SrfJ, a *Salmonella* Type III Secretion System Effector Regulated by PhoP, RcsB, and IolR

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Virulence-related type III secretion systems are present in many Gram-negative bacterial pathogens. These complex devices translocate proteins, called effectors, from the bacterium into the eukaryotic host cell. Here, we identify the product of *srfJ*, a *Salmonella enterica* serovar Typhimurium gene regulated by SsrB, as a new substrate of the type III secretion system encoded by *Salmonella* pathogenicity island 2. The N-terminal 20-amino-acid segment of SrfJ was recognized as a functional secretion and translocation signal specific for this system. Transcription of *srfJ* was positively regulated by the PhoP/PhoQ system in an SsrB-dependent manner and was negatively regulated by the Rcs system in an SsrB-independent manner. A screen for regulators of an *srfJ-lacZ* transcriptional fusion using the T-POP transposon identified IolR, the regulator of genes involved in *myo*-inositol utilization, as an *srfJ* repressor. Our results suggest that SrfJ is synthesized both inside the host, in response to intracellular conditions, and outside the host, in *myo*-inositol-rich environments.

Type III secretion systems (T3SS) are complex devices that are present in many Gram-negative bacteria, including the animal pathogens *Yersinia* spp., enteropathogenic *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella flexneri*, *Chlamydia* spp., and *Salmonella enterica*, and the plant pathogens or symbionts *Pseudomonas syringae*, *Ralstonia solanacearum*, *Erwinia* spp., *Rhizobium* spp., and *Xanthomonas campestris* (29). T3SS allows secretion and translocation of effector proteins from bacteria to eukaryotic cells. The injected proteins are often able to interfere with host signal transduction pathways to enable bacterial invasion and survival in subcellular niches.

Salmonella enterica is a facultative intracellular bacterium responsible for gastroenteritis and systemic infections in many animals, including humans (45). *S. enterica* virulence depends on two distinct T3SS, T3SS1 and T3SS2. T3SS1 translocates proteins through the plasma membrane of the host cell and is necessary for invasion, whereas T3SS2 is induced intracellularly, injects effector through the membrane of the *Salmonella*-containing vacuole (SCV), and is essential for survival and proliferation inside host cells. Both systems, however, depend on each other for efficient functioning (1). The genes encoding the structural components, many effectors, and some regulators of T3SS1 and T3SS2 are located in two *Salmonella* pathogenicity islands, SPI1 and SPI2, respectively (16, 17, 21, 41, 44, 54). Some effector proteins are encoded by genes outside SPI1 and SPI2.

SsrA (also named SpiR) and SsrB are the sensor and the response regulator, respectively, of a two-component regulatory system encoded by SPI2 that is necessary for the expression of T3SS2 (9, 28, 44). A genetic screen identified 12 genes (named *srfA* to *srfM*, for SsrB-regulated factors) outside SPI2 that were regulated by SsrB (62). These genes were located in horizontally acquired regions and, because of their pattern of expression, were suggested as candidates to encode T3SS2 effectors (61, 62). However, to date, the product of only one of these genes, *srfH* (also named *sseI*), has been demonstrated to be translocated into host cells through T3SS2 (39). One of the most interesting genes identified in this screen was *srfJ*. An *srfJ* null mutant has a slight virulence defect (49). More interestingly, the product of this gene, SrfJ, is similar to human lysosomal glucosylceramidase, an enzyme involved in regulating metabolism of ceramide, one of the most intensely studied classes of sphingolipids (4, 30).

The potential of *srfJ* prompted us to investigate in detail its pattern of expression and the possibility of secretion of its product, SrfJ, through a T3SS. Here, we show that SrfJ can be secreted *in vitro* and that the amino-terminal region of this protein directs translocation into macrophages specifically through T3SS2. *srfJ* is regulated not only by SsrB but also by PhoP, RcsB, and, surprisingly, by IolR, the main regulator of genes involved in *myo*-inositol utilization (33).

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and strain construction. *E. coli* and *S. enterica* serovar Typhimurium strains used in this study are described in Table 1. *Salmonella* strains derive from the mouse-virulent strain ATCC 14028. Transductional crosses using phage P22 HT 105/1 *int201* (53) were used for strain construction (37). To obtain phage-free isolates, transductants were purified by streaking on green plates (6). Phage sensitivity was tested by cross-streaking with the clear-plaque mutant P22 H5.

Bacterial culture. The standard culture medium for *S. enterica* and *E. coli* was Luria-Bertani broth (LB). Solid LB contained 1.5% agar (final concentration). Antibiotics were used at the following concentrations: kanamycin (Km), 50 μg ml^{-1}; chloramphenicol (Cm), 20 μg ml^{-1}; ampicillin (Ap), 100 μg ml^{-1}; and tetracycline (Tc), 20 μg ml^{-1}. Subinhibitory concentrations of polymyxin B sulfate (Sigma) used in some experiments were 1 μg ml^{-1} in LB and 5 μg ml^{-1} in LPM. For some experiments, 40 μg ml^{-1} 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-Gal) or 55.5 mM *myo*-inositol was added. For SPI1-inducing conditions, *Salmonella* strains were grown overnight at 37°C in LB–0.3 M NaCl medium in static conditions. For SPI2-inducing conditions, cells from cultures in LB were washed and diluted 1:125 with minimal medium

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
E. coli strains		
DH5α	*supE44 ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	26
TP610	F⁻ *thi-1 thr-1 leuB6 lacY1 tonA21 supE44 hsdR hsdM recBC lop-11 lig⁺ cya-610*	27
S. enterica serovar Typhimurium strains		
14028	Wild type	ATCC
55130	14028 *pho-24* (PhoP constitutive)	E. A. Groisman
SV4699	14028 *phoP7953*::Tn*10*	Laboratory stock
SV4758	14028 *rcsC55*	19
SV5049	14028 Δ*rcsB*::Cm^r	Laboratory stock
SV5093	14028 Δ*rcsA*::Cm^r	18
SV5136	14028 *ssaV*::Cm^r	Laboratory stock
SV5373	14028 Δ*hilA*	Laboratory stock
SV5379	14028 Δ*prgH*	Laboratory stock
SV5452	14028 Δ*ssrB*::Cm^r	18
SV5470	14028 *ssaV*::*lacZ*	18
SV5599[a]	14028 *srfJ*::3×FLAG Km^r	This study
SV5600[a]	14028 *srfJ*::*lacZ*	This study
SV5604	14028 Δ*prgH ssaV*::Cm^r	Laboratory stock
SV6017	14028 ΔSPI2::Cm^r	Laboratory stock
SV6055	14028 ΔSPI1::Km^r	Laboratory stock
SV6891	14028 Δ*iolR*::Cm^r	This study
Plasmids		
pBAD18	*bla* P$_{BAD}$ promoter	25
pCE36	*aph* FRT *lacZY*⁺ *t$_{his}$* oriR6K	15
pCP20	*bla cat cI*857 λP$_R$ *flp* pSC101 oriTS	7
pKD3	*bla* FRT *cat* FRT PS1 PS2 oriR6K	11
pKD4	*bla* FRT *aph* FRT PS1 PS2 oriR6K	11
pKD46	*bla* P$_{BAD}$ *gam bet exo* pSC101 oriTS	11
pIZ1673	pSIF003-R1 Δ*lacI*	5
pIZ1843	pIZ1673-SrfJ	This study
pIZ1844	pIZ1673-SrfJ(1-42)	This study
pIZ1856	pIZ1673-SrfJ(1-20)	This study
pIZ1897	pIZ1673-SrfJ(1-10)	This study
pIZ1898	pIZ1673-SrfJ(10-42)	This study
pIZ1899	pIZ1673-SrfJ(20-42)	This study
pIZ1973	pBAD18-*iolR*⁺	This study
pIZ1974	pBAD18-*phoP*⁺	This study

