genes compared to the same strains grown in glucose, which represses the *araBAD* promoter (Fig. 2). Neither sugar affected any of the fusions in the absence of pCA114 or with the control plasmid pBAD18 (not shown). These results, together with those from the Δ(*csrA*)::Cam mutant, show that CsrA can play both positive and negative regulatory roles in the control of invasion genes and suggest that the level of CsrA must be tightly controlled to maximize invasiveness.

Role of *csrA* in the invasion gene regulation pathway. One gene under the control of *csrA* is *hilA*, a regulator of other SPI1 genes. To determine whether the effect of CsrA on *hilA* was direct, we used two plasmids, pLS31 and pLS79, that have identical transcriptional fusions of *hilA* to *lacZY* but differ in the amount of upstream sequence they carry (50). The fusion in pLS31 extends to position −497, including the upstream regulatory region, placing it under the control of genetic and

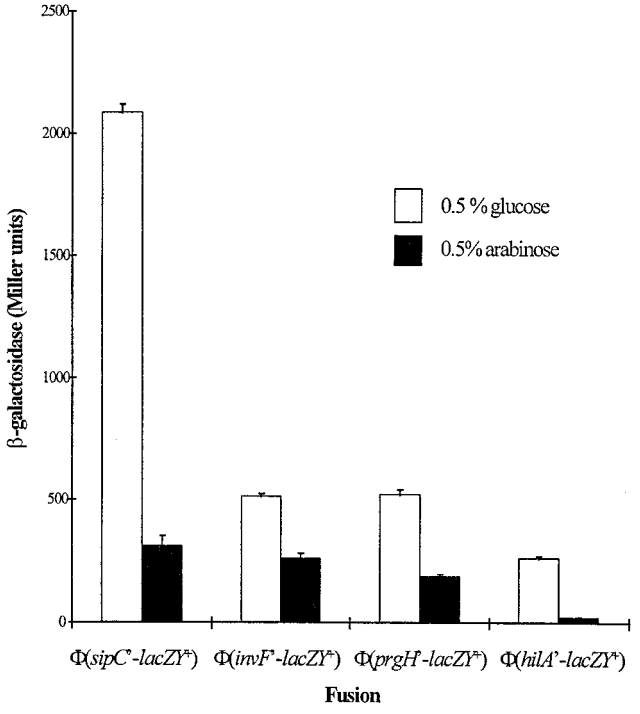

FIG. 2. Effect of *csrA* overexpression on invasion gene expression. *csrA* was expressed under the control of the *araBAD* promoter on plasmid pCA114 in the *ara9* strain background. Arabinose or glucose was added to a final concentration of 0.5% to induce or repress, respectively, *csrA* expression. Values represent mean and standard error for strains tested in triplicate.

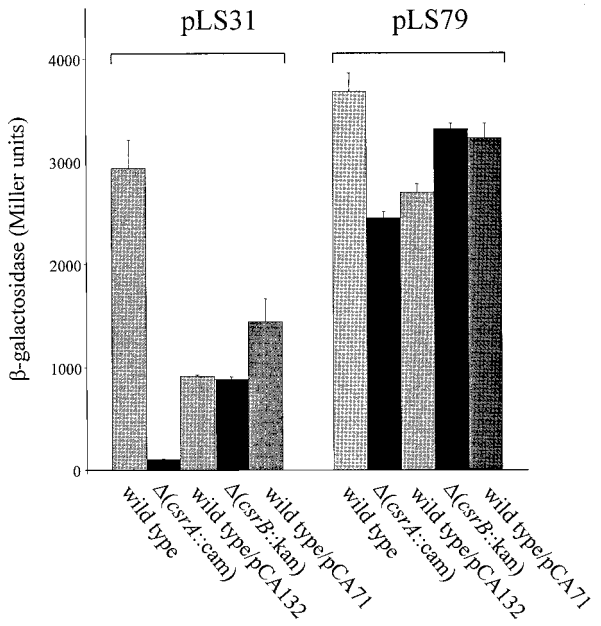

FIG. 3. Control of *hilA* expression by *csrA*. pLS31 and pLS79 carry transcriptional *lacZY* fusions to *hilA*. pLS31 has positions −497 to +420 of *hilA*, and pLS79 has positions −39 to +420 of *hilA*. pCA132 expresses *csrA*, and pCA71 expresses *csrB*. Values represent mean and standard error for strains tested in triplicate.

environmental elements that regulate *hilA*. The pLS79 fusion reaches only to position −39, making fusion expression constitutive. The *hilA* transcription start sites on these two plasmids are identical to each other and to that of chromosomal *hilA*. Since CsrA is predicted to work at the level of message stability, and the messages are the same, a direct action of CsrA on the *hilA-lacZ* fusion message would affect β-galactosidase expression from the two plasmids equally. If, however, CsrA degrades the message of a gene upstream from *hilA* in the regulatory pathway, it should affect *hilA* expression from only pLS31.

