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SirA Orthologs Affect both Motility and Virulence

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The *sirA* gene of *Salmonella enterica* serovar Typhimurium encodes a two-component response regulator of the FixJ family that has a positive regulatory influence on the expression of type III secretion genes involved with epithelial cell invasion and the elicitation of bovine gastroenteritis. SirA orthologs in *Pseudomonas, Vibrio*, and *Erwinia* control the expression of distinct virulence genes in these genera, but an evolutionarily conserved target of SirA regulation has never been identified. In this study we tested the hypothesis that *sirA* may be an ancient member of the flagellar regulon. We examined the effect of a *sirA* mutation on transcriptional fusions to flagellar promoters (*flhD, fliE, fliF, flgA, flgB, fliC, fliD, motA*, and *fliA*) while using fusions to the virulence gene *sopB* as a positive control. SirA had only small regulatory effects on all fusions in liquid medium (less than fivefold). However, in various types of motility agar plates, *sirA* was able to activate a *sopB* fusion by up to 63-fold while repressing flagellar fusions by values exceeding 100-fold. Mutations in the *sirA* orthologs of *Escherichia coli, Vibrio cholerae, Pseudomonas fluorescens*, and *Pseudomonas aeruginosa* result in defects in either motility or motility gene regulation, suggesting that control of flagellar regulons may be an evolutionarily conserved function of *sirA* orthologs. The implications for our understanding of virulence gene regulation in the gamma *Proteobacteria* are discussed.

The animal and plant pathogens of the gamma subdivision of *Proteobacteria* cause vast amounts of agricultural damage and human disease. These pathogens include members of the genera *Pseudomonas, Erwinia, Escherichia, Vibrio*, and *Salmonella*. The individual species among these genera are very diverse in some respects. They include free-living species, symbionts, commensals, plant pathogens, and animal pathogens. However, they also have striking similarities. For instance, a single locus has been identified as a transcriptional regulator of genes involved with secondary metabolism and/or virulence in all five genera. The locus is known as *gacA* in *Pseudomonas* species, *varA* in *Vibrio cholerae, expA* in *Erwinia carotovora, uvrY* in *Escherichia coli*, and *sirA* in *Salmonella enterica* serovar Typhimurium. These five genes (*sirA, varA, gacA, expA*, and *uvrY*) are orthologs based on the following criteria. They are highly conserved (each pair wise comparison shows at least 54% amino acid identity). The genomic context of each gene is conserved (each is located directly upstream of *uvrC*). Finally, in the four genera studied, the genes have similar functions (regulation of secondary metabolism and/or virulence; see below). For simplicity, the *sirA* orthologs of all species (*uvrY, varA, gacA*, and *expA*) will be referred to as *sirA* throughout this report.

By sequence homology, *sirA* orthologs encode a two-component response regulator of the FixJ family. The *E. coli* SirA ortholog is phosphorylated by a sensor kinase named BarA (53). Genetic evidence suggests that the SirA orthologs of *Salmonella, Erwinia*, and *Pseudomonas* are also phosphorylated by proteins orthologous to BarA of *E. coli*. The BarA ortholog is known as BarA in *Salmonella*, ExpA in *Erwinia*, and GacS, LemA, or PheN in *Pseudomonas* (7, 12, 22, 29, 31, 42, 50, 56, 57). For simplicity, the *barA* orthologs of all species (*expA* and *lemA*/*gacS*/*pheN*) will be referred to as *barA* throughout this report.

The phenotypes of *sirA* mutants suggest that SirA is near the top of a virulence gene regulatory cascade in each of the pathogens listed above. In *V. cholerae*, the *sirA* ortholog is required for expression of the ToxR regulon and colonization of the murine intestine (74). The *sirA* ortholog is required for extracellular enzyme production and plant virulence in *Erwinia carotovora, Pseudomonas syringae, Pseudomonas aereofaciens, Pseudomonas marginalis*, and *Pseudomonas viridiflava* (10, 12, 20, 42, 43). *Pseudomonas aeruginosa* requires the *sirA* ortholog for proper expression of the LasR and RhlR quorum-sensing cascade (55), which influences *rpoS* expression (38), extracellular virulence factor production (24, 51, 71), biofilm formation (16), twitching motility (28), and virulence in plant, animal, and nematode models (54, 61). Switching between the pathogenic wild-type and nonpathogenic phenotypic variant forms of *Pseudomonas tolaasii* involves a reversible DNA rearrangement within the *barA* (*pheN*) locus (30). A *sirA* ortholog is also required for swarming motility in *Pseudomonas syringae* (35) and expression of antifungal compounds and extracellular enzymes by *Pseudomonas fluorescens* (23, 39). In both *E. coli* and *P. fluorescens*, the *sirA* ortholog affects the expression of *rpoS*, which regulates oxidative stress resistance (49, 53, 70). In *Azotobacter vinelandii*, the *sirA* and *barA* orthologs regulate polymer synthesis (9).

In *Salmonella* serovar Typhimurium, *sirA* is required for optimal invasion of epithelial cells (32) and elicitation of fluid secretion and neutrophil migration into bovine ligated ileal loops (bovine gastroenteritis) (2). To do this, SirA positively regulates a pathogenicity island that encodes a type III secretion system (SPI1 for *salmonella* pathogenicity island 1). This secretion system directly injects effector proteins into the cytosol of host cells (11). Alteration of host cell signaling ensues, which can lead to uptake of bacteria into the host cell via

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TABLE 1. Strains and plasmids used