[a] Derivatives of these strains were used as indicated in the text.

at pH 5.8 (LPM) containing 80 mM 2-(N-morpholino)ethanesulfonic acid (pH 5.8), 5 mM KCl, 7.5 mM (NH₄)₂SO₄, 0.5 mM K₂SO₄, 0.1% Casamino Acids, 38 mM glycerol, 337.5 μM K₂HPO₄-KH₂PO₄ (pH 7.4), and 8 μM MgCl₂, and then incubated overnight at 37°C with shaking. For some experiments, the concentration of MgCl₂ or the pH of the medium was modified as indicated. Arabinose was used at a final concentration of 0.2%.

DNA amplification with PCR. Amplification reactions were carried out in a Perkin Elmer Gene-Amp PCR system 2400 (Perkin Elmer Cetus). The final volume of reactions was 50 μl, and the final concentration of MgCl₂ was 1.5 mM. Reagents were used at the following concentrations: deoxynucleoside triphosphates (dNTPs), 300 μM; primers, 0.3 μM; and *Taq* polymerase (KAPA HiFi DNA Polymerase; Kapa Biosystems), 1 U per reaction. The thermal program included the following steps: (i) initial denaturation for 5 min at 95°C; (ii) 25 cycles of denaturation (98°C, 20 s), annealing (57°C, 15 s), and extension (72°C, 30 s); and (iii) final incubation at 72°C for 5 min to complete the extension. Primers are listed in Table 2. PCR constructs were sequenced with an automated DNA sequencer (Stab Vida, Oeiras, Portugal) to confirm that the sequence was correct.

Plasmids. Plasmids used in this study are listed in Table 1. Plasmids expressing CyaA′ fusions were derivatives of pIZ1673, a modification of pSIF003-R1 (58) (a gift from I. Rosenshine, The Hebrew University of Jerusalem) that was constructed by deletion of the *lacI* gene using enzymes HpaI and SphI. Therefore, expression of CyaA fusions from derivatives of pIZ1673 is no longer dependent on isopropyl-β-D-thiogalactopyranoside (IPTG). To construct these plasmids, DNA from strain 14028 was used as a template for PCR amplification with the primers listed in Table 2. The amplified fragments were digested with BamHI and ligated with BamHI-digested and dephosphorylated pIZ1673. To detect successful constructs (plasmid with insert in the correct orientation), the ligation mixture was transformed into *E. coli* TP610 and transformants were selected in MacConkey agar supplemented with Ap and 1% maltose. TP610 is a *cya* mutant strain (22) that can be complemented with the basal adenylate cyclase activity that possesses CyaA from *Bordetella pertussis* in the absence of calmodulin. Transformants with empty plasmids or with inserts in the wrong orientation give white colonies in MacConkey-maltose, whereas plasmids with inserts in the correct orientation give red colonies in the same medium. Derivatives of plasmid pBAD18 were used for complementation studies. To construct pIZ1973 and pIZ1974, *iolR* and *phoP*,

TABLE 2 Oligonucleotides used in this study

Oligonucleotide and use	Sequence (5′–3′)
Insertion in *srfJ*	
srfJP1	CAGATCGACTCCTGCCGCCATAGCAACGTACTGGCGCCTGGTGTAGGCTGGAGCTGCTTC
srfJP2	ATGAAAGGCAGACTCATCTCTTCCGATCCGTATCGTCAGCCATATGAATATCCTCCTTAG
Insertion in *iolR*	
iolRP1	TGGTTATTTACGAAATTTTCGTTCTATTAGAGTATCATGCGTGTAGGCTGGAGCTGCTTC
iolRP2	AATGCCGATGATCGCTAAATACGATCATCGGCTTGTTTTTCATATGAATATCCTCCTTAG
Epitope tagging of *srfJ*	
srfJP1flag	GTCAGGCGCCAGTACGTTGCTATGGCGGCAGGAGTCGATCGACTACAAAGACCATGACGG
srfJP2flag	TTCGCATACAGGTCGCTTCATTAAATCCCAGCTTCATCATCATATGAATATCCTCCTTAG
Construction of pIZ1843	
srfJbampsif5′	GAATGGATCCAGGAGGTTCCCTATGAAAGGCAGACTCATCTC
srfJbampsif3′	CAAGGGATCCAGATCGACTCCTGCCGCCATAG
Construction of pIZ1844	
srfJbampsif5′	
srfJ42bampsif3′	CAAGGGATCCGTAACGCGTGGCGCGGTAAGAC
Construction of pIZ1856	
srfJbampsif5′	
srfJ20bampsif3′	CAAGGGATCCCCGCACGCTCAACAAGGAATTG
Construction of pIZ1897	
srfJbampsif5′	
srfJ10bampsif3′	CAAGGGATCCACGGATCGGAAGAGATGAGTC
Construction of pIZ1898	
srfJ10bampsif5′	GAATGGATCCAGGAGGTTCCCTATGCCGTATCGTCAGCAATTCCTTG
srfJ42bampsif3′	
Construction of pIZ1899	
srfJ20bampsif5′	GAATGGATCCAGGAGGTTCCCTATGGCGGTCTCTTTTTCGCATCG
srfJ42bampsif3′	
Construction of pIZ1973	
iolReco5′	CTAGGAATTCAGGAGGTCATGCATGTCTAAACATCAAAC
iolRhind3′	CATGAAGCTTTTACTCCGTCGCCAGCGCCAG
Construction of pIZ1974	
phoPeco5′	CTAGGAATTCAGGAGGAAGAGATGATGCGCGTACTG
phoPhind3′	CATGAAGCTTTTAGCGCAATTCAAAAAGATATC
ST-PCR	
tpop1	GCCTTCTTATTCGGCCTTGAATTGATCATATGCGG
tpop2	CTTTTTCCGTGATGGTAACC
st1	GGCCACGCGTCGACTAGTAC
stACGCC	GGCCACGCGTCGACTAGTACNNNNNNNNNNNACGCC
stGATAT	GGCCACGCGTCGACTAGTACNNNNNNNNNNNGATAT

respectively, were PCR amplified with the primers indicated in Table 2, which introduce EcoRI and HindIII sites. Digestions with these enzymes were used for oriented cloning in pBAD18.

Mammalian cell culture. RAW264.7 cells (murine macrophages; ECACC no. 91062702) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum; 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin were included in the culture media. Cells were maintained in a 5% CO_2 humidified atmosphere at 37°C.