We found that the loss of *csrA* greatly (28-fold) diminished fusion expression from pLS31 but had only a small effect (an approximately 30% decrease) on pLS79 (Fig. 3). As expected, the overexpression of *csrA* from pCA132 also reduced the expression of the fusion, and did so more strongly in pLS31 than in pLS79. We further examined the effects of indirectly manipulating CsrA levels by altering the expression of *csrB*. We used a Δ(*csrB*)::Kan mutant, in which free CsrA should increase, and a strain overexpressing *csrB* from pCA71, in which more CsrA should be bound to CsrB and thus inactive. In both cases, we found that expression from pLS31 was reduced (threefold for *csrB* loss and twofold for overexpression), while that from pLS79 was affected only slightly. Therefore, if CsrA works posttranscriptionally in serovar Typhimurium as it does in *E. coli*, by altering message stability, these results suggest that the primary effect of CsrA on *hilA* is indirect, through the control of a gene or genes that control *hilA*. The small effects of *csrA* on pLS79 might mean that CsrA can interact directly with *hilA* message as well; alternatively, they might suggest that pLS79 maintains a partial binding site for one or more transcriptional regulators under the control of CsrA.

To further examine the control of *hilA* by CsrA, we tested the effects of *csrA* loss and overexpression on the known regulators of *hilA*. HilD, a member of the AraC/XylS family of transcriptional regulators, derepresses *hilA*. Since its action requires the region upstream from the *hilA* promoter, it is postulated to directly activate *hilA* transcription (50). We examined first the effects of *csrA* loss on *hilD* expression using Northern analysis. Because the Δ(*csrA*)::Cam mutant grows so poorly, making RNA isolation from unsuppressed strains difficult, we used for these experiments the Δ(*csrA*)::Cam *sup8* derivative, shown above to express invasion genes identically to the unsuppressed strain. A specific *hilD* message of approximately 1.2 kb was observed for the wild-type strain (Fig. 4) but was absent in the Δ(*csrA*)::Cam *sup8* strain, indicating that *csrA* is required for the expression of *hilD*. We next tested the effect of *csrA* overexpression on this same message. When *csrA* was expressed from pCA114, under the control of the arabinose-inducible *araBAD* promoter, in a strain grown in arabinose, no message could be detected. However, an isogenic strain grown under the same conditions, but carrying only the pBAD18 vector without *csrA*, produced an obvious 1.2-kb band. These results show that *csrA* controls *hilD* and, similar to its effects on downstream invasion genes, can act as both a positive and a negative regulator of *hilD*. We next examined *hilC* message. Similar to HilD, HilC is an activator of *hilA* of the AraC/XylS family (50). When probed for *hilC* message, the wild type produced three distinct bands of approximately 1.0 to 1.4 kb, but these bands were absent from the Δ(*csrA*)::Cam *sup8* strain (Fig. 4). When *csrA* was overexpressed from pCA114, the bands were also absent but were present in the isogenic strain carrying pBAD18. These results, taken together, show that either too much or too little CsrA reduces the expression of both *hilD* and *hilC* and further suggest that the action of CsrA on *hilA* is indirect, through its control of *hilD* and *hilC*. We tested three additional regulators of *hilA* for control by CsrA and found the message levels of none of these genes (*sirA, barA*, and *phoP*) to be affected by either loss or overexpression of *csrA* (data not shown).

Within SPI1, both HilA and InvF control the *sip* operon (13, 14). HilA directly stimulates *sip* expression and also does so indirectly, by increasing InvF, which in turn activates *sip*, probably through the use of an alternative promoter (13). Thus, the control of *sip* by HilA has both *invF*-dependent and -independent components. We found CsrA to exhibit this same pattern of control over *sip* (Table 3). An in-frame deletion of *invF* reduced expression of the *sipC* fusion 13-fold. Expression was, however, additionally reduced an average of 11-fold in the Δ*invF* Δ(*csrA*)::Cam double mutant. This reduction shows that at least some portion of the control of *sip* by CsrA does not require InvF.