Strain or plasmid	Genotype or description	Source, construction, or reference
MG1655	Wild-type *Escherichia coli*	*E. coli* Genetic Stock Center
RG133	MG1655 *uvrY33*::Tn*5*	This study
14028	Wild-type *S. enterica* serovar Typhimurium	American Type Culture Collection
BA746	14028 *sirA3*::cam	2
BA1526	14028 *sopB1526*::MudJ	2
BA1726	14028 *sirA3*::cam *sopB1526*::MudJ	2
0395	*Vibrio cholerae*	Stephen Calderwood (74)
SW33S	0395 *varA*::*aphA-3*	Stephen Calderwood (74)
PAO1	*Pseudomonas aeruginosa*	Dieter Haas (55)
PAO6281	PAO1 *gacA*::Sm/Sp	Dieter Haas (55)
CHAO	*Pseudomonas fluorescens*	Dieter Haas (39)
CHA89	CHAO *gacA*::kan	Dieter Haas (39)
SM10λpir	*E. coli thi-1 thr leu tonA lacY supE recA*::RP4-2-*tet*::Mu-1*kan*::Tn*7* integrant λpir	48
S17-1λpir	*E. coli recA pro hsdR* <RP4-2-*tet*::Mu-1*kan*::Tn*7*> λpir	60
BW20767	*E. coli* RP4-2-*tet*::Mu-1*kan*::Tn*7* integrant *leu-63*::IS*10 recA1 creC510 hsdR17 endA1 zbf-5 uidA* (Δ*Mlu*I)::*pir*$^+$ *thi*	46
RG200	14028 *flhD*$^+$/*flhD*::*lacZY* integrant	This study
RG201	BA746 *flhD*$^+$/*flhD*::*lacZY* integrant	This study
RG202	14028 *fliA*$^+$/*fliA*::*lacZY* integrant	This study
RG203	BA746 *fliA*$^+$/*fliA*::*lacZY* integrant	This study
RG207	14028 *fliC*$^+$/*fliC*::*lacZY* integrant	This study
RG208	BA746 *fliC*$^+$/*fliC*::*lacZY* integrant	This study
RG211	14028 *fliE*$^+$/*fliE*::*lacZY* integrant	This study
RG212	BA746 *fliE*$^+$/*fliE*::*lacZY* integrant	This study
RG213	14028 *flgA*$^+$/*flgA*::*lacZY* integrant	This study
RG214	BA746 *flgA*$^+$/*flgA*::*lacZY* integrant	This study
pRE112	Suicide vector, Cam^r, *sacB* R6K *ori*	17
pRE112*uvrY*	pRE112 *uvrY*$^+$	This study
pRE112*uvrY33*::Tn*5*	pRE112 *uvrY33*::Tn*5*	This study
pWSK29	Low-copy-number cloning vector, Amp^r, pSC101	69
pBA305	pWSK29 *sirA*$^+$	2
pSB401	*luxCDABE* transcriptional fusion vector, Tet^r, p15A	72
pRG19	*S. enterica* serovar Typhimurium *motA*::*luxCDABE*	1885 to 2689 of accession no. D3640 into Δ*Eco*RI site of pSB401
pRG34	*S. enterica* serovar Typhimurium *fliA*::*luxCDABE*	250 to 899 of accession no. AB010947 into the filled-in Δ*Eco*RI site of pSB401
pRG38	Serovar Typhimurium *flhD*::*luxCDABE*	499 to 1501 of accession no. D43640 into Δ*Eco*RI site of pSB401
pRG39	Serovar Typhimurium *fliC*::*luxCDABE*	440 of accession no. X51740 to 619 of accession no. D13689 into Δ*Eco*RI site of pSB401 (≈800 bp)
pRG46	Serovar Typhimurium *fliD*::*luxCDABE*	Same as pRG39 (*fliC*) fragment but in reverse orientation in pSB401
pRG51	Serovar Typhimurium *flgA*::*luxCDABE*	445 of accession no. D13703 to 4200 of accession no. D25292 into Δ*Eco*RI site of pSB401 (≈850 bp)
pRG52	Serovar Typhimurium *flgB*::*luxCDABE*	Same as pRG51 (*flgA*) fragment but in reverse orientation in pSB401
pRG53	Serovar Typhimurium *fliE*::*luxCDABE*	600 of accession no. M24462 to 481 of accession no. M84993 into filled-in Δ*Eco*RI site of pSB401 (962 bp)
pRG54	Serovar Typhimurium *fliF*::*luxCDABE*	Same as pRG53 (*fliE*) fragment but in reverse orientation in pSB401
pBA409	Serovar Typhimurium *sopB*::*luxCDABE*	4 to 657 of accession no. AF021817 into Δ*Eco*RI site of pSB401
pRG26	*E. coli flhD*::*luxCDABE*	1310 to 392 of accession no. 1788200 into Δ*Eco*RI site of pSB401
pRG25	*E. coli fliA*::*luxCDABE*	1825 to 994 of accession no. 1788229 into Δ*Eco*RI site of pSB401
pRG14	*E. coli motA*::*luxCDABE*	360 of accession no. 1788200 to 10448 of accession no. 1788189 into Δ*Eco*RI site of pSB401
pVIK112	Promoterless *lacZY* suicide vector, Kan^r	34

macropinocytosis (6, 21). This invasion event is also associated with the elicitation of inflammation and fluid secretion into ligated bovine ileal loops (41; reviewed in references 62 and 68).

Although the biochemical details of SPI1 gene regulation are not known, genetically it appears that there is a regulatory heirarchy. SirA, which is encoded outside of SPI1, positively regulates another regulatory gene, *hilA*, that is encoded within SPI1 (2, 32). HilA then activates the genes that make up the structural components of the SPI1 type III secretion system and yet another regulator, *invF* (8). The entire *sirA*/*hilA*/*invF* cascade is required for the efficient expression of secreted