Construction of mutants and of a chromosomal *lacZ* fusion. Disruption and replacement of *srfJ* with a Km resistance gene and of *iolR* with a Cm resistance gene were performed as described previously (11). Briefly, the Km resistance gene from plasmid pKD4 or the Cm resistance gene from plasmid pKD3 was PCR amplified with primers srfJP1 and srfJP2 or with primers iolRP1 and iolRP2, respectively. The sequences of the primers used are shown in Table 2. The PCR products were used to transform the wild-type strain carrying the Red recombinase expression plasmid pKD46. The antibiotic resistance cassette introduced by the gene-targeting procedure was eliminated by recombination using the FLP helper plasmid pCP20 (11). The FRT site (FLP recombinase recognition target) generated by excision of the antibiotic resistance cassette in *srfJ* was used to integrate plasmid pCE36 to generate a transcriptional *lacZ* fusion (15).

Chromosomal gene epitope tagging. Addition of a DNA fragment encoding the 3× FLAG epitope tag at the 3′ end of *srfJ* (designated SrfJ-3×FLAG) was carried out as described previously (57) using primers srfJP1flag and srfJP2flag.

β-Galactosidase assays. Levels of β-galactosidase activity were assayed for stationary-phase cultures in LB or in minimal medium as described previously (40) using the CHCl₃/SDS permeabilization procedure.

Western blotting and antibodies. *Salmonella* strains were grown under different conditions. Usually, cultures in LB medium were diluted and grown in different media. The bacteria were then pelleted by centrifugation and resuspended in SDS-PAGE sample buffer. Proteins from the same numbers of bacteria were separated by gradient SDS-PAGE (Mini-Protean TGX precast gels; 4 to 15%; Bio-Rad) and electrophoretically transferred to nitrocellulose filters for Western blot analysis using anti-Flag M2 monoclonal antibodies (1:5,000; Sigma), anti-GroEL polyclonal antibodies (1:20,000; Sigma), or anti-DnaK (8E2/2) monoclonal antibodies (1:5,000; Assay Designs). Goat anti-mouse horseradish peroxidase (HRP)-conjugated antibodies (1:5,000; Bio-Rad) and goat anti-rabbit HRP-conjugated antibodies (1:10,000; GE Healthcare) were used as secondary antibodies.

Protein secretion analysis. *Salmonella* strain SV5599 (14028 expressing SrfJ-3×FLAG) and *prgH* or *ssaV* derivatives were grown overnight in LPM at pH 5.8, followed by 1 h of incubation at pH 7.2 or overnight incubation in LB supplemented with 55.5 mM *myo*-inositol. Whole cells and culture supernatants were separated by centrifugation at $13,000 \times g$ for 10 min. The supernatants were filtered (0.22-μm pore size) and incubated for 30 min on ice with 0.02% deoxycholate. Proteins were then precipitated by adding trichloroacetic acid at a final concentration of 10% (vol/vol), followed by incubation at −20°C for 5 min, placement on ice for 15 min, and then centrifugation ($13,000 \times g$, 4°C, 15 min). The supernatant was discarded and the pellet was incubated with 300 μl of cold acetone for 15 min on ice. After a new centrifugation step, the pellet and the whole cells were processed for electrophoresis and Western blotting.

Bacterial infection of cultured cells. RAW 264.7 cells were plated in 24-well plates at 1.5×10^5 cells per well and incubated for 24 h at 37°C with 5% CO_2. Bacteria were grown in LB medium for 24 h at 37°C with shaking and were added at a multiplicity of infection (MOI) of 250. Bacteria were centrifuged onto the cell monolayer at $200 \times g$ for 5 min and then incubated at 37°C with 5% CO_2. The cell culture was washed twice with phosphate-buffered saline (PBS) 1 h postinfection, overlaid with DMEM containing 100 μg ml⁻¹ gentamicin, and incubated for another hour. The culture was then washed twice with PBS, covered with DMEM with gentamicin (16 μg ml⁻¹), and incubated for 14 h.

Protein translocation assays. Following the infections described above, the translocation of SrfJ-CyaA fusions into the eukaryotic cells was monitored by measuring the levels of cyclic AMP (cAMP). The infected cells were lysed and the levels of cAMP in the lysates were determined using a colorimetric direct cAMP enzyme immunoassay kit (Arbor Assays) according to the manufacturer's instructions.

Mutagenesis with T-POP. Strain 14028 of *S. enterica* serovar Typhimurium was mutagenized with T-POP, a derivative of Tn*10*dTc (48). Pools of 5,000 colonies, each carrying an independent T-POP insertion, were then prepared and lysed with phage P22 HT. The lysates were used to transduce strain SV5600 (14028 *srfJ*::*lacZ*), with selection of Tc-resistant transductants on LB plates supplemented with X-Gal, Tc, and Km.

Molecular characterization of T-POP insertions. A semirandom, two-step PCR protocol, ST-PCR (8), was used to amplify genomic regions adjacent to the T-POP insertions. The first reaction was carried out on bacterial colonies with primers tpop1 and stACGCC or stGATAT (Table 2) in a final volume of 25 μl using a thermal program with the following steps: (i) initial denaturation for 2 min at 94°C; (ii) 6 cycles of denaturation (94°C, 30 s), annealing (42°C, 30 s, −1°C for each cycle), and extension (72°C, 3 min); (iii) 25 cycles of denaturation (94°C, 30 s), annealing (65°C, 30 s), and extension (72°C, 3 min); and (iv) final incubation at 72°C

for 7 min to complete extension. The second reaction was carried out with primers tpop2 and st1 using 1 μl of a 5-fold dilution of the product of the first reaction. The thermal program for the second reaction was (i) initial denaturation for 30 s at 94°C; (ii) 30 cycles of denaturation (94°C, 30 s), annealing (56°C, 30 s), and extension (72°C, 2 min); and (iii) final incubation at 72°C for 7 min to complete extension. The final product was sequenced using primers tpop2 and st1. Sequence analysis was performed with molecular biology algorithms from the National Center for Biotechnology Information at www.ncbi.nlm.nih.gov.

RESULTS

***srfJ* is expressed under SPI2-inducing conditions.** In the course of an infection, *S. enterica* receives different environmental cues that lead to expression of T3SS1 or T3SS2 and translocation into the host cells of their cognate effectors. Different host environments can be partially mimicked *in vitro* using different culture conditions: overnight incubation with low aeration in LB medium supplemented with 0.3 M NaCl favors expression of T3SS1, and culture in LPM medium at pH 5.8 with good aeration induces expression of T3SS2. A *lacZ* transcriptional fusion was used to study the pattern of expression of *srfJ*. Data shown in Fig. 1A indicated that *srfJ* was transcribed specifically under T3SS2-inducing conditions. Increases in pH or Mg^{2+} concentration dramatically reduced transcription of *srfJ*, indicating that these were relevant factors in the regulation of the expression of this gene. The results at the transcriptional level were confirmed at the protein level using an SrfJ-3×FLAG fusion and immunoblotting with an anti-FLAG antibody (Fig. 1B).

SrfJ is secreted into the culture media under SPI2-inducing conditions. Although coexpression of *srfJ* with T3SS2 (shown in the previous section) and regulation by SsrB (62) are consistent with a role of SrfJ as a substrate of T3SS2, secretion of this protein had not been previously shown. A derivative of strain 14028 of *S. enterica* serovar Typhimurium expressing a SrfJ-3×FLAG fusion was used to test secretion into the culture medium. Initial attempts to detect the protein in the supernatants of overnight cultures in LPM (pH 5.8) were unsuccessful. Secretion of many T3SS2 effectors into the culture medium can be triggered by shifting the pH of the culture medium from 5.8 to 7.2 during the last hour of incubation (65). Indeed, these conditions allowed detection of SrfJ in the culture supernatant. DnaK, a cytosolic protein used as a control, was not detected, as expected (Fig. 2). Interestingly, secretion of SrfJ was significantly reduced in an *ssaV* mutant (SsaV is an essential component of T3SS2), suggesting that secretion of SrfJ was, at least partially, dependent on T3SS2 under these *in vitro* conditions.