One possible conclusion from the above results is that CsrA controls invasion genes solely by its effects through *hilA*. CsrA, however, might additionally exert control of invasion genes

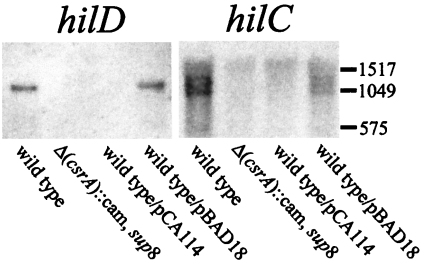

FIG. 4. Regulation of *hilD* and *hilC* by *csrA*. Northern analysis was performed using equal amounts of total bacterial RNA prepared from each of four strains and probes generated from *hilD* or *hilC*. pCA114 carries *csrA* under the control of the *araBAD* promoter on pBAD18. Strains with both plasmids were grown with 0.5% arabinose added to the medium for promoter induction.

TABLE 3. *csrA*-mediated regulation of *invF, sipC*, and *prgH*

Strain/plasmid	β-Galactosidase[a] (Miller units)		
	Φ(*sipC'-lacZY*$^+$)	Φ(*invF'-lacZY*$^+$)	Φ(*prgH'-lacZY*$^+$)
Wild type	2,700 ± 110	1,400 ± 60	890 ± 24
Δ(*csrA*)::Cam	200 ± 9	25 ± 1	72 ± 3
Δ*invF*	200 ± 5		
Δ*invF* Δ(*csrA*)::Cam	18 ± 0		
hilA339::Kan	200 ± 10	120 ± 3	3 ± 0
hilA339::Kan Δ(*csrA*)::Cam	32 ± 11	32 ± 5	2 ± 0
Wild type/*hilA*$^{+b}$	4,700 ± 61	1,800 ± 24	1,300 ± 7
Δ(*csrA*)::Cam/*hilA*$^{+b}$	870 ± 120	500 ± 19	1,400 ± 210

[a] Mean and standard error for strains tested at least in triplicate.
[b] The plasmid used was pVV214.

independently of *hilA*. To test this possibility, we examined the effect of *csrA* on *sipC, invF*, and *prgH* expression in a *hilA* mutant strain and in a strain that constitutively expresses *hilA* from plasmid pVV214 (4). If CsrA works only by controlling *hilA*, it should have no effect in either case, since in the former HilA is absent and in the latter its level remains constant. As shown in Table 3, the loss of *hilA* alone reduced expression of these three fusions 12- to 13-fold. Expression of *sipC* and *invF*, however, was further reduced in strains containing both the Δ(*csrA*)::Cam and *hilA339*::Kan mutations (six-fold for *sipC* and fourfold for *invF*). This shows that *csrA* has positive regulatory effects on *sipC* and *invF* even in the absence of HilA and so can regulate these genes independently of its role in controlling *hilA*. Further, constitutive expression of *hilA* from pVV214 failed to completely suppress the effect of *csrA* on these two genes. Expression of both *sipC* and *invF* was significantly lower in the Δ(*csrA*)::Cam mutant with pVV214 than in the wild-type strain with the same plasmid, showing that a portion of *csrA*-mediated control is independent of *hilA*. In contrast, expression of *prgH* in the Δ(*csrA*)::Cam *hilA339*::Kan double mutant was indistinguishable from that in the *hilA339*::Kan mutant alone. Similarly, constitutive *hilA* expression fully suppressed the effect of *csrA* on *prgH*. Thus, unlike the case for *sipC* and *invF*, the control of *prgH* by *csrA* appears to function solely through *hilA*.