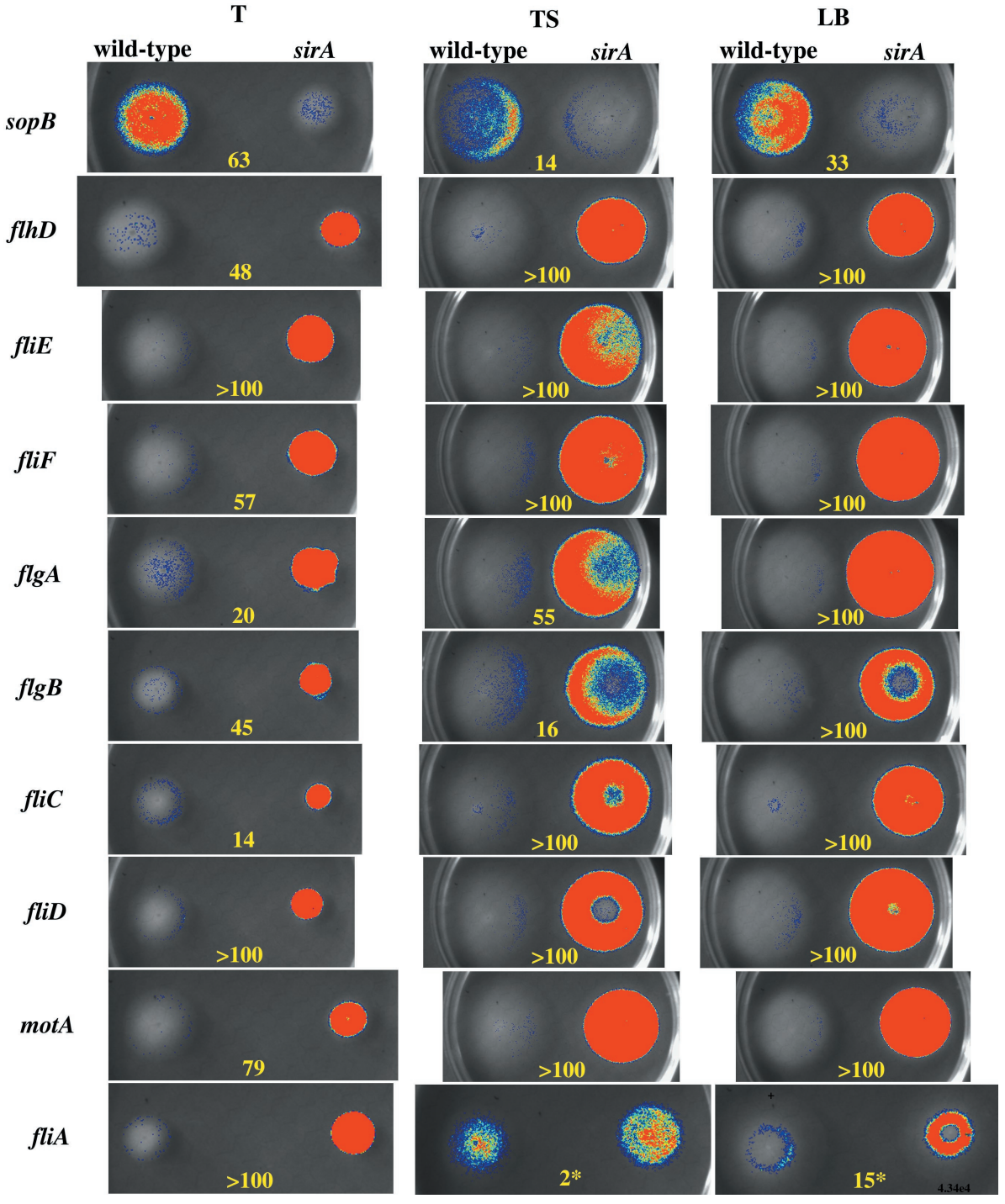

FIG. 1. SirA-dependent regulation of serovar Typhimurium *sopB* and flagellar regulon components during chemotaxing through three different types of 0.3% motility agar plates (T, TS, and LB) at 37°C. Each plate compares the expression of a particular promoter fusion in wild-type serovar Typhimurium (14028) compared to the isogenic *sirA* mutant (BA746). The plate type is indicated at the top of each column and the promoter being tested is indicated at the left of each row. Luminescence is pseudocolored, with blue indicating low intensity and red indicating high intensity. The fold difference between each pair of strains is indicated numerically. Each plate contains tetracycline for plasmid maintenance. These results are representative of at least five independent experiments. *, The *fliA* fusion gives variable results on TS and LB motility agar. See text for details.

substrates that are encoded both inside and outside of SPI1, with InvF potentially being the direct regulator (2, 8, 15, 18).

Despite the realization that *sirA* is required for virulence in several bacterial species, two observations led us to hypothesize that the primary function of *sirA* had not yet been discovered. First, *sirA* is found in both pathogens and non pathogens. Second, *sirA* is encoded within an evolutionarily conserved region of the genome, yet in every case, the virulence genes

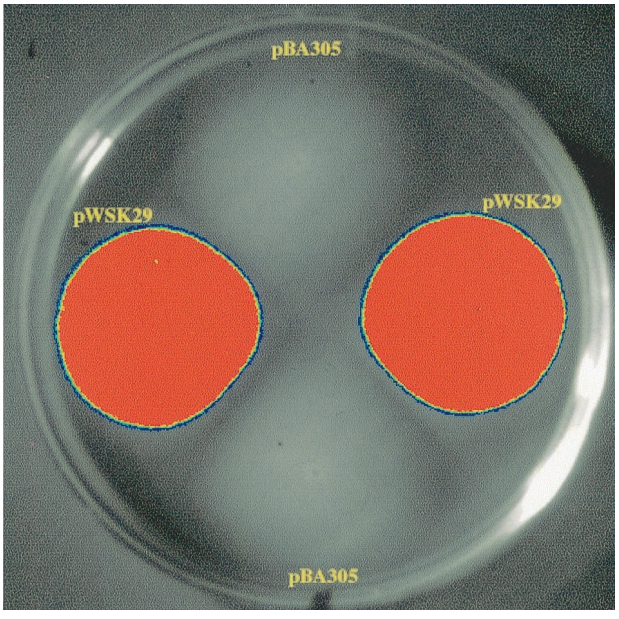

FIG. 2. Complementation of the serovar Typhimurium *sirA* mutation with regard to repression of the chemotaxis gene *motA*. A low-copy-number plasmid encoding the *Salmonella sirA* gene pBA305, or the vector control, pWSK29, was electroporated into a Serovar *Typhimurium sirA* mutant carrying the *motA*::*luxCDABE* fusion (BA746/pRG19). Each strain was inoculated in duplicate on a 0.3% TS motility agar plate containing ampicillin and tetracycline and grown at 37°C overnight. Luminescence is pseudocolored as in Fig. 1. The presence of the *sirA* plasmid but not the vector control repressed the *motA* transcriptional fusion by greater than 100-fold.

that *sirA* regulates are specific to each pathogen and probably acquired by horizontal transfer. This strongly suggested that *sirA* was present in these genomes before the acquisition of the virulence genes that it now controls. Given that flagellar regulons can influence virulence gene expression in a variety of species (13, 19, 26, 27, 33, 44, 59, 75) and that *sirA* is physically located between flagellar regions II and IIIa of the *E. coli* and *S. enterica* serovar Typhimurium genomes (32, 45), we hypothesized that SirA may be an ancient member of flagellar regulons. In this report, we have determined that SirA does indeed regulate flagellar promoters of serovar Typhimurium and *E. coli*. In addition, mutations in the *sirA* orthologs of *V. cholerae, P. aeruginosa*, and *P. fluorescens* result in motility defects, suggesting that SirA is a member of the flagellar regulons in these species as well.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown in Luria-Bertani (LB) medium or on LB supplemented with 1.5% agar (EM Science) unless otherwise indicated. Motility assays were performed with plates containing agar concentrations varying between 0.25 and 0.35% (EM Science) in either LB medium, T medium (1% tryptone; Difco), TS medium (T plus 1% NaCl), or TSG medium (TS plus 0.2% glucose), as indicated. M9 minimal glucose medium was made as described (47). Ampicillin, tetracycline, chloramphenicol, kanamycin, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were added at 100, 20, 30, 60, and 80 μg/ml, respectively, when appropriate.