The N-terminal portion of SrfJ directs translocation into macrophages through T3SS2. We decided to test the translocation of SrfJ into host cells. To this end, we used a sensitive method based on the generation of fusions with the catalytic domain of CyaA from *Bordetella pertussis*, a calmodulin-dependent adenylate cyclase. Calmodulin is not produced in bacteria but is present in eukaryotic host cells. After infection of eukaryotic cell cultures with bacteria expressing a CyaA′ fusion, translocation is detected as an increase in the concentration of cAMP, whose production is catalyzed by adenylate cyclase. Analysis of the sequence of SrfJ, a protein of 447 amino acids, revealed the presence of a C-terminal domain similar to glucosylceramidases starting at amino acid 43. Since translocation signals of T3SS effectors are usually located in the N-terminal portion of the protein, we generated a CyaA fusion with
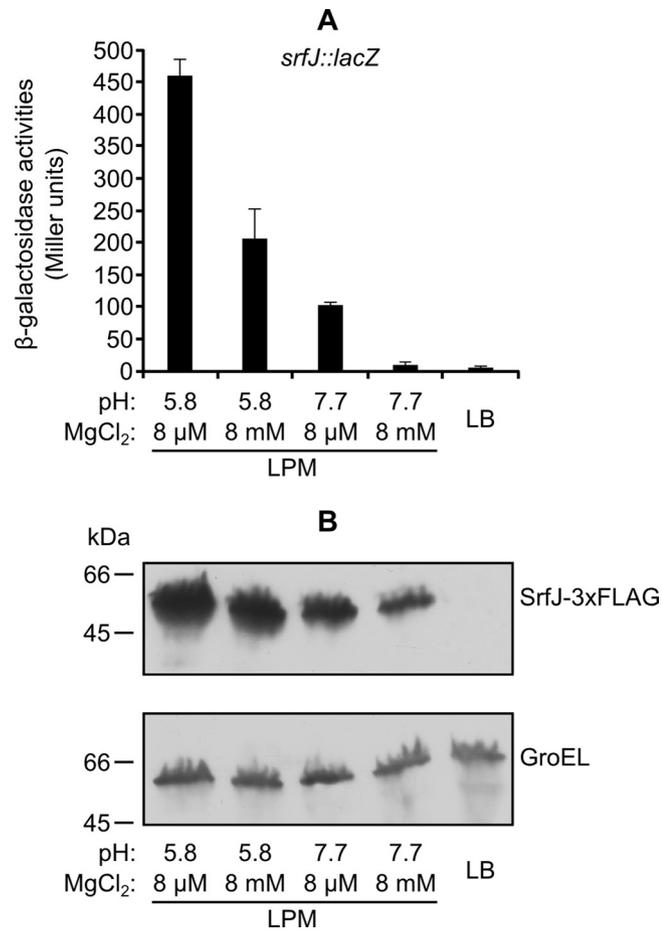
FIG 1 Expression of *srfJ* in different media. Bacteria were incubated overnight at 37°C with shaking in LPM or without shaking in LB with 0.3 M NaCl (LB). Different pH and Mg^{2+} concentrations were used as indicated. (A) β-Galactosidase activities were measured from cultures of an *S. enterica* strain carrying a chromosomal *srfJ::lacZ* transcriptional fusion. Means and standard deviations from duplicate experiments are represented. (B) Expression at the protein level was studied by Western blotting using a strain expressing SrfJ-3×FLAG. Extracts from this strain were resolved by SDS-PAGE (4 to 15% gradient), and a monoclonal anti-FLAG antibody was used for immunoblotting (upper panel). Polyclonal anti-GroEL antibody was used to get a loading control (lower panel).

amino acids 1 to 42 of SrfJ expressed from a constitutive promoter in a plasmid. A derivative of *S. enterica* serovar Typhimurium 14028 carrying this plasmid was used to infect cultures of RAW264.7 macrophages. An increase in the level of cAMP in the culture 16 h postinfection indicated translocation of the fusion into the cytosol of the host cells (Fig. 3A). Fragment 1-20 of SrfJ, but not fragment 1-10, 10-42, or 20-42, was also able to direct translocation of CyaA into macrophages. Fusions with fragments 1-42 and 1-20 were also translocated in a strain lacking T3SS1 (ΔSPI1), but they were no longer translocated in a strain lacking T3SS2 (ΔSPI2) (Fig. 3B). These results suggest that the N-terminal 20 amino acids of SrfJ are enough to direct translocation into macrophages specifically through T3SS2.

Expression of *srfJ* is regulated positively by SsrB and PhoP and negatively by RcsB. The *srfJ*::*lacZ* transcriptional fusion and the SrfJ-3×FLAG fusion were used to search for regulators of this gene. The expression of the fusions was analyzed in LPM using *Salmonella* strains with mutations in genes encoding known virulence regulators: PhoP, SsrB, HilA, and RcsB. In addition to null mutations, the activating mutations *pho-24* and *rcsC55* were also used. As seen in Fig. 4A and B, PhoP and SsrB were necessary for expression of *srfJ* in LPM. Expression of *srfJ* was increased in a *pho-24* background, confirming that PhoP was a positive regulator of this gene. Additional confirmation was provided by complementation analysis. Plasmid pIZ1974, carrying the *phoP* gene under the control of the arabinose-inducible P_{BAD} promoter, was constructed for this purpose. Arabinose-induced *phoP* expression, but not the empty vector (pBAD18), complemented the effect of *phoP* disruption and activated *srfJ* expression (Fig. 4C). The *phoP* mutant used in our experiments was the product of a Tn*10* insertion that is expected to have a polar effect on *phoQ*. Partial complementation due to expression of *phoP* alone is explained by overexpression of the transcriptional regulator PhoP, which can activate its target genes in a PhoQ-independent manner (34). In addition, overactivation of the Rcs system by the *rcsC55* mutation prevented *srfJ* transcription, suggesting that RcsB is a negative regulator of *srfJ*. This conclusion was confirmed using a ΔrcsB:: Cm^r mutation: introduction of this null mutation in the *rcsC55* mutant restored wild-type levels of *srfJ::lacZ* transcription (Fig. 4D). In contrast, a ΔrcsA::Cm^r mutation was unable to suppress the effect of the *rcsC55* mutation on the expression of *srfJ*::*lacZ*,
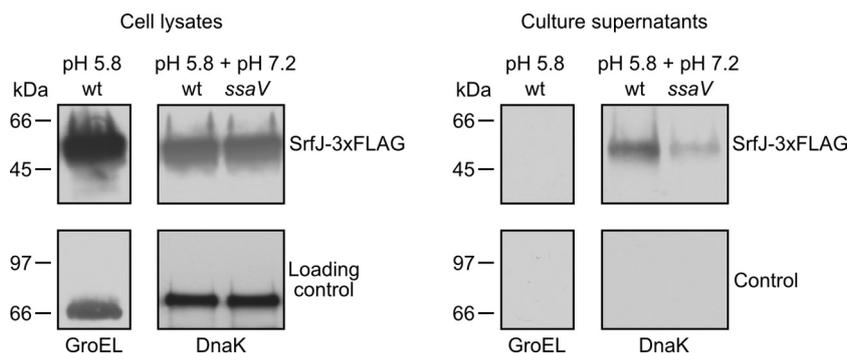