DISCUSSION

The *csrA*/*B* regulatory system globally affects *E. coli* by altering the stability of a variety of specific mRNA targets. CsrA enhances message degradation, whereas the regulatory RNA CsrB binds to CsrA, sequestering it and thus antagonizing its effect (38). Here we identify two new roles for CsrA in *S. enterica* serovar Typhimurium, control over growth and control of SPI1 invasion genes. The finding of a growth defect in the *csrA* mutant was surprising: it was unexpected that these two closely related species would differ in their control of a process as fundamental as growth. Additionally, our previous work with serovar Typhimurium strains that overproduce CsrB showed them to grow normally (3). It has recently been reported that a *csrA rpoS* strain of *E. coli* grows poorly in media containing acetate and that suppressors are mutants of glycogen biosynthesis (54). None of the suppressors that we have isolated in the serovar Typhimurium *csrA* mutant, however, fall into this class (not shown). One possible explanation for the growth defect is that in serovar Typhimurium, additional genes have come under the control of *csrA*, the inappropriate expression of which is detrimental to growth. Several pathogenicity islands, including SPI1, could represent potential new targets of *csr* regulation, since they are present in *Salmonella* but not in *E. coli* (7, 44, 51, 55, 56). However, none of the invasion gene mutations that we tested, of both regulators and effectors of the type III secretion apparatus, affected growth of the *csrA* mutant, showing that inappropriate expression of at least these SPI1 genes cannot account for the growth defect.

We show here that *csrA* positively regulates the expression of SPI1 genes. CsrA controlled *hilA*, a regulator of the other SPI1 genes tested, and probably did so primarily by controlling regulators of *hilA*, including *hilD* and *hilC*. CsrA was also required for expression of *invF* and *sipC* independent of HilA. InvF is necessary for full expression of *sipC*, presumably by activating transcription of the *sip* operon (13, 14). Thus, the simplest model for positive control of invasion genes by CsrA has a single regulator that controls both *hilC*/*hilD* and *invF* and, in turn, is controlled by CsrA (Fig. 5). Alternatively, it is possible that CsrA independently affects *hilC, hilD, hilA, invF*, and *sipC* through the control of a number of genes in the regulatory pathway. Since CsrA works by degrading messages, its target for positive control would likely be a repressor of invasion genes, exerting its effect by decreasing the half-life of the repressor message. No repressor of *invF* has yet been identified, nor has the control of *hilC* or *hilD* been described.

Our results show that CsrA can also have negative effects on invasion gene expression. The overexpression of *csrA* had repressive effects on SPI1 genes similar to the effects of a *csrA* null mutation. Results obtained from overexpression experiments must be judged with caution, but the biological role of CsrA as a repressor is supported by the finding that its antagonist, CsrB, can activate SPI1 genes (3). These results raise the question of how CsrA can both activate and repress the same genes. Such dual control could be achieved if the regulatory activity of CsrA were directly related to its effective concentration. CsrA, for example, might have different affinities for its

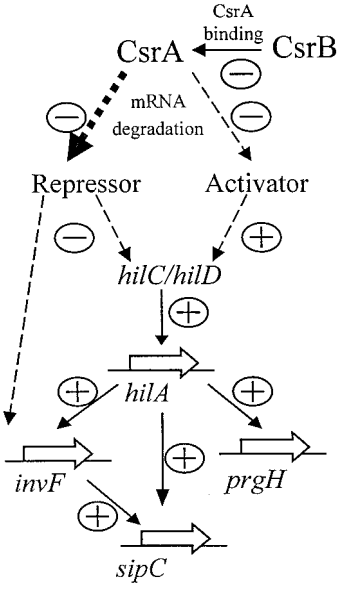

FIG. 5. A model for regulation of SPI1 genes by CsrA. Under conditions conducive to invasion, active CsrA concentration is kept low, either by decreased production or by binding to CsrB. At this concentration, CsrA preferentially degrades a repressor (heavy dashed line) that affects *hilC* and *hilD* and independently *invF*. For simplicity, positive control by CsrA is shown as resulting from the message degradation of a single repressor. Alternatively, CsrA might have more than one target in the invasion gene regulation pathway. Under invasion-repressing conditions, CsrA concentration increases, degrading the message of an activator that works through *hilD* and/or *hilC*, thus reducing the expression of invasion genes. Solid arrows represent known routes of regulation; dashed arrows show the hypothetical route of SPI1 gene control by CsrA.