Construction of an *E. coli uvrY*::Tn*5* mutant (RG133). The *E. coli* ortholog of *sirA, uvrY*, was disrupted with a Tn*5* insertion. To do this, the region of DNA surrounding *uvrY* (ca. 860 bp upstream and 200 bp downstream of *uvrY*) was amplified using *Pfu* Turbo DNA polymerase (Stratagene) with MG1655 as the template DNA. The forward primer was BA402 (ATCTCTGAGAATACGGT CAATTTCCAC), and the reverse primer was BA256 (AACCGTTACATCAA TTTGCTGGATC). The resulting PCR product was cloned using pCR-Blunt II-Topo (Invitrogen). The *uvrY* fragment was subsequently removed by *Xba*I and *Sac*I restriction and ligated into the mobilizable *sacB* suicide vector pRE112 (Cam^r) digested with *Xba*I and *Sac*I to give plasmid pRE112*uvrY*. This plasmid was mutagenized in vitro using the EZ::TN insertion kit (Epicentre Technologies). The mutagenized plasmids were transformed into S17λpir, selecting for kanamycin and chloramphenicol resistance. Transformants with *uvrY*::Tn*5* insertions were identified by PCR screening with the reverse primer of EZ::TN (Epicentre) and primer BA256. This screening strategy identifies only Tn*5* insertions in which the Kan^r gene of Tn*5* is oriented opposite to *uvrY*. Insertion points were confirmed using DNA sequencing with the forward and reverse primers of EZ::TN (Epicentre). A single insertion in the 56th codon of *uvrY* was chosen for further study and designated *uvrY33*::Tn*5*. To recombine this allele into the MG1655 chromosome, SM10λpir carrying pRE112*uvrY33*::Tn*5* was mated with MG1655, selecting for kanamycin resistance on M9 minimal medium. To select against plasmid integrants, the transformants were pooled, incubated at 37°C in shaking LB broth for 8 h, and plated on LB-kanamycin lacking NaCl but containing 5% sucrose (17). PCR screening with primers BA256 and BA402 confirmed the absence of the *uvrY*$^+$ allele and the presence of the *uvrY33*::Tn*5* allele. One isolate was designated RG133 and kept for further study.

Reporter constructions. To examine the regulation of flagellar genes, both episomal *luxCDABE* and chromosomal merodiploid *lacZY* transcriptional fusions were constructed. pSB401 is a reporter vector containing a p15A origin of replication, a tetracycline resistance marker, and a promoterless *luxCDABE* operon from *Photorhabdus luminescens* (72). Upstream of the luciferase operon is an *Eco*RI fragment containing a *luxI* promoter from *Vibrio fischeri*. This fragment was removed and replaced with regulatory regions of interest. The regulatory regions were amplified using *Pfu* Turbo DNA polymerase (Stratagene) with 14028 as the template (Table 1). The resulting PCR products were gel purified using Qiagen gel extraction columns and cloned using pCR-Blunt II-Topo (Invitrogen). The cloning site of pCR-Blunt II-Topo is flanked by *Eco*RI sites, so the *Eco*RI fragment of each clone was gel purified and ligated into the Δ*Eco*RI site of pSB401. The *fliA* and *fliE* promoters contain an internal *Eco*RI site, so the blunt-ended PCR product was ligated directly into pSB401 that had been digested with *Eco*RI and filled in using the Klenow fragment of DNA polymerase. The *fliF* promoter DNA fragment is identical to that of *fliE* except that they are in opposite orientations with respect to *luxCDABE*. The *flgA* and *flgB* fusions, as well as the *fliC* and *fliD* fusions, are also identical DNA fragments cloned in the opposite orientation with respect to *luxCDABE*. The reporter plasmids were placed into the appropriate strains using electroporation with a Bio-Rad Gene Pulser II.

Chromosomal merodiploid *lacZY* transcriptional fusions were constructed to the promoters of *flhD, fliA, fliC, fliE*, and *flgA*. For *flhD, fliC*, and *flgA*, this was done by removing the promoter region from the appropriate pSB401-based *luxCDABE* fusion plasmid (pRG38, pRG39, and pRG51 respectively) by *Eco*RI digestion and inserting it into the *Eco*RI site of the suicide vector pVIK112 (34). In the case of *fliA* and *fliE*, a blunt-ended PCR product (identical to that described above for construction of pRG34 and pRG53, respectively) was ligated directly into the *Sma*I site of pVIK112. BW20767 carrying the resulting plasmids was mated with wild-type and *sirA* mutant serovar Typhimurium, selecting for plasmid integrants by kanamycin resistance on M9 minimal medium. Transconjugants were designated RG200 to RG214 (Table 1) and examined for *sirA*-dependent gene regulation in TS motility agar containing kanamycin and X-Gal (80 μg/ml, final concentration).

Assay of luciferase activity. Luciferase activity was measured after growth of the bacteria under three types of conditions: shaking liquid culture, standing liquid culture, and motility agar. Shaking cultures were 5-ml cultures in tubes (18 by 150 mm) rotating at 50 rpm at a nearly horizontal angle. At various time points, the optical density of these cultures at 550 nm was measured using a Spectronic 20D+ or a Beckman DU-64 spectrophotometer. Samples (10 μl) were then taken for measurement of luciferase activity in a Turner Designs TD-20/20 luminometer. Results are expressed as relative light units per second. The standing liquid culture is a 1:50 subculture of an overnight culture which is left standing without agitation at 37°C for 6 h (40). At the 6-h point, luciferase activity was measured in a 10-μl sample with the Turner Designs TD-20/20 luminometer. All luminometer samples were oxygenated by "ratcheting" the sample tube across a tube rack prior to insertion into the luminometer.

Expression of luciferase activity in motility agar plates was imaged and quantitated using a Hamamatsu C2400-32 intensified charge-coupled device camera with an Argus 20 image processor. Images were captured with a Macintosh G4 computer and Adobe Photoshop 5.0 software. Comparison of light intensity between two strains within the same image is very accurate within a 2-log linear