FIG 2 SrfJ is secreted into the culture medium through T3SS2. A derivative of strain 14028 of *S. enterica* serovar Typhimurium expressing SrfJ-3×FLAG either was grown overnight in LPM at pH 5.8 or was grown overnight in LPM at pH 5.8, the pH shifted to 7.2, and the incubation continued for an additional hour. After centrifugation, the supernatants were filtered and precipitated. The supernatants and aliquots of the pellets (cell lysates) were submitted to SDS-PAGE and immunoblotting with anti-FLAG antibodies (upper panels) and with anti-GroEL or anti-DnaK antibodies (lower panels). The same experiment was carried out in an *ssaV* mutant (lacking T3SS2), as indicated. wt, wild type.
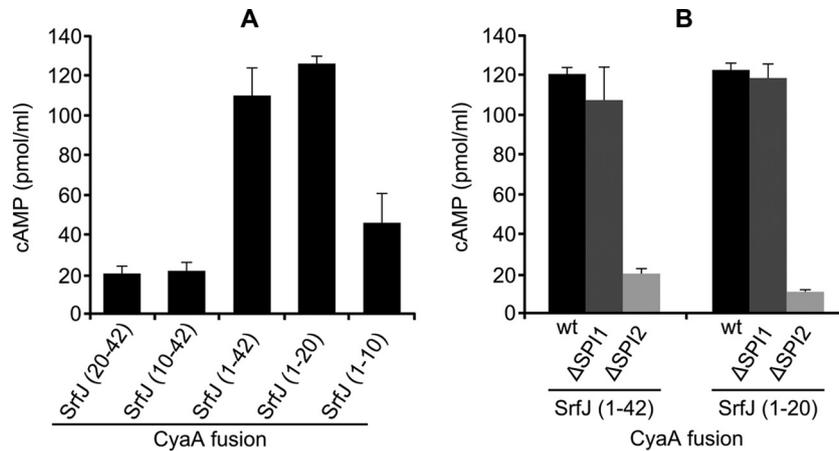
FIG 3 The 20 N-terminal amino acids of SrfJ are able to direct translocation into macrophages through T3SS2. (A) Different fragments of SrfJ were expressed in the wild-type strain of *S. enterica* serovar Typhimurium in fusion with the catalytic domain of CyaA from *B. pertussis*. RAW264.7 cells were infected with bacteria grown for 24 h in LB at 37°C with shaking, and the concentrations of cAMP were measured 16 h postinfection. Numbers in parentheses indicate the amino acids of SrfJ included in the fusion. The results are represented as the means from four independent experiments. Error bars represent standard deviations. (B) Fragments containing amino acids 1 to 42 and 1 to 20 from SrfJ fused to the catalytic domain of CyaA from *B. pertussis* were expressed in the wild-type (wt) strain of *S. enterica* serovar Typhimurium, in a strain lacking T3SS1 (ΔSPI1), and in a strain lacking T3SS2 (ΔSPI2). Cultures from these bacteria were used to infect RAW264.7 cells, and the concentrations of cAMP were measured 16 h postinfection. Means and standard deviations from two independent experiments are represented.

indicating that the coregulator RcsA does not participate in the regulation of *srfJ* transcription. *pho-24 ssrB* and *pho-24 rcsC55* double mutants were constructed to carry out epistasis analysis. The results shown in Fig. 4 suggested that PhoP activates *srfJ* through SsrB, whereas RcsB acts independently. Since both the Rcs and the PhoP/PhoQ systems are activated by cationic antimicrobial peptides, we decided to study the effect of subinhibitory concentrations of polymyxin B on *srfJ* expression. No significant modifications in the expression of *srfJ*::*lacZ* were detected after addition of polymyxin B to LB–0.3 M NaCl or to LPM (data not shown). This result is consistent with the opposite effects that these regulatory systems have on *srfJ*.

A T-POP-based screen identifies IolR as a negative regulator of *srfJ* transcription. We took advantage of the *srfJ*::*lacZ* fusion and the T-POP transposon to look for new regulators of *srfJ*. A pool of random T-POP insertions was generated in strain SV5600, a derivative of strain 14028 of *S. enterica* serovar Typhimurium carrying an *srfJ*::*lacZ* fusion. Tc-resistant isolates were selected on LB plates supplemented with Tc and the chromogenic indicator X-Gal. Most colonies were white, as expected, since *srfJ* shows a very low level of expression in this medium. However, 15 colonies out of 20,000 screened were blue. All were also blue in the absence of tetracycline, suggesting that their phenotypes were due to disruption of a regulatory gene by T-POP and not to the Tc-dependent overexpression of an adjacent gene(s) from a T-POP promoter (48). To identify the location of the T-POP insertion, a semirandom, two-step PCR protocol (described in Materials and Methods) was used to amplify the DNA adjacent to the transposon for sequencing. All of the isolates carried the T-POP transposon in the same gene: STM14_5307 (STM4417 in strain LT2), also known as *iolR* (33). To confirm the results, an *iolR* null mutant was constructed using λ Red recombineering, and the expression of *srfJ*::*lacZ* was measured in wild-type and *iolR* backgrounds. The *iolR* mutation caused a >200-fold increase in β-galactosidase activity of LB–0.3 M NaCl cultures (Fig. 5A). This increase was confirmed by immunoblotting using the SrfJ-3×FLAG fusion

(Fig. 5B). Epistasis analysis showed that this regulation was independent of SsrB and PhoP (Fig. 5A and B). Similar experiments carried out in LPM (pH 5.8) showed that the *iolR* mutation was unable to increase expression of *srfJ* in this medium (Fig. 5C and D). Interestingly, this mutation increased expression of *srfJ* in a *phoP* or an *ssrB* background (compare data in Fig. 4 and 5). Additional support for the conclusion that IolR was a repressor of *srfJ* transcription was provided by complementation analysis. As seen in Fig. 5E, derepression of *srfJ*::*lacZ* transcription in LB–0.3 M NaCl caused by disruption of *iolR* was abolished by a derivative of plasmid pBAD18 expressing wild-type *iolR* from a P_{BAD} promoter (plasmid pIZ1973) but not by the empty vector. Complementation was confirmed at the protein level in the strains expressing the SrfJ-3×FLAG fusion (Fig. 5F).