various target messages. During growth under conditions favorable to invasion, CsrA could reach a concentration sufficient to affect only messages for which it had the highest affinity, presumably including repressors of invasion genes. Increasing the concentration of free CsrA, by either the loss of CsrB or the overexpression of CsrA, would lead to the control of additional genes for which it had lower affinity, including those that stimulate invasion (Fig. 5). Thus, lower concentrations of CsrA would lead to the degradation of repressor messages, while higher concentrations would cause degradation of both repressor and activator messages. Maximal invasion would therefore be achieved with a low but constant level of CsrA. Similarly, it is possible that CsrA has positive effects when it interacts with some cofactor, produced in limiting amounts, but negative effects when it does not. Such control systems would provide a rapid and extremely sensitive means to alter invasion gene expression in response to environmental conditions by changing the concentration of CsrA, CsrB, or both. Although all of the work described here examines the effects of CsrA by changing the level of *csrA* expression, it is possible that control is also achieved by altering CsrA activity. The concentration and the activity of this regulator are presumably closely related, since production of more CsrA is a likely means to overcome CsrB binding and gain higher levels of active protein. It is, however, possible that additional means exist to control CsrA by altering its activity independent of its concentration.

The regulatory RNA CsrB presumably prevents the interaction of bound CsrA with its target messages (38). It would therefore be expected that phenotypes caused by the loss of CsrA could be approximated by the overproduction of CsrB. We have shown here, however, that a *csrA* mutant grew poorly and was unable to invade epithelial cells, while strains that overproduce CsrB did not exhibit these same phenotypes (3). It is clear that overexpression of CsrB is not equivalent to the complete loss of CsrA. These seemingly conflicting findings imply that the concentration of CsrA required to affect the positive control of growth and of invasion is little changed by overproduced CsrB. It is possible that the affinity of CsrA for target messages in these pathways is much higher than its affinity for CsrB, thus allowing some active CsrA to be present despite the increased concentration of CsrB. It is also possible that the binding of CsrA to CsrB does not inactivate the protein; CsrB-bound CsrA might still be able to bind target messages. In this case, CsrB would not sequester CsrA, but each CsrB RNA would instead amass 18 to 20 CsrA molecules, effectively decreasing the CsrA concentration.

CsrA and CsrB have been integrated into the complex system of invasion gene regulation, but the means by which the expression of *csrA* and *csrB* is controlled is unknown. It has recently been shown that in *P. fluorescens*, GacA, the SirA homologue, and RsmA, the CsrA homologue, exhibit an opposing, postranscriptional control of genes that requires the same nucleotide sequence in the region of the ribosome binding site (8). It has been proposed that RsmA recognizes this site, allowing it to bind to and degrade specific messages, and that GacA exerts it control indirectly by either repressing *rsmA* or activating *rsmB*. It has also been shown that the two component-regulator GacA/GacS controls the untranslated regulatory RNA PrrB, similar in structure to CsrB (1). In serovar Typhimurium, an analogous system of *csr* control would have the two-component regulator BarA/SirA repressing *csrA* or activating *csrB*. In support of this, we found that multicopy expression of *csrB* completely suppressed the invasion defect of a *barA* null mutant. It is unlikely, however, that BarA/SirA could work solely by activating *csrB*, since a *barA* mutant had a more severe invasion phenotype than did a *csrB* mutant (3). It remains possible, however, that the regulatory effects of BarA/SirA stem from repression of *csrA*.

Several environmental cues are also known to affect the expression of invasion genes, but it is not known which, if any, might be mediated through *csr*. In *E. coli*, many *csr*-regulated genes are involved with carbohydrate biosynthesis, making nutrient status a candidate, but the environmental signals to which *csr* responds have not been identified (49). In *Erwinia carotovora*, secretion of virulence proteins under control of *rsmA/B* is also controlled by a quorum sensing mechanism (10), suggesting that bacterial density or nutrient limitation could provide the signal. In serovar Typhimurium, the integration of invasion genes into the *csr* control scheme could mean that any of the environmental signals that influence SPI1 gene expression, including pH, osmolarity, and growth phase, is the *csr* signal. If so, bacterial exposure to the intestinal lumen would induce the expression of invasion genes at a time most productive for virulence.

ACKNOWLEDGMENTS

We thank Russ Maurer, Catherine Lee, and Virginia Miller for generous gifts of strains and plasmids, and we thank Heran Darwin for technical assistance. We also thank Paul Orndorff, Russ Maurer, and Dorothy Debbie for critical review of the manuscript.

This work was supported by a CVM grant to C.A.

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Editor: A. D. O'Brien