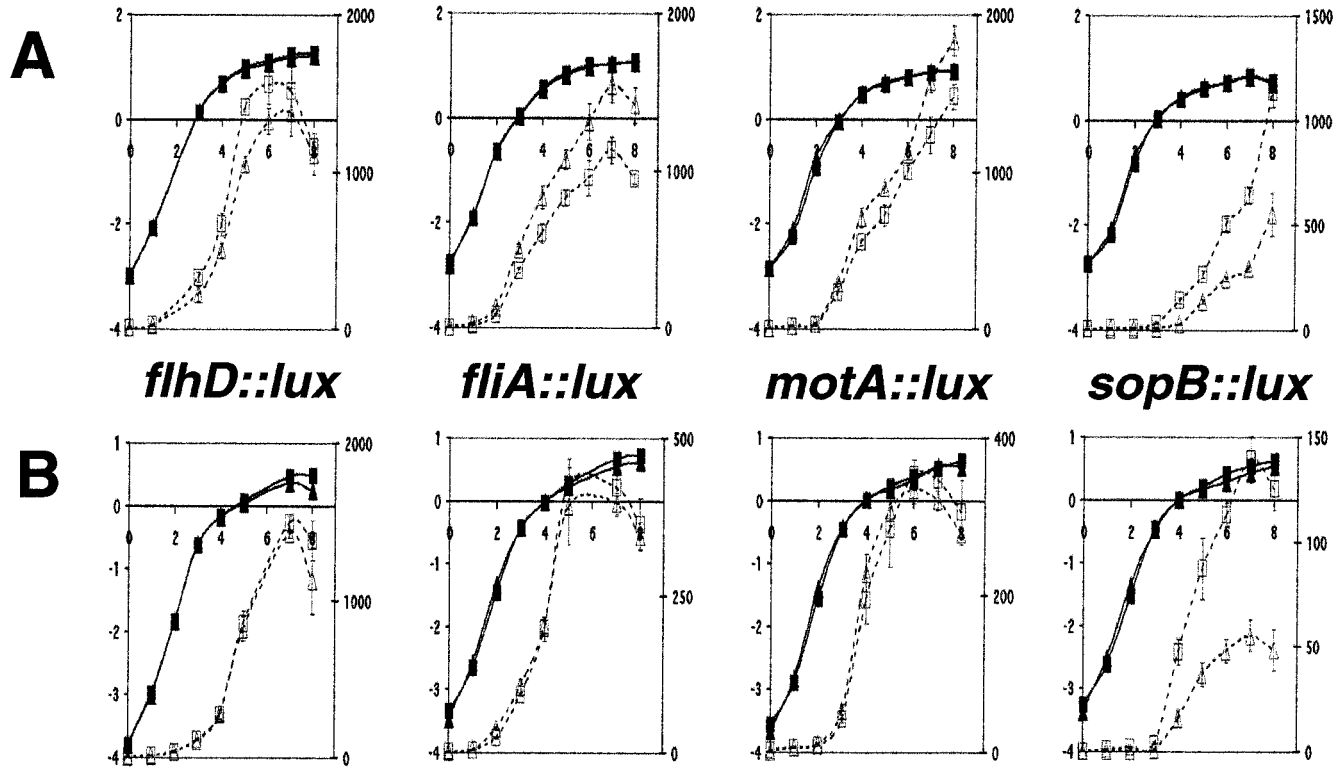

FIG. 3. SirA-dependent regulation of serovar Typhimurium *sopB* and flagellar regulon promoter fusions in shaking liquid medium. The expression of the *flhD, fliA, motA*, and *sopB* promoters was measured using plasmid-based transcriptional fusions to luciferase. Each fusion was placed into wild-type (14028) or *sirA* mutant (BA746) *Salmonella* strains. The fusion being measured is indicated. Two types of growth media were used: (A) LB and (B) TS. Solid symbols, natural log of the optical density of the culture at 550 nm, left side *y* axis; open symbols, luciferase activity, in RLU per second, right side *y* axis. Time (hours) is indicated on the *x* axis. Squares indicate the wild-type background, and triangles represent the *sirA* mutant background. Experiments were performed on three occasions. Values shown are the mean ± standard deviation of triplicate cultures from one representative experiment.

range. However, the optimal intensifier setting required to get each sample into the linear range varies from plate to plate. Therefore, all results are expressed as fold differences in luminescence between two strains on the same plate. Comparisons of light intensity between different images are not valid. Comparing gene expression of strains growing on the same plate also prevents plate-to-plate variations in thickness, moisture content, etc.

RESULTS

SirA dramatically affects the flagellar regulon during growth in motility agar. There are three levels to flagellar biosynthesis. The level 1 proteins, FlhD and FlhC, form a heterotetramer that is required for transcriptional activation of the level 2 genes, which encode the hook-basal body complexes and the alternative sigma factor FliA. The FliA sigma factor allows expression of the level 3 genes, which encode the filament protein, hook-associated proteins, motor proteins, and chemotaxis proteins (36, 37). The level 3 genes are further subdivided into level 3a and level 3b to distinguish those that have some *fliA*-independent expression (level 3a) from those that do not (level 3b) (45). To examine the effect of a *sirA* mutation on the expression of these genes, we constructed plasmid-based transcriptional fusions to genes representing each level of the regulon (Table 1).

Cloning of regulatory regions into pSB401 results in fusions to a promoterless *luxCDABE* operon of *Photorhabdus luminescens* (72, 73). This operon encodes both luciferase (LuxAB) and the enzymes that synthesize the substrate (LuxCDE), so that light is produced in response to gene expression. The level 1 fusion is to the *flhDC* operon. Level 2 is represented by fusions to the *fliA, fliE, fliF, flgA*, and *flgB* promoters. The *fliD* promoter represents level 3a, and the *fliC* and *motA* promoters represent level 3b. Each plasmid-based fusion was electroporated into both wild-type and *sirA* mutant serovar Typhimurium strains (14028 and BA746).

By monitoring luciferase activity in these strains, SirA was found to have repressing effects on all levels of the flagellar regulon (Fig. 1). The repressing effect was maximal while the bacteria were actively chemotaxing through motility agar (Fig. 1). Under these conditions, the *sirA* mutant expressed at least 100-fold more luciferase activity than the wild type from all of the level 1, 2, and 3 flagellar fusions (Fig. 1). Interestingly, despite the high levels of *sirA*-dependent flagellar gene regulation, the *sirA* mutant is nearly identical to the wild type with regard to swarm size.

The *fliA* fusion was unique in that it did not show a simple regulatory pattern. The *fliA* fusion demonstrated a standard *sirA*-dependent repression when grown in T motility agar but was variable in both TS and LB motility agar. In TS motility agar, the *fliA* fusion was largely unaffected by *sirA*, with assay variability ranging between threefold repression and fourfold activation. In LB motility agar, the results varied widely from

experiment to experiment, with values ranging between 15-fold repression and 61-fold activation by *sirA* (Fig. 1). No other flagellar gene fusion behaved this way, and the basis for the variability is unknown. A merodiploid chromosomal *lacZY* fusion to *fliA* is consistently repressed by *sirA* (see below).

SirA activates the virulence gene *sopB* in motility agar. To date, SirA has never been found to have a repressing effect on any gene in any species. We wanted to determine if the repressing behavior of SirA on the flagellar fusions was due to the growth of *Salmonella* in motility agar or was unique to the flagellar genes. Therefore, a luciferase transcriptional fusion was constructed to the *Salmonella* virulence gene *sopB*. This fusion was placed into both wild-type and *sirA* mutant serovar Typhimurium, and expression was examined during growth in T, TS, and LB motility agar. In TS agar, the *sopB* fusion was expressed at 14-fold higher levels in the wild type than in the *sirA* mutant (Fig. 1). In LB, the effect was 33-fold, and in T agar, the effect was 63-fold (Fig. 1). This demonstrates that SirA positively regulates *sopB* regardless of growth medium and that the repressing effect of SirA is restricted to the flagellar fusions.

Regulatory effects of a *sirA* mutation can be complemented by plasmid-encoded *sirA*. The *sirA* gene is directly upstream of *uvrC*, which raised the possibility that the effects of the *sirA3*::cam mutation are due to polarity on downstream genes. To confirm that the regulatory effects of the *sirA3*::cam mutation are not due to secondary mutations or polarity effects on downstream genes, a complementation experiment was performed. A low-copy-number plasmid encoding the *sirA* gene of serovar Typhimurium (or the vector control) was electroporated into a serovar Typhimurium *sirA3*::cam mutant (BA746) carrying the *motA*::*luxCDABE* fusion plasmid (pRG19). The presence of the *sirA* plasmid but not the vector control fully repressed the *motA* transcriptional fusion (Fig. 2). This demonstrates that *sirA* is responsible for the regulatory effect on *motA*. Complementation was used previously to confirm the regulatory role of *sirA* on *sopB* (2).