***myo*-inositol induces transcription of *srfJ* but not secretion into the medium.** The *iolR* gene product was described as a negative regulator of genes involved in *myo*-inositol utilization in *S. enterica* serovar Typhimurium (33). The presence of *myo*-inositol is expected to prevent binding of IolR to promoters. Therefore, we tested the effect of *myo*-inositol on the expression of *srfJ* at both the transcriptional and protein level. As seen in Fig. 6A and B, addition of 55.5 mM *myo*-inositol to LB–0.3 M NaCl induced expression of *srfJ* to the same level as that obtained for the *iolR* mutant in LB–0.3 M NaCl in a PhoP-, SsrB-, and RcsB-independent manner. SrfJ-3×FLAG was not detected, however, in the supernatant of cultures in LB–0.3 M NaCl with *myo*-inositol (Fig. 6B), indicating that *myo*-inositol induces expression but not secretion under these conditions. The effect of *myo*-inositol on *srfJ* expression seems to be specific, since transcription of *ssaV*, another SsrB-regulated gene used for comparison, was not affected by *myo*-inositol (Fig. 6A). Finally, we compared the growth of the wild-type strain to that of a strain lacking *srfJ* in minimal medium with *myo*-inositol as the sole carbon source. No differences were observed (data not shown), indicating that SrfJ is not involved in the utilization of *myo*-inositol.
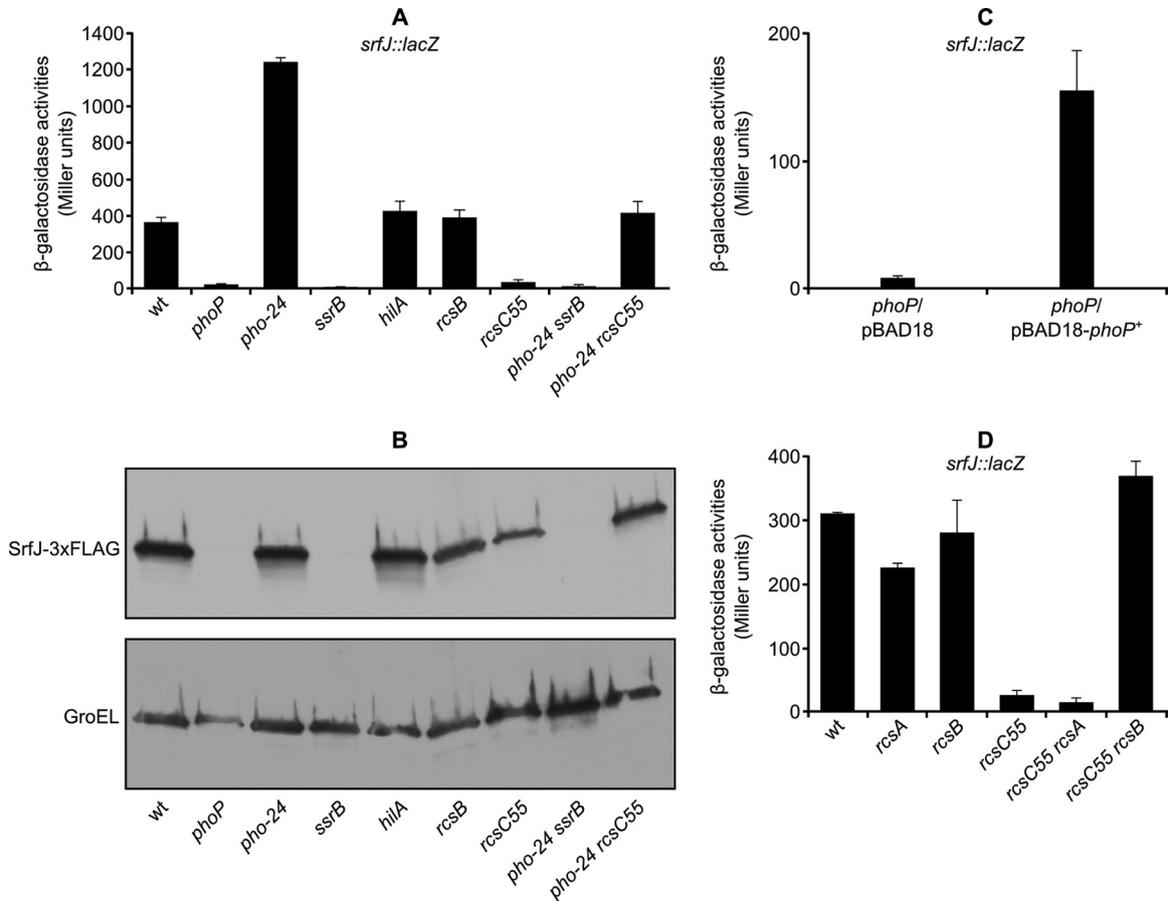
FIG 4 *srfJ* is regulated by PhoP, SsrB, and RcsB. (A) β-Galactosidase activities were measured from cultures in LPM of several *S. enterica* strains: 14028 (wild type [wt]), null mutants (*phoP*, *ssrB*, *hilA*, and *rcsB*), mutants with constitutive activation of the PhoP/PhoQ or the Rcs system (*pho-24* and *rcsC55*, respectively), and double mutants (*pho-24 ssrB* and *pho-24 rcsC55*), all of them carrying a chromosomal *srfJ*::*lacZ* transcriptional fusion. Means and standard deviations from duplicate experiments are represented. (B) Expression at the protein level was studied by Western blotting using strains expressing SrfJ-3×FLAG. Extracts from these strains were resolved by SDS-PAGE (4 to 15% gradient), and a monoclonal anti-FLAG antibody was used for immunoblotting (upper panel). A polyclonal anti-GroEL antibody was used to get a loading control (lower panel). (C) A plasmid expressing wild-type *phoP* (*phoP*⁺) under a P$_{BAD}$ promoter was used to complement the effect of a null *phoP* mutation on *srfJ* transcription. The empty vector (pBAD18) was used as a control. Means and standard deviations of β-galactosidase activities obtained from strains carrying a chromosomal *srfJ*::*lacZ* transcriptional fusion grown in LPM supplemented with 0.2% arabinose are represented. (D) To study the role of RcsB and RcsA in the regulation of *srfJ*, β-galactosidase activities were measured from cultures in LPM of wild-type (wt) *S. enterica* or mutants, as indicated, all of them carrying a chromosomal *srfJ*::*lacZ* transcriptional fusion. Means and standard deviations from duplicate experiments are represented.

DISCUSSION

Characterization of the whole catalogue of effector proteins is an important step in the understanding of the pathogenesis of bacteria that rely on T3SS for their interaction with the host. Although the components of the secretion apparatus are well conserved between distantly related bacteria, the secreted proteins are usually very different. This is a hurdle in the identification of effector proteins, in spite of the increasing availability of whole-genome sequences. Proteomic and genetic screens have led to the identification of many effectors in different bacteria but the catalogue of effectors is still growing, indicating that it is not completed yet (12, 20, 43, 46, 51, 52, 55).

In this work, we provide evidence suggesting that SrfJ, the product of an SsrB-regulated gene, is a new substrate of *Salmonella* T3SS. Regulation by SsrB is a feature shared by most T3SS2 effectors encoded inside or outside SPI2. Therefore, all *srf* genes (62) were, in principle, candidates to encode such an effector. For instance, SrfH, also called SseI, was shown to be translocated

through T3SS into the host cytosol (39), where it binds the actin-cross-linking protein filamin and colocalizes with polymerizing actin in the cytoskeleton and with TRIP6 (38, 63). There was no evidence of translocation for the other *srf* gene products. Previous work in our laboratory showed that the *srfABC* operon is not expressed in coordination with SPI2; therefore, its products, SrfA, SrfB, and SrfC, are not expected to be secreted through T3SS2 (18). In addition, no translocation was detected for SrfK using the CyaA fusion system (our unpublished results).

Translocation of SrfJ into macrophages was dependent on T3SS2 and independent of T3SS1. In addition, secretion of SrfJ into the culture medium occurred only under SPI2-inducing conditions, although a shift from pH 5.8 to 7.2 was essential to detect the protein in the supernatant of the cell cultures. Experiments by Yu et al. (65) suggest that the SPI2-encoded proteins SsaL, SsaM, and SpiC form a complex that is required for secretion of translocon proteins and suppress secretion of effectors at pH 5.0. Exposure to pH 7.2 after growth at pH 5.0 causes dissociation and
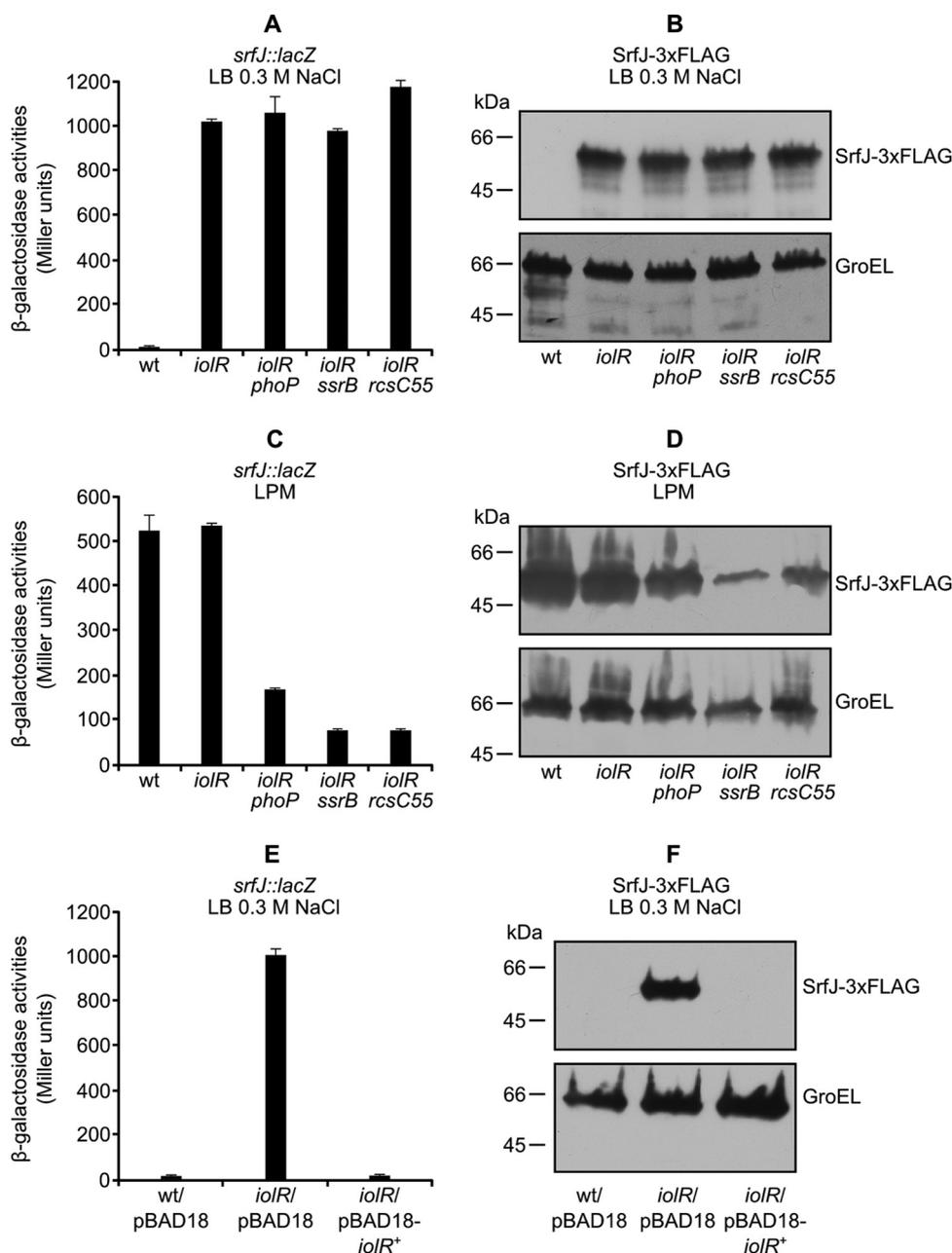
FIG 5 IolR represses *srfJ*. Expression of *srfJ* at the transcriptional level was monitored using a chromosomal *srfJ*::*lacZ* fusion. β-Galactosidase activities were measured in LB–0.3 M NaCl (A) or LPM (pH 5.8) (C) cultures from strains with different genetic backgrounds: the wild type (wt) and *iolR*, *iolR phoP*, *iolR ssrB*, and *iolR rcsC55* mutants. Means and standard deviations from duplicate experiments are represented. Expression of *srfJ* at the protein level was monitored using an SrfJ-3×FLAG fusion. An anti-FLAG antibody was used to detect the fusion protein by immunoblotting in extracts from cultures in LB–0.3 M NaCl (B) or LPM (pH 5.8) (D) of *S. enterica* serovar Typhimurium strains with the indicated genetic backgrounds (upper panels). An anti-GroEL antibody was used as a loading control (lower panels). Complementation of the effect of the *iolR* mutation on *srfJ* transcription observed in LB–0.3 M NaCl cultures expressing wild-type *iolR* (*iolR*⁺) from a P_BAD promoter was demonstrated at the transcriptional level (E) and the protein level (F).

degradation of the complex and allows effector secretion. The same requirement was previously observed for other T3SS2, like SseF and SseJ (65), but not for other effectors, like SteA, that can also be secreted through T3SS1 (5).

In most T3SS effectors studied, a small N-terminal domain is enough to direct translocation, although a conserved sequence has been identified in only a few cases (39). As a consequence, computational methods used to detect secretion signals are only par-

tially accurate (2, 36, 50, 60, 64). Our experiments with SrfJ fragments fused to CyaA showed that the minimal signal sequence necessary for translocation is contained in the N-terminal 20 amino acids of this protein. This short sequence was enough to direct translocation of CyaA specifically through T3SS2, suggesting that in this case interaction with a chaperone, as has been described for other effectors, was dispensable.

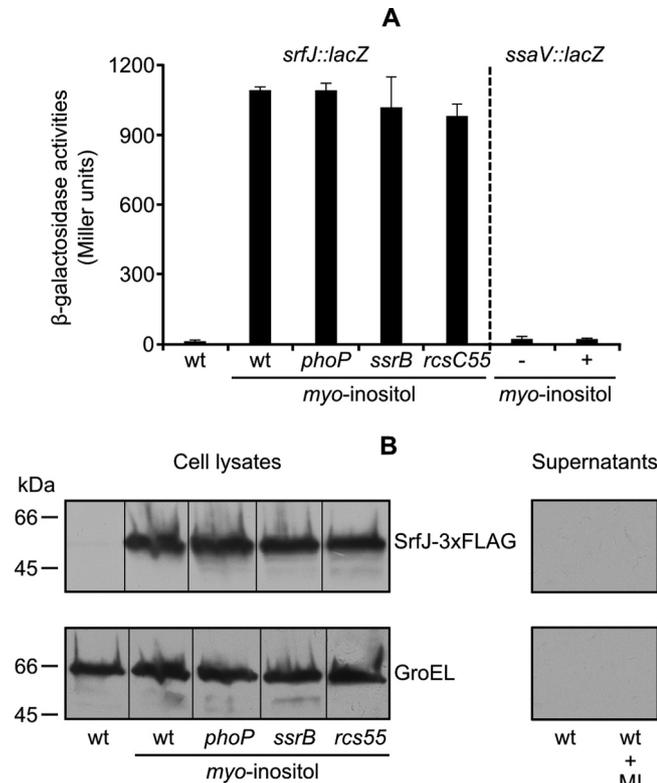As expected for a T3SS2 effector, expression of *srfJ* is coordi-