SirA is less active in liquid media. The activity of each transcriptional fusion was examined throughout the growth curve in either agitated LB broth or agitated TS medium at 37°C (Fig. 3). In both media, *sirA* had only small effects on virulence and flagellar gene expression. The level 2 and 3 flagellar fusions were slightly repressed by *sirA*, but never by more than twofold. SirA had no detectable effect on the *flhD* fusion under these conditions. The *sopB* virulence gene fusion was activated threefold by *sirA*. Therefore, under these conditions, the activity of SirA appears to be minimal, although the magnitude of repression of the flagellar genes mirrors the magnitude of activation of *sopB*.

Serovar Typhimurium intestinal virulence genes require low-oxygen conditions for maximal expression (40). A common in vitro technique to obtain maximal serovar Typhimurium virulence gene expression is to allow standing subcultures to reach the late exponential or early stationary phase of growth (40). Without agitation, these cultures rapidly become microaerophilic and express the invasion genes of SPI1. We hypothesized that the effect of *sirA* on virulence and flagellar genes would be higher under these conditions than in the agitated LB cultures. Therefore, the reporter fusions were subcultured into LB broth and allowed to stand at 37°C without agitation for 6 h before measurement of luciferase activity. Under these conditions, *sirA* had very little repressing effect on the flagellar genes (less than twofold) and had a fivefold positive effect on the virulence gene *sopB* (Fig. 4). This fivefold activation of *sopB* is similar in magnitude to previous reports on the activation of secreted effector genes by SirA (2, 32, 44). Clearly the *sirA* gene has much larger regulatory phenotypes in motility agar than in either agitated or standing liquid medium.

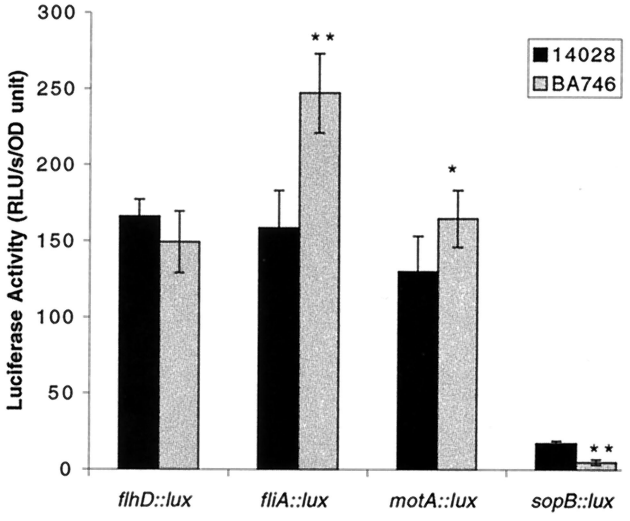

FIG. 4. Effect of *sirA* on the expression of *sopB* and the flagellar regulon under oxygen-limiting conditions. The activity of each promoter fusion was measured in serovar Typhimurium wild-type (14028) and *sirA* mutant (BA746) backgrounds during growth in standing LB cultures and indicated as RLU per second per optical density unit. Data are means ± standard error of three independent experiments of triplicate cultures. Statistically significant differences (*, $P < 0.05$; **, $P < 0.01$) between wild-type and *sirA* mutant activity are indicated.

Chromosomal *lacZY* fusions are also regulated by *sirA*. To examine the effects of *sirA* on the flagellar regulon with a second methodology, we constructed and tested chromosomal *lacZY* fusions to all levels of the serovar Typhimurium flagellar regulon: *flhD* (level 1), *fliA, fliE*, and *flgA* (level 2), and *fliC* (level 3). Examining the regulation of these genes in motility agar requires that they be able to swim, and therefore functional merodiploids were created. This was done by placing the promoter regions of these genes into the *Eco*RI site of the suicide vector pVIK112, which creates *lacZY* transcriptional fusions (34). BW20767 carrying the resulting plasmids was mated with wild-type and *sirA* mutant Typhimurium strains, selecting for kanamycin resistance and counterselecting for prototrophy. Transconjugants were then examined for *sirA*-dependent gene regulation in motility agar containing the colorimetric β-galactosidase substrate X-Gal (Fig. 5). It is difficult to quantitate the degree of blue color in the motility agar, but a qualitative assessment indicated that *sirA* has a repressing effect on all levels of the flagellar regulon (Fig. 5). A previously described chromosomal *sopB*::MudJ insertion (which creates a *lacZY* transcriptional fusion) was also examined in this manner (BA1526 compared to BA1726 [2]). As expected, the *sopB*::MudJ insertion was positively regulated by *sirA* in motility agar (Fig. 5). However, *sirA* has more dramatic effects on the plasmid-based luciferase fusions than it has on the chromosomal *lacZY* fusions. This could be due either to copy number effects

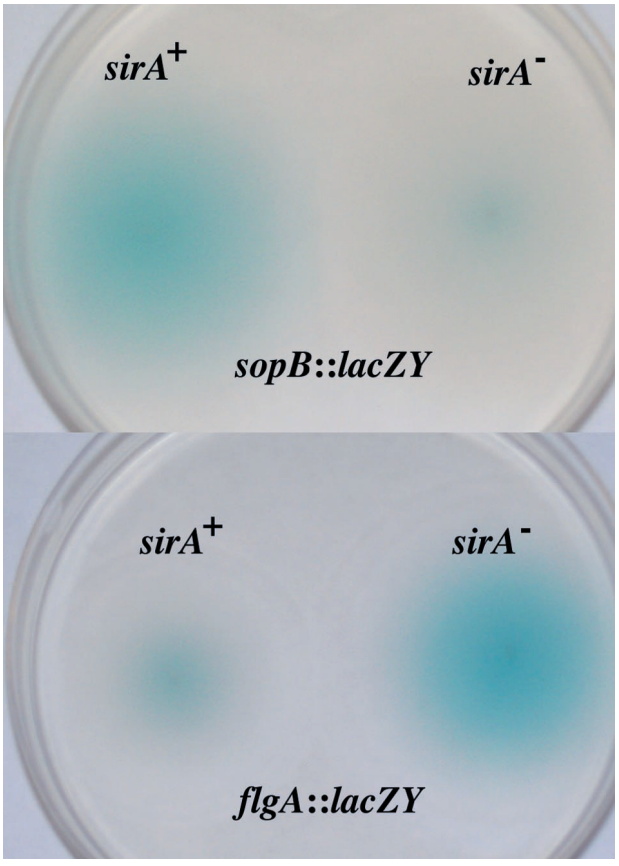

FIG. 5. SirA-dependent regulation of *sopB* and *flgA* chromosomal *lacZY* fusions during chemotaxing through 0.3% TS agar containing kanamycin and X-Gal at 37°C. Each plate compares the β-galactosidase activity of a *sopB*::MudJ fusion or a chromosomal merodiploid *flgA*$^+$/*flgA*::*lacZY* promoter fusion in wild-type (*sirA*$^+$) and *sirA* mutant (*sirA*$^-$) *Salmonella* backgrounds. Fusions to *flhD, fliA, fliC*, and *fliE* demonstrated a repression similar to that seen with *flgA* (not shown).

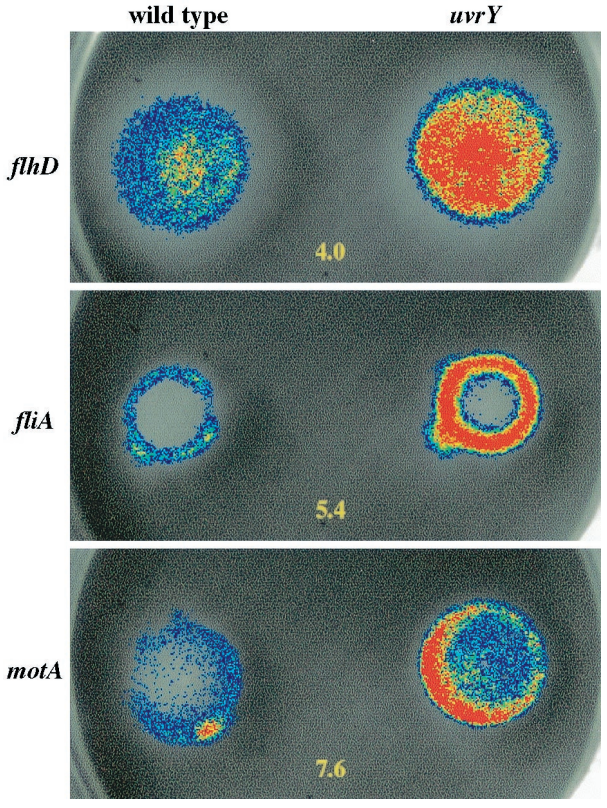

FIG. 6. SirA-dependent regulation of *E. coli* flagellar promoters during chemotaxing through 0.25% TS agar at 37°C. Each plate compares the expression of a particular promoter fusion to *luxCDABE* in wild-type *E. coli* (MG1655) compared to the isogenic *uvrY (sirA)* mutant (RG133). The promoter being tested is indicated at the left of each row. Luminescence is pseudocolored as in Fig. 1. The fold difference between each pair of strains is indicated numerically. Each plate contained tetracycline for plasmid maintenance. These results are representative of at least three independent experiments.

of the plasmid-based fusions or to the accumulation of blue precipitate when using X-Gal, which would mask repressing effects. In either case, both the chromosomal *lacZY* fusions and the plasmid-encoded *luxCDABE* fusions indicate that *sirA* positively regulates the virulence gene *sopB* and negatively regulates the flagellar regulon of serovar Typhimurium.

SirA-dependent regulation of the flagellar regulon is evolutionarily conserved. We hypothesized that regulation of the flagellar regulon may be an evolutionarily conserved function of SirA orthologs in the gamma proteobacteria. Therefore, we first examined the regulation of the *E. coli* flagellar regulon by the *E. coli* ortholog of *sirA*, which is named *uvrY*. Transcriptional fusions to *E. coli* flagellar genes and a *uvrY* mutant of *E. coli* were constructed (see Materials and Methods). The *E. coli* fusions were electroporated into both wild-type *E. coli* (MG1655) and the isogenic *uvrY* mutant (RG133). As was the case in serovar Typhimurium, the flagellar gene fusions of *E. coli* are repressed by *uvrY*, although the magnitude of the repression is not as great (Fig. 6). Also, like the *sirA* mutant of serovar Typhimurium, the *uvrY* mutant of *E. coli* does not have a motility defect.

To further examine the issue of SirA orthologs being evolutionarily conserved members of flagellar regulons, we examined the motility phenotypes of *sirA* mutants in three other species: *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* (in which the *sirA* ortholog is named *gacA*) and *Vibrio cholerae* (in which the *sirA* ortholog is named *varA*). All three species were found to have motility defects compared to the wild type (Fig. 7). These results are unlike those obtained with *E. coli* and serovar Typhimurium, in which *sirA* does not confer a motility defect, but suggest that *sirA* (*gacA*/*varA*) regulates motility genes, either positively or negatively, in these species as well. Further studies in *Pseudomonas* and *Vibrio* will be required to determine the precise role that GacA and VarA have in flagellar gene expression.

DISCUSSION

Numerous members of the gamma proteobacteria require *sirA* orthologs to cause disease. In each species, the *sirA* ortholog controls the expression of unique virulence genes. Because the virulence genes are unique to each species and often appear to be recent horizontal acquisitions, we hypothesized that regulation of these genes must be a relatively new function

for the *sirA* orthologs (1). If true, *sirA* orthologs must have a more ancient and evolutionarily conserved function(s) that remains to be discovered (assuming that those functions have not been lost). Identification of the conserved functions of *sirA* orthologs is very important because it may provide clues to the environmental and/or physiological signals that lead to SirA activation. This was recently demonstrated with the *phoPQ* regulatory locus of *S. enterica* serovar Typhimurium, in which the identification of Mg^{2+} transporters as part of the PhoPQ regulon led to the discovery that PhoQ is a sensor of extracellular cation concentrations (25, 67). The unidentified signal(s) leading to activation of SirA orthologs is rather paradoxical. The gamma proteobacterial pathogens cause disease in organs as different as lungs and intestines and organisms as diverse as plants and animals. What signal could be common to a plant, a lung, and an intestinal tract? And why would any signal that is so common be so important?

Recently, it was discovered that the *sirA* orthologs of *P. fluorescens* and *E. coli* regulate the evolutionarily conserved gene *rpoS* (49, 53, 70). Therefore, regulation of *rpoS* appears to be the first example of an evolutionarily conserved function for *sirA* orthologs. In this study we have determined that *sirA* orthologs from *E. coli* and serovar Typhimurium have repressive effects on the flagellar genes of these species. Motility defects in *sirA* mutants of *P. fluorescens, P. aeruginosa*, and *V. cholerae* confirmed that control of flagellar regulons is an evolutionarily conserved function of *sirA* orthologs.

In *S. enterica* serovar Typhimurium, SirA was found to repress all levels of the flagellar regulon while activating virulence gene expression. SirA had much larger effects on virulence and flagellar fusions when the bacteria were grown in motility agar rather than in liquid medium. It is unclear whether growth in motility agar is directly stimulating SirA activity or whether the effect is indirect, potentially by removing the competitive effects of other regulators. However, the presence of high levels of SirA activity in motility agar suggests a physiological activation signal rather than a host-derived signal.