FIG 6 *myo*-inositol induces expression, but not secretion, of *srfJ* in LB–0.3 M NaCl. (A) Expression of *srfJ* at the transcriptional level was monitored using a chromosomal *srfJ*::*lacZ* fusion. Strains carrying the indicated mutations were cultured in LB–0.3 M NaCl medium or LB–0.3 M NaCl medium supplemented with *myo*-inositol. Expression of *ssaV* in LB–0.3 M NaCl medium (−) or the same medium supplemented with *myo*-inositol (+) was monitored using a chromosomal *ssaV*::*lacZ* transcriptional fusion. Means and standard deviations from duplicate experiments are represented. (B) Expression and secretion of *srfJ* at the protein level was monitored using an SrfJ-3×FLAG fusion. An anti-FLAG antibody was used to detect the fusion protein by immunoblotting in cell lysates and in concentrated supernatants from cultures in LB–0.3 M NaCl medium of *S. enterica* serovar Typhimurium strains with the indicated mutations (upper panel). An anti-GroEL antibody was used as the loading control (lower panel). wt, wild type; MI, *myo*-inositol.

nated with expression of SPI2. In fact, high expression is observed in LPM (pH 5.8), a medium that mimics some of the conditions found inside the SCV and activates some regulators that are activated within the phagosome. Our experiments reveal that both acidic pH and Mg^{2+} limitation are important factors in the regulation of *srfJ*. Transcription of *srfJ* is positively regulated by the two-component systems PhoP/PhoQ and SsrA/SsrB and negatively regulated by the RcsC/RcsD/RcsB phosphorelay system. Our epistasis experiments suggest that, whereas PhoP regulation is SsrB dependent, RcsB acts independently from the other two regulators. Positive regulation by PhoP is consistent with expression in LPM (pH 5.8), since the PhoQ sensor is activated in response to low Mg^{2+} concentrations and acidic pH (31, 47). Regulation of SPI2 genes by the two-component system PhoP/PhoQ occurs because PhoP controls expression of *ssrB* and *ssrA* at transcriptional and posttranscriptional levels, respectively (3). Here, we show that the same regulatory cascade affects *srfJ*, a T3SS2-related gene located outside SPI2. The Rcs system is also involved in the control of *Salmonella* virulence (13, 14, 19, 42) through the

regulation of many genes, including SPI1 and SPI2 genes (35, 59). A general regulatory overlap between Rcs and PhoP/PhoQ in the control of *Salmonella* virulence was already shown (18). The regulation of *srfJ* by both systems is a new element contributing to this overlap.

Using a T-POP-based screen, we have identified IolR as an unexpected regulator of *srfJ*. IolR is the main regulator of five operons, encompassing the genes *iolA-iolB*, *iolE-iolG1*, *iolC1-iolC2*, *iolD1-iolD2-iolG2*, and *iolI2-iolH*, which are involved in the utilization of *myo*-inositol as a carbon source (33). Transcription of these operons is repressed in minimal medium with glucose and induced in minimal medium with *myo*-inositol. Deletion of *iolR* resulted in stimulation of the transcription of the *iol* operons in minimal medium with glucose, suggesting that the IolR protein was a repressor. In fact, IolR is similar to proteins belonging to the RpiR family of transcriptional regulators. The *iol* operons and the *iolR* gene are located in a 22.6-kb genomic island. Sequence comparisons using BLAST identified this island in the genomes of *S. enterica* serovar Typhimurium strains LT2, 14028, D23580, UK-1, T000240, ST4/74, and SL1344; *S. enterica* serovar Paratyphi B strain SPB7; *S. enterica* serovar Agona strain SL483; *S. enterica* serovar Weltevreden strain 2007-60-3289-1; and *E. coli* ED1a. Most genes in the island are absent from the genomes of *Salmonella bongori* and certain *S. enterica* strains from serovars Typhi, Paratyphi, Gallinarum, Enteritidis, Dublin, and Diarizonae. Interestingly, *srfJ* is present, downstream of *iolG1*, in the strains that contain the island and is absent from the genomes of the strains that lack the island.

The IolR repressor is expected to be inactivated in the presence of *myo*-inositol. Consistent with this notion, addition of this sugar to LB–0.3 M NaCl, a medium where *srfJ* exhibits a very low level of expression, had the same effect as the *iolR* null mutation and resulted in a more than 200-fold increase in *srfJ* transcription. The *iolR* mutation, or the addition of *myo*-inositol, failed to induce expression of *srfJ* in LPM. This result suggests that IolR can repress *srfJ* when SsrB is inactive but not when SsrB is active (LB–0.3 M NaCl versus LPM). In fact, the *iolR* mutation significantly increased transcription of *srfJ* in LPM when tested in an *ssrB* background.

Our results show that *srfJ* can be expressed in two different physiological conditions: (i) intracellularly inside the SCV (conditions mimicked by LPM) in coordination with T3SS2 and (ii) in the presence of *myo*-inositol, a carbohydrate abundant in soil and plants. Whereas intracellular expression is consistent with SrfJ being a T3SS2 effector, the significance of *myo*-inositol-dependent expression is unclear. In fact, SrfJ is not secreted in LB–0.3 M NaCl supplemented with *myo*-inositol in spite of its high level of expression, probably owing to the lack of T3SS2 under these conditions. One possibility is that accumulation of SrfJ before ingestion by the host animal (i.e., in environments where *myo*-inositol is present) is advantageous for early secretion as soon as bacteria enter the host cells and the T3SS2 is mounted. However, an independent role for SrfJ in environments where *myo*-inositol is present cannot be excluded. In support of this idea is the fact that *srfJ* and the *myo*-inositol utilization island are present in *E. coli* ED1a (10, 56), a human host-specific commensal strain belonging to genetic group B2 that is avirulent in a mouse model of extraintestinal infection and does not possess an identified virulence-related T3SS.

Finally, the identification of a new T3SS effector opens the way

to physiological studies. SrfJ is especially interesting because it contains a C-terminal domain that is similar to human lysosomal glucosylceramidase (also known as acid β-glucocerebrosidase or acid β-glucosidase). Both proteins share 30% amino acid sequence identity over 447 residues and show structural similarities in the overall fold and the active-site environment (32). The human enzyme catalyzes the hydrolysis of glucosylceramide to β-glucose and ceramide (23). Ceramide is a lipid molecule composed of sphingosine and a fatty acid. The formation of ceramide within the cell membrane alters biophysical characteristics of membranes and leads to the generation of ceramide-enriched membrane domains. These domains have a general function in signal transduction through amplification of receptor- and stress-mediated signaling events (66). Well-known events modulated by ceramide include differentiation, proliferation, and apoptosis. In addition, ceramide and ceramide-enriched membrane domains have been involved in host responses to many pathogens (24).

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