At this time, SirA has not been biochemically demonstrated to bind directly to any promoter in any species. Therefore, we do not know at what level SirA exerts its influence on the flagellar regulon. The simplest hypothesis is that SirA represses the master regulator of the flagellar regulon *flhDC*, which leads to decreased expression of all the flagellar gene fusions examined in this study. It is also possible that SirA only indirectly affects *flhDC* by controlling the expression of another regulator that directly modulates *flhDC* expression. Further genetic and biochemical studies are required to determine precisely how SirA affects the flagellar regulon.

There are also multiple scenarios by which SirA could simultaneously affect both motility and virulence genes. The simplest hypothesis is that SirA affects flagellar and SPI1 promoters independently. A second formal possibility is that SirA activates expression of a regulatory gene within SPI1, such as *hilA*, the product of which represses the flagellar regulon. While possible, this scenario seems unlikely, since both *sirA* and the flagellar apparatus appear to have been present in the *Salmonella* genome much longer than the proposed regulatory intermediate within SPI1. The third possibility is that SirA directly regulates only the flagellar regulon, and the flagellar regulon somehow affects the expression of SPI1. Although this latter hypothesis does not correlate with the positive role for *fliZ* in SPII expression without postulating yet another regulatory intermediate, it remains very intriguing because of recent studies in which the expression of virulence genes can be affected by mutations in flagellar genes. For instance, mutations in motility genes have been identified in numerous screens for avirulent mutants in a wide variety of species (reviewed in reference 52). However, it has largely been assumed that these

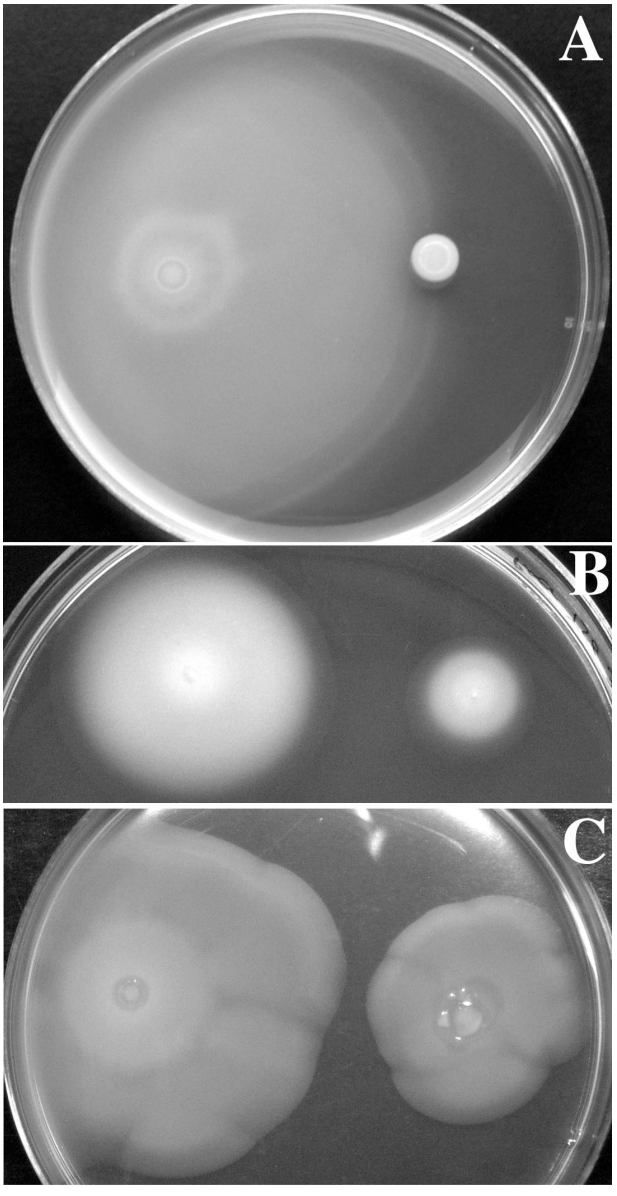

FIG. 7. Motility phenotypes of *sirA* ortholog mutants of various species. (A) *Pseudomonas fluorescens* in 0.28% T agar at 22°C. The wild-type CHAO is on the left and the *gacA* (*sirA*) mutant CHA89 is on the right. (B) *Pseudomonas aeruginosa* in 0.28% TSG agar at 37°C. The wild-type PAO1 is on the left and the *gacA* (*sirA*) mutant PAO6281 is on the right. (C) *Vibrio cholerae* in 0.35% TS agar at 37°C. The wild-type O395 is on the left and the *varA* (*sirA*) mutant SW33S is on the right.

mutants are avirulent simply because they cannot swim or properly chemotax to appropriate locations within their host. Only in the last few years has it become increasingly apparent that these mutants may be avirulent for reasons other than a lack of motility per se. Instead, these mutants may be avirulent because the flagellar regulon is required for the expression of virulence genes that were not previously recognized as part of the flagellar regulon. This was demonstrated in serovar Typhimurium, in which the *fliZ* gene and the direction of flagellar rotation were found to play a role in regulating the expression of invasion genes encoded within SPI1 (19, 33, 44). In *V. cholerae* it has been noted that motility phenotypes correlate with virulence gene expression (13, 26). In *Xenorhabdus nematophilus, flhDC* was found to be required for more than just motility and virulence in a nematode model. Instead, *flhDC* was also required for lipolysis and hemolysis in plate assays, suggesting that the flagellar regulon of this species regulates virulence genes in addition to motility genes (27). The most dramatic example of virulence gene expression being influenced by the flagellar regulatory cascade is found in *Yersinia enterocolitica*, in which the *yplA* gene encodes a phospholipase involved with virulence (58). This gene requires *flhDC* for expression, and the YplA gene product is actually secreted through the flagellar basal body (59, 75). All of these observations suggest that some component(s) of the flagellar regulon may play an active role in regulating virulence genes and/or secondary metabolism in a variety of gram-negative bacteria. Clearly there is a regulatory triad between *sirA*, the flagellar genes, and the virulence genes of several gamma proteobacterial species that needs to be further studied.

Interestingly, this triad appears to be similar to that of *Bordetella* species, which are members of the beta proteobacteria. In *Bordetella*, the *bvgAS* operon encodes the BvgA response regulator, which is phosphorylated by the sensor kinase BvgS (63–65). In the active state (the Bvg^+ phase), numerous virulence genes are activated and motility genes are repressed (3–5, 14). In the Bvg^- phase, the organism is motile but not virulent. While it might seem that SirA and BarA are simply distantly related orthologs of BvgA and BvgS, respectively, the evolutionary history is not so clear. In fact, *E. coli* encodes another locus, named *evgAS*, that is more likely to be orthologous to *bvgAS* (66). The function of *evgAS* is unknown. What can be concluded is that the regulatory phenotypes discovered for the *barA*/*sirA* system of *Salmonella* are similar to those of the *bvg* system of *Bordetella*.

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